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COMPARTMENTALIZED D2-DOPAMINE RECEPTORS: ROLES IN SIGNALING & POTENTIAL MECHANISM OF ANTIPSYCHOTIC DRUG ACTION

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COMPARTMENTALIZED D2-DOPAMINE RECEPTORS: ROLES IN SIGNALING & POTENTIAL MECHANISM OF ANTIPSYCHOTIC DRUG ACTION

BY

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ABSTRACT

Dopamine is the most widely distributed catecholamine neurotransmitter in the brain. D2-dopamine receptors (D2R) are one member of a receptor class, known as G-protein coupled receptors, which transduce cellular signals upon an interaction with dopamine. Historically, it was believed that in schizophrenic patients, an excess of dopaminergic signaling at D2R was the cause of psychotic symptoms. Thus, antipsychotics reversed psychosis by blocking excessive dopaminergic signaling from D2R. Sixty years after the introduction of antipsychotic drugs, the connection between the anti-dopaminergic activity of these drugs at D2R and the suppression of psychotic symptoms remains unknown. Understanding the ways that different molecules mediate changes in the ability of D2R to signal and internalize, and thereby affect its cellular compartmentalization is critical to our development of potent antipsychotic agents with fewer side effects.

Upon dopamine binding to the receptor, D2R is thought to generate an intracellular signal or signals by activating a heterotrimeric α-β-γ G-protein that is a component of the receptor complex. In contrast to other G protein beta subunits, Gβ5 is a unique beta subunit that has not been shown to interact with other heterotrimeric G proteins *in vivo*. Previously, it was assumed that regulators of G-protein signaling (RGSs, specifically the R7 RGS family of proteins) and Gβ5 are required for the physiological

activity of Gβ5. Here we show that no R7 RGS proteins are required for Gβ5 to interact with both compartmentalized and non-compartmentalized forms of D2R. Additionally, we have identified one way that Gβ5 may modulate D2R signaling, by specifically blocking the internalization of the receptor in response to dopamine without disrupting the G protein signaling.

All currently available antipsychotics have been demonstrated to bind to and inhibit D2R receptors. Despite their strong anti-dopaminergic action, the efficacy of antipsychotic agents is limited by the serious side effects that these drugs produce, such as tardive dyskinesia and metabolic syndromes. Clozapine, a uniquely efficacious antipsychotic drug, produces reductions in schizophrenic symptoms without manifesting the common adverse effects of other antipsychotics. Therefore we have reexamined the effects of clozapine on D2R biochemistry, specifically how cellular compartmentalization of D2R may be affected by antipsychotic treatment. We found that all antipsychotics tested significantly increased the surface localization, as well as enhancing the solubility, of D2R; with the sole exception of clozapine. This new paradigm may explain clozapine's unique therapeutic efficacy. This discovery could lead to the developments of new therapeutic agents that produce less significant side effects, and more potent reduction of schizophrenic symptoms than the currently available antipsychotic agents

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graduate experience. Lastly, I thank my family and friends for their support throughout my graduate education.

DEDICATION

To the girl who showed me the mountains

and the woman who showed me California

-*T.S.Y.*

PREFACE

This dissertation was completed in the manuscript format. It is divided into two major manuscripts that relate to the understanding how cellular microenvironments and protein-protein interactions can affect the signaling of the D2-Dopamine receptor. The first manuscript is a description of how a unique G protein beta subunit, Gβ5, can influence the signaling of the D2-Dopamine receptor. This manuscript was prepared according to the format guidelines of the Journal of Neurochemistry. The second manuscript identifies a novel mechanism of action of antipsychotic agents and a distinctive confirmation of the D2-Dopamine receptor that is produced by the antipsychotic clozapine, an exceptionally potent antipsychotic drug. This last manuscript was prepared according to the format of the Journal of Biological Chemistry.

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Functional interactions between G protein beta 5 and D2-dopamine receptors

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ABSTRACT

G beta 5 (Gbeta5, Gβ5) is a unique G protein β subunit that is thought to be expressed as an obligate heterodimer with R7 regulator of G protein signaling (RGS) proteins instead of with G gamma (Gγ) subunits. However, here we provide data suggesting that Gβ5 can functionally interact with D2R independently of R7 RGS proteins. We found that D2R coexpression enhances the expression of Gβ5, but not that of the G beta 1 (Gβ1) subunit, in HEK293 cells, and that the enhancement of expression occurs through a stabilization of Gβ5 protein. We had previously demonstrated that the vast majority of D2R either expressed endogenously in the brain or exogenously in cell lines segregates into detergent-resistant biochemical fractions. We report that when expressed alone in HEK293 cells, Gβ5 is highly soluble, but is retargeted to the detergent-resistant fraction after D2R coexpression. Furthermore, an in-cell biotin transfer proximity assay indicated that D2R and Gβ5 segregating into the detergent-resistant fraction specifically interacted in intact living cell membranes. Dopamine-induced D2R internalization was blocked by coexpression of Gβ5, but not Gβ1. However, Gβ5 coexpression had no effect on agonist-induced internalization of MOR, cell surface D2R levels, dopamine-mediated recruitment of β-arrestin to D2R, or the amplitude of D2R-G protein coupling and had only a negligible effect on the deactivation kinetics of D2R-activated G protein signals. The latter data suggest that direct functional interactions between D2R and Gβ5 that are not mediated by endogenously expressed R7 RGS proteins.

Keywords: G Beta 5, D2 Dopamine Receptor, G proteins, R7 RGS proteins, Detergent Resistant Membrane Compartments, Receptor Regulation

Abbreviations: ANOVA, analysis of variance; AP, attenuated acceptor peptide with sequence GLNDIFEAQKIE; Arr-BL, β arrestin-2 biotin ligase fusion construct; BL, E. coli biotin ligase, BirA; BRET, bioluminescence resonance energy transfer; cDNA, complementary DNA; D2R-AP, D2R construct with the biotin ligase acceptor peptide insertion into the 3rd cytoplasmic loop; D2R, D2-dopamine receptor; D4R, D4-dopamine receptor; DAMGO, [D-Ala(2), N-Me-Phe(4), Gly(5)-ol]-enkephalin; DR, dopamine receptor; FK506, FKBP binding protein; FKBP-AP, FK506 binding protein with an attached attenuated acceptor peptide; FLAG, FLAG epitope tag; FRB-BL, FKBP-rapamycin binding protein, biotin ligase construct; FRB, FKBP-rapamycin binding protein; GAP, GTPase accelerating protein; GDP, guanosine diphosphate; GGL, Gγ-like domain; GPCR, G protein coupled receptor; GTP, guanosine-5'-triphosphate; GTPase, guanosine-5'-triphosphatase; Gαo, G protein alpha (o) subunit; Gβ1, G protein beta 1 subunit; Gβ5-BL, G protein beta 5-biotin ligase fusion construct; Gβ5, short isoform of the G protein beta 5 subunit; Gβ5L, long isoform of the G protein beta 5 subunit; Gβγ-Venus, Gβγ dimer tagged with Venus; Gβγ, G protein beta-gamma dimer; Gγ, G protein gamma subunit; HEK293, human embryonic kidney cells 293 stably expressing the SV40 T-antigen; HRP, horseradish peroxidase; KRAS–BL, BirA biotin-ligase fusion construct with the membrane-targeting domain with the sequence KKKKKKSKTKCVIM from the protein KRAS; masGRK3ct-NanoLuc, Gβγ binding peptide from GRK3 fused to an enhanced

renilla luciferase; MOR, mu opioid receptor; PBS, phosphate-buffered saline; PVDF, polyvinylidene fluoride; RGS, regulator of g protein signaling; RGS9-2, regulator of g protein signaling 9-2; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; t½, half-life of agonist response; TCA, trichloroacetic acid; TX100, Triton X-100; V5, V5 epitope tag; ΔBRET, BRET signal values corrected with baseline signal

INTRODUCTION

The D2-dopamine receptor (D2R), is a G protein coupled receptor (GPCR) that is a major target of drugs used to alleviate symptoms of schizophrenia, Parkinson's disease and depression (Missale et al. 1998; Neve et al. 2004). Many of the cellular actions of GPCRs are mediated via the activation of intracellular heterotrimeric G proteins, which consist of a Gα subunit and a protein dimer consisting of Gβ and γ subunits. When an activated GPCR encounters a trimeric G protein, it catalyzes the exchange of GTP for GDP at Gα, leading to the dissociation Gα subunit from a Gβγ dimer. The activated GTP-bound Gα subunit and the free Gβγ dimer regulate the activity of diverse cellular effector molecules. Signal termination is mediated by the intrinsic GTPase activity of the Gα, which hydrolyzes the bound GTP to GDP, allowing it to re-associate with the Gβγ dimer (Bockaert and Pin 1999; Lagerström and Schiöth 2008).

Five different G protein Gβ subunits have been identified thus far, of which the first four share 80-90% homology (Slepak 2009). The fifth, Gβ5, is an atypical member, and shares only about 50% sequence homology with the first four members. Two alternatively spliced isoforms of Gβ5 have been described. The "short" isoform (Gβ5) is broadly expressed in neural, neuroendocrine and excitable tissues while the long isoform (Gβ5L) has only been found expressed in retinal photoreceptors. Severe phenotypes associated with the Gβ5 knockout mice, indicate Gβ5 likely has many important and diverse cellular functions. For example, Gβ5 knockout mice have impaired brain development and exhibit multiple neurological abnormalities (33–35). In addition, these mice have altered

metabolism and abnormal weight regulation, presumably via actions in the central nervous system (Wang et al. 2011)

The GTPase activity of Gα G proteins is enhanced by RGS (regulator of G protein signaling) proteins and thus RGS proteins accelerate the rate of GPCR signal termination. All RGS proteins have a conserved core "RGS domain" which is necessary and sufficient for their GTPase accelerating protein (GAP) function (Ross and Wilkie 2000). Many RGS proteins also possess additional C- and N-terminal domains (Sethakorn et al. 2010; De Vries et al. 1999) that mediate diverse functions.

For example, R7 RGS family proteins contain a Gγ-like (GGL) domain that has been shown to specifically bind Gβ5 subunits and enhance GAP function (32, 38–41). In fact, it is thought that *in vivo*, Gβ5 does not form G protein Gβγ dimers and that complex formation between Gβ5 and the GGL domain-containing R7 RGS proteins is necessary for stabilizing both Gβ5 and R7 RGS proteins (32, 38–41). The genetic ablation of Gβ5 resulted in the loss of all R7 RGS proteins (39), and conversely, Gβ5 protein was not detected in the retina of a triple knockout mouse line lacking the R7 RGS proteins, RGS6, RGS7, and RGS11 (42). Furthermore, the Gβ5 long isoform (Gβ5l) that forms a complex with the R7 RGS protein, RGS9-1, was absent from the photoreceptors of RGS9 knockout mice (Chen et al. 2000).

However, it has not been demonstrated that Gβ5 exists solely as a heterodimer with R7 RGS proteins in all tissues where Gβ5 may be expressed. Alternative

proteins, not abundantly expressed in retinal cells, could contribute to stabilizing Gβ5 expression in other regions.

Previously, it has been shown that the complexes of Gβ5 and R7 RGS proteins can target to D2R and other GPCRs but these interactions are thought to occur through protein domains, such as the DEP domain, that are present within R7 RGS proteins (44–48).

Here we report that Gβ5 can functionally interact with D2R in HEK293 cells and that D2R coexpression stabilizes Gβ5 to enhance Gβ5 expression. Moreover, the D2R-Gβ5 interaction likely occurs independently of R7 RGS proteins suggesting that Gβ5 may have additional cellular functions in addition to its established role as a component of the R7-RGS/Gβ5 complex.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents were purchased from Sigma-Aldrich, Fisher Scientific or from suppliers that have been specifically identified below.

Cell Culture and Transfection

Human embryonic kidney cells (HEK293, American Type Culture Collection) were grown in Dulbecco's Modified Eagle Medium supplemented with 10% v/v fetal bovine serum, penicillin (100 units/ml) and streptomycin sulfate (100 µg/ml). Mammalian expression plasmids containing the appropriate cDNA constructs were transiently transfected using LTX transfection reagent (Life Technologies) according to the manufacturer’s instructions. Total transfected DNA was

maintained between groups by co-transfecting empty plasmid vector pcDNA 3.1+ (Life Technologies).

cDNA Constructs

All plasmid constructs utilized below were created using standard techniques in molecular biology. The N-terminal FLAG-tagged version of the long form of the human D2-dopamine receptor (D2R) (Kearn et al. 2005), the N-terminal FLAG-tagged D4-dopamine receptor (D4R) (Marley and von Zastrow 2010), the Gβ5 short isoform construct (Kovoor et al. 2000), the FLAG-tagged D2R construct with the biotin ligase acceptor peptide insertion into the 3rd cytoplasmic loop (D2R-AP) (Sharma et al. 2013), the *E. coli* BirA biotin-ligase fusion construct with the membrane targeting domain of KRAS (KRAS-BL, and the β-arrestin-2 biotin ligase fusion construct (Arr-BL) (Sharma et al. 2013) have previously been described. The D2R-AP construct consists of the FLAG-tagged D2R into which an attenuated acceptor peptide sequence (GLNDIFEAQKIE) is inserted between amino acids at position 305 and 306 in the 3rd cytoplasmic loop. KRAS-BL consisted of the following peptide sequences in order from the N to the C-terminus: the V5 epitope-tag, the BirA *E. coli* biotin ligase enzyme (BL), a GSGSG linker and a membrane targeting peptide sequence (KKKKKKSKTKCVIM) from the protein KRAS. Arr-BL consisted of the following peptide sequences in order from the N to the C terminus, β-arrestin-2, a GSGSG linker, and the BirA an *E. coli* biotin ligase enzyme (BL). The N-terminal FLAG-tagged human G protein Gβ1subunit was obtained from the Missouri S&T cDNA Resource Center (catalog #: GNB010FN00). The G protein beta 5-biotin ligase

fusion (Gβ5-BL) was created by attaching the BirA biotin ligase enzyme to the N-terminus of the full-length Gβ5 short isoform via a two amino acid linker. Thus, the fusion protein in order from N to C terminus consists of BirA, an Arg-Tyr linker, the Gβ5 short isoform, and a V5 epitope tag. The cDNA for the *E. coli* biotin ligase, BirA (BL), was provided by Dr. Alice Ting (Massachusetts Institute of Technology) and was amplified by PCR. Diagrams of these constructs are provided in Fig. 4A and 7A.

Triton X-100 Biochemical Fractionation of Proteins

The method for the Triton X-100 (TX100) biochemical fractionation of proteins has been adapted from our previous publication (Celver et al. 2012). Briefly, 48 hr post transfection cells were lysed in TX100 lysis buffer (phosphate-buffered saline, in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 2 KH_2PO_4, pH 7.4 (PBS) containing 2% v/v of the non-ionic detergent, Triton X-100) and a 1× concentration of SigmaFast Protease inhibitor (made according to manufacturer's instructions, Sigma-Aldrich) for 1 hr at 4 ºC The samples were centrifuged (10,000 g, 10 min at 4 ºC) to pellet the insoluble proteins. Supernatant proteins (i.e. the TX100-soluble fraction) were precipitated by the addition of trichloroacetic acid (TCA, final concentration 10% v/v). Supernatant proteins were washed 3× with ice-cold 95% v/v acetone (4 °C). Both the TX100-soluble and the insoluble proteins were re-suspended in equal volumes of SDS sample buffer (2% w/v SDS, 0.01% w/v bromophenol blue, 8 M urea, 20 mM dithiothreitol, 50 mM Tris-HCl, pH 6.8). Samples were sonicated 25× for approximately 0.5 s at power setting of 10 for ~0.5 s to reduce sample viscosity

prior to loading using a sonicator (XL-2000, qSonica). Equal volumes of the samples were then resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the relative levels of protein expression were then compared by Western blotting.

Protein Degradation Assay

To determine the effect of D2R on the rate of degradation of Gβ5 we used cycloheximide, a translational inhibitor, to block protein synthesis and then measured the amount of Gβ5 present in cells at 3 and 6 hr after the addition of cycloheximide. 2.5×10^5 HEK293 cells were transfected with appropriate cDNA plasmids containing Gβ5 with or without D2R in a 24 well plate. At 48 and 51 hr post-transfection selected wells were treated with 100 μM cycloheximide (time = 6 and 3 hr cycloheximide treatment, respectively). After incubation for 3 hr (54 hr post-transfection) all cell samples were harvested in media from multi-well plates using a micropipetter. Cells were spun down for 5 minutes at 300 × g using a bench top centrifuge and carefully washed 3 × with cold (4 °C) PBS. Washed cells were then lysed by sonication on ice after being resuspended in equivalent volumes of SDS sample buffer. Protein samples were then incubated for 15 min at 65 °C resolved by SDS-PAGE.

Biotinylation of D2R-AP by Biotin Ligase Fusion Proteins

We utilized an in cell biotin transfer assay to detect whether or not Gβ5 interacted with detergent soluble or insoluble forms of D2R. The plasmids containing cDNAs for D2R-AP, Gβ5-BL or KRAS-BL as described in Figure 5A were

transfected into HEK293 cells in biotin-free media. 48 hr post-transfection, 10 μM biotin was added to the media. After 2 minutes of incubation at 25 °C, the cells were washed 3 × using ice-cold (4 °C) 1 × PBS. The cells were then vigorously resuspended in ice-cold 2% v/v TX100 lysis buffer and incubated for 1 hr at 4 °C, with vortexing every 15 minutes. Soluble and insoluble proteins were then harvested using the method as described in the "Triton X-100 Biochemical Fractionation of Proteins" section.

We also used the biotinylation assay to detect recruitment of β-arrestin 2 to D2R after dopamine stimulation. The plasmids for D2R-AP and Arr-BL with or without Gβ5 (Fig. 8A) were transfected into HEK293 cells growing in biotin-free media. 48 hours post-transfection, cells were treated with 10 μM dopamine or vehicle for 30 min. Subsequently, the media containing dopamine was removed, the cells were washed 2 × with cold (4 °C) PBS, and then were treated with 10 μM biotin in 1 × PBS for 2 minutes. Cells were then washed 3 × with ice-cold PBS and then lysed in SDS sample buffer.

Samples were then resolved by SDS PAGE. The proteins were then transferred to methanol-wetted polyvinylidene fluoride (PVDF) membranes and probed with streptavidin conjugated to horseradish peroxidase (HRP). These procedures and reagents are described in the "Western Blotting" section below.

Western Blotting

Proteins were resolved by gel electrophoresis and then transferred to methanol-wetted PVDF membranes by Western blotting apparatus (IBI Scientific) using electrophoresis buffer (25 mM Tris base, 192 mM glycine, 20% v/v methanol, pH

~8.3). For antibody-based detection, PVDF membranes (Immobilon-FL, EMD Millipore) were blocked by incubation with 5% w/v nonfat dry milk reconstituted in PBS (1 hr at 20 ºC). For the detection of biotinylated proteins, membranes were blocked by incubation in 3% w/v bovine serum albumin in PBS (1 hr at 20 ºC).

V5 epitope-tagged proteins (KRAS-BL and Gβ5-BL) were detected by incubating blots with an HRP-conjugated anti-V5 antibody (Invitrogen/Life Technologies, 1:5000 in 5% w/v nonfat milk in PBS). FLAG epitope-tagged protein bands (D2R, D4R, and MOR) were detected by incubating blots with an HRP-conjugated mouse monoclonal anti-FLAG M2 antibody (Sigma-Aldrich, 1:5000 dilution in 5% w/v nonfat milk in 1 × PBS). Biotinylated protein (D2R-AP) bands were detected by incubating blots with HRP-conjugated streptavidin (1:10,000 in 1 × PBS containing 3% w/v BSA). Gβ5 was detected by incubating blots with rabbit polyclonal antibody CT215 (1:5000 in PBS containing 5% w/v nonfat milk) (Watson et al. 1996; Watson et al. 1994). After incubation with the primary antibodies or HRP-conjugated streptavidin the blots were washed 3× in PBS containing 0.1% v/v tween-20 (PBS-T). If the primary antibody was not directly conjugated to HRP the membrane was then incubated with appropriate HRP-conjugated secondary antibodies (Jackson ImmunoResearch, Inc.) and washed 4× in PBS.

Chemiluminescent signals produced by the HRP enzyme were obtained using Supersignal West Femto substrate and detected using a Chemidoc XRS Molecular Imager (Bio-Rad Laboratories). To directly compare the signals from the TX100-soluble and insoluble fractions of a cell sample, the proteins from

these fractions were loaded onto the same SDS-PAGE gel and transferred to a single immunoblot. Protein samples were serially diluted and the signals quantified to ensure that the concentrations used for experiments was in the linear range of the signal-protein function.

Fast Kinetic BRET Assay

The agonist effects of dopamine on G protein signaling in cells expressing D2R was measured using a fast kinetic BRET assay (Masuho et al. 2013). BRET was measured between masGRK3ct-NanoLuc and Gβ1γ2-Venus in living cells as previously described BRET measurements were made at room temperature using a microplate reader (POLARstar Omega, BMG Labtech) equipped with two emission photomultiplier tubes, with a maximum of 50 milliseconds resolution. The BRET ratio is calculated by dividing the light emitted by Gβ1γ2-Venus (535 nm) over the light emitted by masGRK3ct-NanoLuc (475 nm). The average baseline value recorded prior to agonist stimulation was subtracted from BRET ratio values, and the resulting difference (ΔBRET) was obtained. The amplitude of the dopamine response was the maximal ΔBRET ratio achieved after application of 10 nM dopamine. The time constants for signal deactivation were derived from single exponential fits of the deactivation curve following application of 100 μM haloperidol. Kinetic analysis and curve fitting were performed using pCLAMP 6 software (Molecular Devices).

Receptor Internalization Assay

To determine the effect of overexpression of Gβ subunits (Gβ1 or Gβ5) on receptor internalization we used an ELISA-based assay to determine the amount

of receptor present at the plasma membrane after the application of dopamine. Day 1, 5×10^4 HEK293 cells were transfected with appropriate cDNA plasmids containing D2R with or without Gβ1 or Gβ5 or MOR with or without Gβ5, in a 96-well plate. 48 hours post-transfection cells were treated with a saturating concentration (50 μM) of dopamine in the case of D2R or DAMGO in the case of MOR for 45 minutes. The media was then aspirated, and cells were gently washed 3 times with cold (4 °C) PBS. Cells were then fixed with 4% v/v formaldehyde in PBS, and then washed 3 times with PBS. Wells were blocked for 30 minutes with 5% nonfat milk dissolved in PBS. Surface receptor was then probed for using HRP-conjugated mouse monoclonal anti-FLAG M2 antibody (1:5,000 dilution in 5% nonfat milk in PBS) for 1 hour at 37 °C and then washed 3 × with PBS. Supersignal West Femto chemiluminescent substrate (Pierce-Thermo Fisher Scientific) was then applied to each well and signals were detected and quantified using a multi-well plate compatible luminometer (Glomax) and attached computer.

Data Analysis

Signals from the target protein bands were quantified using ImageJ image processing and analysis software (National Institutes of Health, Bethesda, MD, http://rsbweb.nih.gov/ij/). Statistical analyses were performed using Microsoft Excel or GraphPad Prism 4 software (GraphPad Software, Inc.). Images were collected using exposure settings that did not saturate any of the pixels acquired by the camera. The signals resulting from detergent-soluble and insoluble preparations of a protein, respectively, were expressed as a fraction of the total

signal and Student's t-test for independent means of unequal variance was used to determine if the amounts of signal from the target protein bands in each experimental group were significantly different. When testing the significance of means for more than 2 experimental groups, one-way ANOVA was used to first determine group statistical significance and only followed by Tukey's post-hoc analysis if the initial comparison was found to be significant.

RESULTS

Coexpression of D2R in HEK293 cells enhances the detergent-resistance of Gβ5 even in the absence of exogenous coexpression of R7 RGS proteins.

We had previously shown that the vast majority of D2-dopamine receptors (D2R) expressed endogenously in the brain, or exogenously in the plasma membrane of cell lines, segregates into cellular fractions that are resistant to solubilization in non-ionic detergents (Celver et al. 2012) and we showed that this detergent-resistant D2R fraction is functional and responds to dopamine (Sharma et al. 2013). Furthermore, coexpression of D2R produces translocation of putative D2R interacting proteins, such as the RGS9-2/Gβ5 complex and G proteins, from detergent-soluble to detergent-resistant membrane fractions when the latter proteins are expressed in cell lines (Celver et al. 2012; Sharma et al. 2013). In fact, interactions with D2R, which is expressed at relatively high concentrations in the striatum compared to the cortex (Missale et al. 1998; Neve et al. 2004), could explain why we and others have found that the endogenous striatal RGS9-2/Gβ5 complex is resistant to detergent-extraction, but the same complex when expressed in cell lines is highly soluble (Celver et al. 2012; Mancuso et al. 2010).

To provide further support for the idea that targeting to D2R can contribute to enhanced detergent resistance of D2R-interacting proteins the striatum, we compared the detergent-solubility of Gβ5 endogenously expressed in mouse striatum and the cortex. We found that the percent of striatal Gβ5 that was extracted into cold solutions (4 °C) of the non-ionic detergent Triton X-100 was almost halved (from ~40% to 20%), relative to Gβ5 extracted from the cortex (Celver et al. 2012).

One explanation for the increased detergent-resistance of striatal Gβ5 is that D2R, which we have shown is highly resistant to detergent solubilization, is expressed at high concentrations in the striatum compared to the cortex and Gβ5 is then targeted to the detergent-resistant striatal D2R through an interaction with RGS9-2 or other R7 RGS proteins (Celver et al. 2012). Therefore, in a control experiment using HEK293 cells, we tested if D2R could enhance the detergent-resistance of Gβ5 independently of exogenously expressed R7 RGS proteins. We found that coexpression of D2R with Gβ5 in HEK293 cells significantly increased the percent of Gβ5 that segregated into the TX100-insoluble cellular fraction (from ~40% to 70%), even in the absence of exogenously coexpressed R7 RGS protein constructs (Fig. 1A and B). This is a surprising result, because while endogenous expression of R7 RGS proteins in HEK293 cells has been suggested via RNA interference (Laroche et al. 2010), a microarray analysis of mRNA levels of GPCR related signaling proteins expressed in these cells did not detect statistically significant levels of mRNA for any of the R7 RGS proteins (Atwood et al. 2011). Thus, transiently expressed Gβ5 protein, is likely

to vastly exceed the endogenously expressed levels of R7 RGS family members in HEK293 cells.

Coexpression of Gβ5, on the other hand, did not significantly affect the TX100-solubility of D2R protein (% D2R that was soluble when expressed alone was 21.8 ± 4.7, and after Gβ5 coexpression was 30.8 ± 5.4, n = 4, $p > 0.05$, data not shown). Figures 1C and D illustrate, as reported earlier (Celver et al. 2012; Sharma et al. 2013), that the majority (>70%) of coexpressed D2R protein segregates into the TX100-insoluble fraction.

Dopamine pretreatment (10 μM for 30 min) had no effect the TX100-solubilitiy of Gβ5.

We report that the closely related D2-like dopamine receptor, D4R, also segregates into the TX100-insoluble cellular fraction and that Gβ5 is similarly retargeted to the TX100-insoluble cellular fraction after D4R coexpression (Fig. 1A, B, C and D).

The above phenomenon is specific to dopamine receptors as we have previously reported that coexpression of the mu opioid receptor (MOR) did not affect the detergent-solubility of either component of the R7 RGS protein-Gβ5 complex (Celver et al. 2012).

The other, more canonical G protein Gβ subunits are intrinsically resistant to detergent solubilization (Rehm and Ploegh 1997), and thus a similar D2R-mediated retargeting of other Gβ subunits, such as Gβ1, to the TX100-insoluble cellular fraction was not observed (Fig. 1E and F).

D2R coexpression specifically enhances the expression and stability of Gβ5.

In addition to translocating Gβ5 to the TX100-insoluble fraction we observed that the coexpression of D2R simultaneously and dramatically increased the cellular expression of Gβ5 protein (Fig. 1A, 2A and B, and 3A).

The actions of D2R in increasing Gβ5 expression levels were specific. First, coexpression of D2R increased expression levels of Gβ5 by more than 400%, but, in contrast, coexpression of another GPCR, the mu opioid receptor (MOR) did not significantly alter expression levels of Gβ5 (Fig. 2A and B).

Second, the expression level of the G protein Gβ subunit, Gβ1, was instead, significantly decreased after D2R coexpression (Fig. 2C and D).

To explore if D2R-mediated stabilization of Gβ5 contributed to the enhanced Gβ5 expression observed after D2R expression, we treated HEK293 cells expressing Gβ5 alone, or coexpressing D2R and Gβ5, with cycloheximide, a protein translation/synthesis inhibitor, and the decay of the cellular Gβ5 protein signal after cycloheximide treatment for 3 and 6 hr was monitored by Western blotting. We found that coexpression of D2R significantly decreased the decay of the Gβ5 signal observed at both 3 and 6 hr (Fig. 3). For example, after 6 hr of cycloheximide treatment, the levels of Gβ5 protein in cells expressing Gβ5 alone had decayed to less than 30%, but in cells coexpressing D2R greater than 60% of the original Gβ5 signal remained (Fig. 3). Thus, D2R coexpression significantly inhibited the cellular degradation of Gβ5.

An "in-cell biotin proximity biotinylation assay" indicates physical interactions in living cells between D2R and Gβ5 molecules that segregate into the detergent-insoluble cellular fraction.

Traditional coimmunoprecipitation techniques (Berggard et al. 2007) for probing for either direct or indirect physical interactions between D2R and Gβ5 first require solubilizing the proteins in non-ionic detergents that preserve protein-protein interactions. Since the vast majority of D2R segregates into a cellular fraction that is insoluble in non-ionic detergents (e.g. TX100) it was not feasible for us to probe for DR interactions using coimmunoprecipitation. Furthermore, we were unable to coimmunoprecipitate D2R and Gβ5 molecules segregating into the TX100-soluble fraction, possibly due to the relatively low concentration of D2R molecules that segregate into this fraction.

Thus, to assess D2R and Gβ5 interactions, we utilized a novel in-cell proximity biotinylation assay involving the *E. coli* biotin ligase, BirA (Fernandez-Suarez et al. 2008; Sharma et al. 2013), which specifically biotinylates a unique "acceptor peptide" sequence, not present in mammalian proteins. An attenuated biotinylation acceptor peptide substrate sequence (denoted here as AP) was inserted into the 3rd cytoplasmic loop of D2R (D2R-AP), while the BirA biotin ligase enzyme (BL) was fused to either Gβ5 (Gβ5-BL) or a peptide motif from KRAS (KRAS-BL) (Fig. 4A). The D2R-AP substrate and the biotin ligase enzyme fusions were co-expressed in HEK293 cells cultured in biotin-depleted medium. Following a brief (2 min) treatment of the intact living cells with biotin, the cells were lysed in cold (4 °C) TX100 lysis buffer and separated into TX100-soluble

and insoluble fractions. Biotinylation of D2R-AP provides evidence for interactions between the D2R-AP substrate and biotin ligase-containing fusion that had previously occurred in the intact living cell.

The use of the technique to evaluate the level of interaction between two proteins in living cells has been previously validated. For example, the rapamycin-induced interaction between the FKBP (FK506 binding protein) and FRB (FKBP-rapamycin binding protein) protein pair could be detected by enhanced in-cell biotinylation of the FKBP-AP fusion substrate by an FRB-BL fusion (Fernandez-Suarez et al. 2008). Similarly, we found that the in-cell biotinylation of D2R-AP fusions by a β-arrestin2-BL fusion protein was enhanced by treatment of the cells with dopamine (Sharma et al. 2013).

We had reported earlier that the insertion of the AP-tag into D2R does not greatly affect its detergent solubility (Sharma et al. 2013) and that the vast majority (~80%) of the D2R-AP construct segregated into the TX100-insoluble cellular fraction. We also showed previously, that when D2R-AP fusion substrates and a wide variety of peptide motifs and cellular proteins fused to the biotin ligase enzyme were coexpressed in HEK293 cells, in almost every case, the majority of the biotinylated D2R-AP substrate segregated into the TX100-soluble fraction (Sharma et al. 2013). In other words, biotinylated D2R-AP constructs segregated into the TX100-soluble fraction, even though vast majority of the parent D2R-AP substrate protein localized into the TX100-insoluble fraction (Sharma et al. 2013). These results indicate that the detergent-resistant D2R, though functional and expressed in the plasma membrane, as we

previously showed (Sharma et al. 2013), represents receptor that is compartmentalized from interacting non-specifically with other cellular proteins. On the other hand, the detergent-soluble D2R, which represent a minority of the cellular D2R, likely originates from a more fluid region of the cell membrane and can interact randomly with other cellular proteins according to the fluid mosaic model of Singer and Nicolson (Singer and Nicolson 1972).

In accordance with the above results, we show that the majority (~70%) of D2R-AP that was biotinylated by KRAS-BL segregates into the TX100-soluble fraction (Fig. 4B and C). However, we found that the segregation of D2R-AP biotinylated by Gβ5-BL, more closely matched the segregation of the parent protein with ~70% of biotinylated D2R-AP segregating into the TX100-insoluble fraction (Fig. 4B and C). These results may be interpreted to suggest that 1) D2R segregating into the TX100-resistant cellular fraction (i.e. the majority of the plasma membrane-expressed D2R) is not compartmentalized from Gβ5 as it was from KRAS and many other cellular proteins and, 2) that Gβ5, unlike other cellular proteins, efficiently interacts in living cells with D2R molecules that segregate into both TX100-soluble and insoluble cellular fractions (Fig. 4D).

Effect of coexpression of Gβ5 on cellular coupling between D2R and Gαo G proteins.

We then tested if the coexpression of Gβ5 could alter the cellular functions of D2R. To test the effects of Gβ5 coexpression on D2R-mediated G protein activation we utilized a bioluminescence resonance energy transfer (BRET) based assay, recently developed by Hollins and colleagues(Hollins et al. 2009),

which measures the release of free Gβγ subunits from the activated G protein. The BRET pair that is utilized is the Gβγ dimer tagged with Venus (Gβγ-Venus) and masGRK3ct-NanoLuc, a Gβγ binding protein construct fused to a newly engineered luciferase variant (64). The use of this system to monitor coupling between D2R and associated G proteins has been described in detail in a previously published study (Masuho et al. 2013). The following proteins were coexpressed in HEK293 cells: D2R, the D2R coupled G protein subunit, Gαo, a Gβγ fusion with the yellow fluorescent protein Venus (Gβγ-Venus) and masGRK3ct-NanoLuc, a fusion of Gβγ binding peptide from the protein, GRK3, with a luciferase variant, NanoLuc, are transiently coexpressed in HEK293 cells. Activation of the coexpressed G proteins by dopamine-bound D2R results in the release of the Venus-tagged Gβγ subunits and interaction with the BRET partner, the NanoLuc-tagged masGRK3ct reporter, to produce the BRET signal. Subsequent application of the D2R antagonist, haloperidol, allows for the reversal of G protein activation and results in BRET signal decay which can be well fitted by single exponentials. No significant dopamine-elicited response was observed in cells not transfected with cDNA for either D2R or Gαo (Masuho et al. 2013) indicating that the BRET signal results from the activation of exogenously expressed Gαo G proteins by D2R.

Under our assay conditions the amplitude of the D2R-elicited Gαo response saturates at concentrations higher than 1 μM dopamine (Masuho et al. 2013). Using this assay system and a concentration of dopamine of 1 nM we found that coexpression of either of two different Gβ5 concentrations had no effect on the

amplitude of the dopamine and D2R-elicited BRET response (Fig. 5A and B). The inability to detect an effect of Gβ5 on the D2R-elicted response amplitude was not due to saturation of the BRET signal as we confirmed, in the same cells, that the dopamine concentration (10 nM) was sub-saturating. Thus, Gβ5 has no effect on the efficacy of coupling of D2R to associated Gαo G proteins.

We then examined the effects of Gβ5 coexpression on the deactivation kinetics of D2R-Gαo G proteins signaling where the dopamine signal was reversed by the application of 100μM haloperidol. At the lower level of Gβ5 expression, obtained using Gβ5 cDNA transfection concentrations that were similar to those utilized the biochemistry experiments described above, no significant effect of Gβ5 was observed on the deactivation kinetics (Fig. 5A and C). However, with much higher Gβ5 protein concentrations (> 3X), a small but significant acceleration of the deactivation kinetics was detected.

Coexpression of Gβ5, but not Gβ1, inhibits agonist-induced internalization of D2R, and Gβ5 coexpression does not affect agonist-induced internalization of MOR

To quantify receptor internalization we measured the amount of receptor at the surface of HEK293 cells both before and after agonist treatment through a modification of a previously described enzyme-linked immunosorbent assay (ELISA)-based protocol (see Materials and Methods) (Celver et al. 2010). Coexpression of Gβ5 had no significant effect on the levels plasma membrane expressed D2R or MOR, while coexpression of Gβ1 produced a moderate but significant increase in surface expressed D2R (Fig. 6A and B).

Treatment of cells transiently expressing D2R or MOR for 45 min with super-saturating concentrations (10 μM) of the receptor agonists, dopamine or DAMGO, respectively, significantly reduced cell surface levels of the respective receptors (Fig. 6C and D). Coexpression of Gβ1 had no effect on the loss of cell surface D2R produced by dopamine treatment. In contrast, coexpression of Gβ5 completely blocked the dopamine-induced internalization of D2R (Fig. 6C) but had no effect on DAMGO-induced internalization of MOR (Fig. 6D).

Coexpression of Gβ5 does not affect the dopamine-dependent recruitment of arrestin to D2R.

The canonical model for the agonist-induced internalization of many GPCRs involves the recruitment, to the agonist-bound GPCR, of β-arrestins, which then serve to physically bridge the receptor to the cellular endocytotic machinery (Drake et al. 2006). To determine whether Gβ5 inhibited dopamine-induced D2R internalization by suppressing recruitment of β-arrestin we used the in-cell proximity biotin-transfer assay to evaluate the actions of Gβ5 on this process. In this assay D2R-AP and a fusion construct of β-arrestin2 and the *E. coli* biotin ligase BirA (Arr-BL) (Fig. 7A) are transiently expressed in HEK293 cells and dopamine treatment (10 μM for 30 min) significantly enhances the Arr-BL-mediated biotinylation of D2R-AP (Sharma et al. 2013) (Fig. 7B and C). However, coexpression of Gβ5 had no effect on D2R-AP biotinylation suggesting that Gβ5 did not inhibit recruitment of β-arrestin to D2R.

DISCUSSION

D2R-Gβ5 interactions can occur independently of R7 RGS proteins.

Several lines of investigations have led to the supposition that Gβ5 is found expressed only as a heterodimer with R7 RGS family proteins (32, 38, 39, 42, 43). However, none of these experiments have excluded the possibility that alternative proteins could contribute to stabilizing Gβ5 expression in other tissue. The data presented here, using HEK293 cells, suggest the novel hypothesis that D2R can interact with and stabilize the Gβ5 protein, independently of R7 RGS proteins.

Complexes of Gβ5 and R7 RGS proteins can target to D2R and other GPCRs and these interactions are mediated through the domains present in the R7 RGS protein such as the DEP domain (44–47). However, the D2R-Gβ5 interactions reported here likely occur independently of R7 RGS protein for the following reasons.

Atwood and colleagues have conducted a microarray screen for G protein coupled receptors (GPCR) and related signaling molecules that are endogenously expressed in HEK293 cells and found that the detected levels of transcripts for R7 RGS family members were below levels deemed to be statistically significant (Atwood et al. 2011). Thus, stoichiometrically, the levels of Gβ5 transiently expressed in HEK293 cells likely far exceed the levels of any endogenously expressed R7 RGS proteins.

Furthermore, we have shown that endogenous expression of Gβ5 is not detected in HEK293 cells (Celver et al. 2012), and multiple groups, including ours, have shown that, in heterologous expression systems, the observed GTPase accelerating protein (GAP) function of R7 RGS proteins is dramatically enhanced (over 3 fold) by transient coexpression of Gβ5 (40, 55, 67, 68). The Gβ5 enhancement of GAP function likely occurs through multiple mechanisms including 1) direct conformational alteration of R7 RGS proteins that promote GAP function, 2) through an increase in expression of R7 RGS proteins and 3) by facilitating the interaction of R7 RGS proteins with membrane anchors (Keren-Raifman et al. 2001; Masuho et al. 2011). Thus, if a significant proportion of the exogenously expressed Gβ5 associates with endogenously expressed R7 RGS proteins it is expected that the formation of such a complex should substantially accelerate the deactivation kinetics of D2R-G protein coupling. However, such an acceleration was not observed (Fig. 5A and C).

We have previously reported that when R7 RGS proteins, such as RGS9-2, and Gβ5 are transiently expressed in HEK293 cells, D2R co-expression does not significantly alter protein expression levels of either the R7 RGS protein or Gβ5. In other words when Gβ5 is present in a complex with R7 RGS proteins, D2R coexpression does not enhance or stabilize Gβ5 protein expression. However, here we have reported that D2R coexpression can dramatically enhance levels of transiently coexpressed Gβ5 protein (Fig. 1A, 2A and B, and 3A), indicating that Gβ5 is not in a complex with endogenously expressed R7 RGS proteins.

Thus, our data suggest that, in HEK293 cells, D2R interacts either directly or indirectly with Gβ5, but in a manner that is independent of R7 RGS proteins. It is not clear from our data however if D2R is interacting with the Gβ5 monomer or with a complex of Gβ5 with other cellular proteins such a G protein Gγ subunits.

The D2R-Gβ5 interaction has functional consequences and can bias D2R signaling.

We found that 1) the interaction stabilized and enhanced Gβ5 expression and 2) the interaction inhibited dopamine-induced D2R internalization but did not affect the coupling of D2R to G proteins or the dopamine-mediated recruitment of β-arrestin to D2R.

The biased actions of Gβ5 in altering D2R cellular functions are particularly interesting. It is now apparent that endogenous agonists may stabilize multiple receptor conformation and the agonist-bound receptor conformation that promotes G protein activation may be different from the conformation that allow for agonist-induced internalization of the receptor (Kenakin 2007; Reiter et al. 2012). In fact, biased synthetic D2R agonists have been developed that activate non-canonical G protein-independent cellular signals but do not promote D2R-elicited G protein signals (72). However, we believe that this is the first report of a GPCR-interacting cellular protein that modulates the receptor to abolish agonist-induced internalization but does not affect D2R-G protein coupling.

The abolition of dopamine-induced D2R internalization by Gβ5 was not through suppression of interactions with β-arrestin, as Gβ5 did not alter baseline interactions of D2R with β-arrestin or dopamine-induced recruitment of β-arrestin

to D2R (7B and C). Gβ5 had no effect on MOR internalization indicating that the prevention of D2R-internalization by Gβ5 likely occurs through targeting of Gβ5 to D2R and is not a consequence of non-specific disruption of the cellular internalization machinery. One model that may be suggested is that internalization of D2R requires one or more bridges between D2R and the cellular internalization, that are in addition to that made through β-arrestin, and Gβ5 expression disrupts such additional connections.

The expression of D2R in detergent-insoluble plasma membrane microcompartments (Sharma et al. 2013) and the targeting of Gβ5 to these microcompartments did not require dopamine pretreatment, indicating that Gβ5 is preassembled in a manner that allows Gβ5 to edit the actions of dopamine at D2R.

D2R-Gβ5 interactions specific and are not caused by non-specific aggregation of the two proteins.

Coexpression of Gβ5 did not alter either the cell surface levels of D2R, the fraction of D2R expressed at the cell surface or the amplitude of D2R-G protein coupling, but clearly inhibited dopamine-induced D2R internalization. These observations indicate that the interaction with D2R and stabilization of Gβ5 was not caused by non-specific aggregation of the two proteins.

The majority of the D4-dopamine receptor, which is a member of the D2-like dopamine receptor family, also segregates into detergent-resistant cellular fractions and recruits Gβ5 to the same biochemical fraction. However, these interactions are unique and do not extend to other cell-expressed GPCRs such

as mu opioid receptors (MOR), the vast majority of which are readily solubilized in non-ionic detergents (Celver et al. 2012). In addition, D2R coexpression does not significantly alter the detergent-solubility of Gβ1 (Fig. 1E and F) or enhance cellular Gβ1 expression levels (Fig. 2C and D).

Here we have provided evidence for a novel and specific interaction of Gβ5 that is significant because it suggests that Gβ5 can directly modulate D2R, an important GPCR, to bias D2R to signal canonically through G proteins but can prevent dopamine-induced receptor internalization. In addition our data suggests that Gβ5 may be stabilized by protein partners other than R7 RGS proteins. Nevertheless, these experiments were performed in HEK293 cells where concentrations of both D2R and Gβ5 are likely to be higher than that found in native tissue. Hence, definitive *in vivo* evidence for the above supposition will require further investigations such as the examination of Gβ5 levels in D2R-expressing cells in mice where all four R7 RGS protein genes are knocked out.

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Fig. 1. Targeting of Gβ5 to the TX100-insoluble fraction upon coexpression of D2-like dopamine receptors, D2R and D4R.

A. Representative image of a Western blot depicting the segregation of Gβ5 into TX100-soluble (S) and insoluble (I) biochemical fractions prepared from HEK293 cells transfected with cDNAs for the indicated proteins.

B. Quantification of the relative levels of Gβ5 segregating into TX100-soluble (white bars) and TX100-insoluble (black bars) biochemical fractions expressed as percentage of the total cellular Gβ5 signal from the respective cellular samples (mean ± SEM; n = 4, *p < 0.01, t-test relative to cells expressing Gβ5 alone.

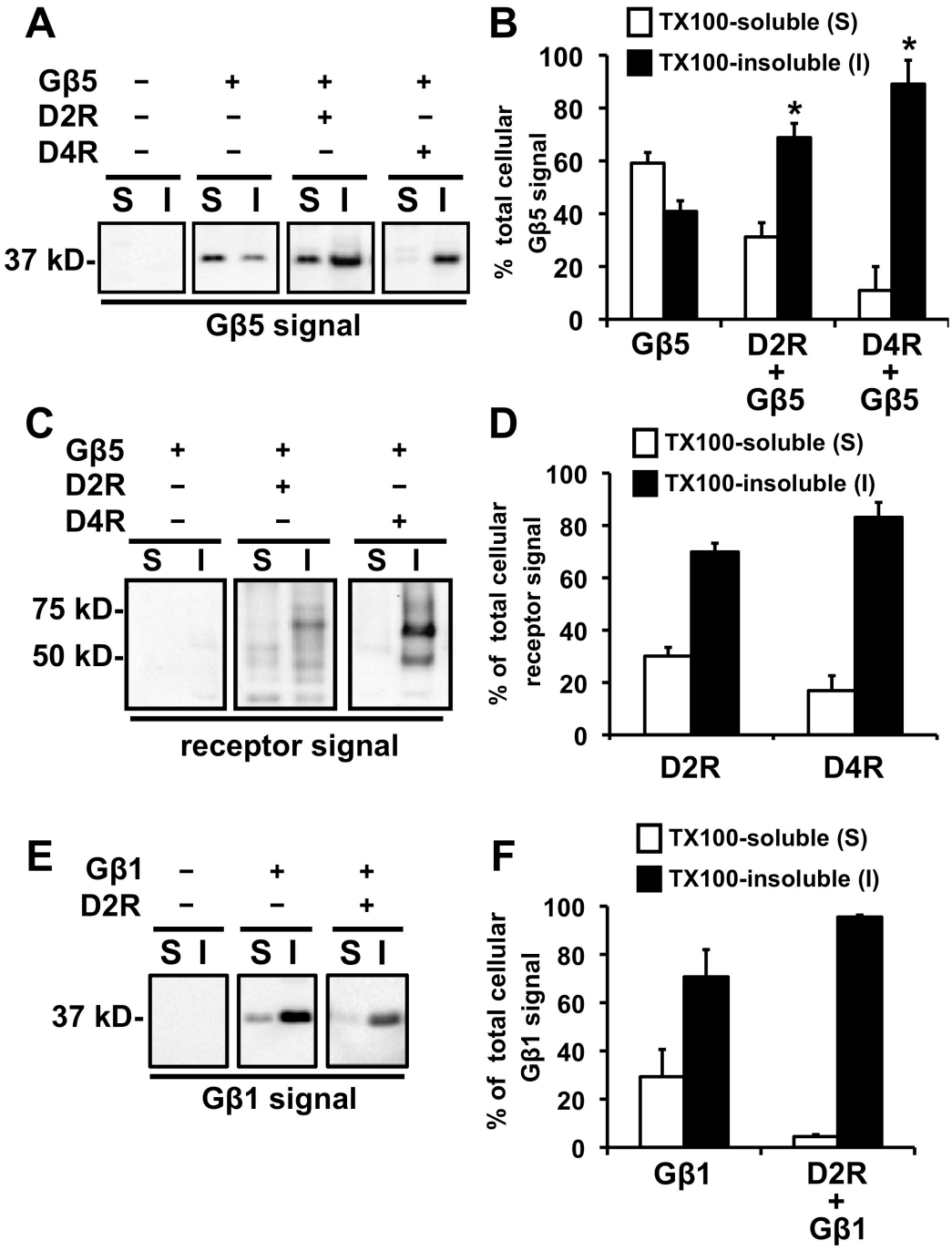
C. Representative image of a Western blot depicting the segregation of the respective FLAG-tagged dopamine receptor proteins, D2R and D4R, into TX100-soluble (S) and insoluble (I) biochemical fractions prepared from HEK293 cells transfected with the indicated cDNAs.

D. Quantification of the relative levels of D2R and D4R segregating into TX100-soluble (white bars) and TX100-insoluble (black bars) biochemical fractions prepared from cell samples indicated in **C** (mean ± SEM; n = 4).

E. Representative image of a Western blot depicting the segregation into TX100-soluble (S) and insoluble (I) biochemical fractions of Gβ1 protein transiently expressed in HEK293 cells and effect of transient coexpression of D2R on such segregation.

F. Quantification of the relative levels of Gβ1 segregating into TX100-soluble (white bars) and TX100-insoluble (black bars) biochemical fractions.

Figure 1.


A
Gβ5 – + + +
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D4R – – – +
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37 kD-
Gβ5 signal
B
TX100-soluble (S)
TX100-insoluble (I)
% total cellular Gβ5 signal
100
80
60
40
20
0
*
*
Gβ5
D2R + Gβ5
D4R + Gβ5
C
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D2R – + –
D4R – – +
S I S I S I
75 kD-
50 kD-
receptor signal
D
TX100-soluble (S)
TX100-insoluble (I)
% of total cellular receptor signal
100
80
60
40
20
0
D2R
D4R
E
Gβ1 – + +
D2R – – +
S I S I S I
37 kD-
Gβ1 signal
F
TX100-soluble (S)
TX100-insoluble (I)
% of total cellular Gβ1 signal
100
80
60
40
20
0
Gβ1
D2R + Gβ1

Fig. 2. Coexpression of D2R enhances the expression of Gβ5 but not of Gβ1 and coexpression of MOR does not significantly alter expression of Gβ5.

A. Representative images from a Western blot depicting the relative levels of expression of Gβ5 protein in HEK293 cells transfected with cDNA for the indicated proteins.

B. Quantification of the relative cellular expression levels of Gβ5 after coexpression of MOR or D2R. The Gβ5 protein signal is expressed as a percent of the signal measured in cells expressing Gβ5 alone (mean ± SEM; n = 4, *p<0.05, ANOVA followed by Tukey's post-hoc test, compared to the levels in cells expressing just Gβ5).

C. Representative images from a Western blot depicting the relative levels of expression of Gβ1 protein in HEK293 cells transfected with cDNA for the indicated proteins.

D. Quantification of the relative cellular expression levels of Gβ1 after coexpression of D2R. The Gβ1 protein signal is expressed as a percent of the signal measured in cells expressing Gβ1 alone (mean ± SEM; n = 3, *p<0.05, t-test, compared to the levels in cells expressing just Gβ5).

Figure 2.

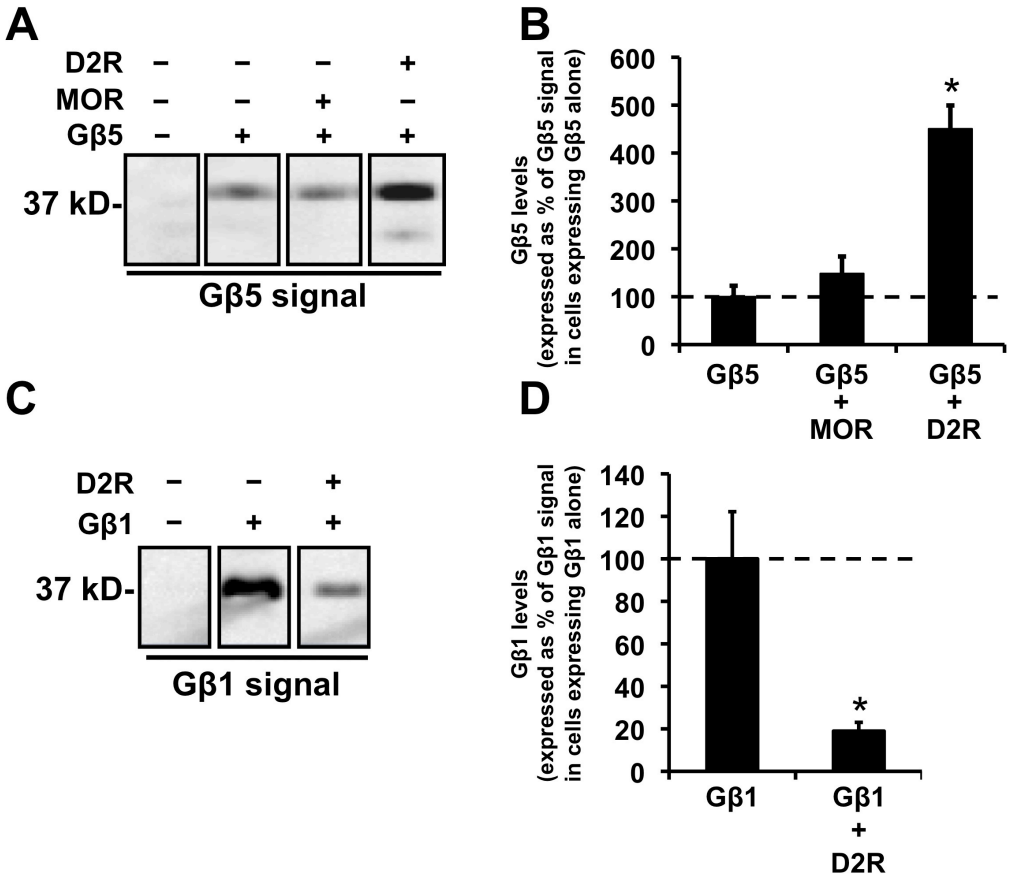

A
D2R – – – +
MOR – – + –
Gβ5 – + + +
37 kD-
Gβ5 signal
B
Gβ5 levels (expressed as % of Gβ5 signal in cells expressing Gβ5 alone)
600
500
400
300
200
100
0
*
Gβ5
Gβ5 + MOR
Gβ5 + D2R
C
D2R – – +
Gβ1 – + +
37 kD-
Gβ1 signal
D
Gβ1 levels (expressed as % of Gβ1 signal in cells expressing Gβ1 alone)
140
120
100
80
60
40
20
0
*
Gβ1
Gβ1 + D2R

Fig. 3. Coexpression of D2R enhances the stability of Gβ5.

A. Representative image of a Western blot which depicts Gβ5 cellular expression levels, from HEK293 cells transiently expressing either Gβ5 alone or Gβ5 coexpressed with D2R, at times, t = 0, 3, or 6 hr after treatment with cycloheximide (100 μM).

B. Quantification of the reduction in cellular Gβ5 levels after treatment of cells with cycloheximide. The Gβ5 levels at times 3 and 6 hr after cycloheximide treatment are expressed as a percentage of the levels of Gβ5 measured in cells that were not treated with cycloheximide (mean ± SEM; n = 4, *$p<0.05$, t-test, comparing to the cells that did not coexpress D2R).

Figure 3.

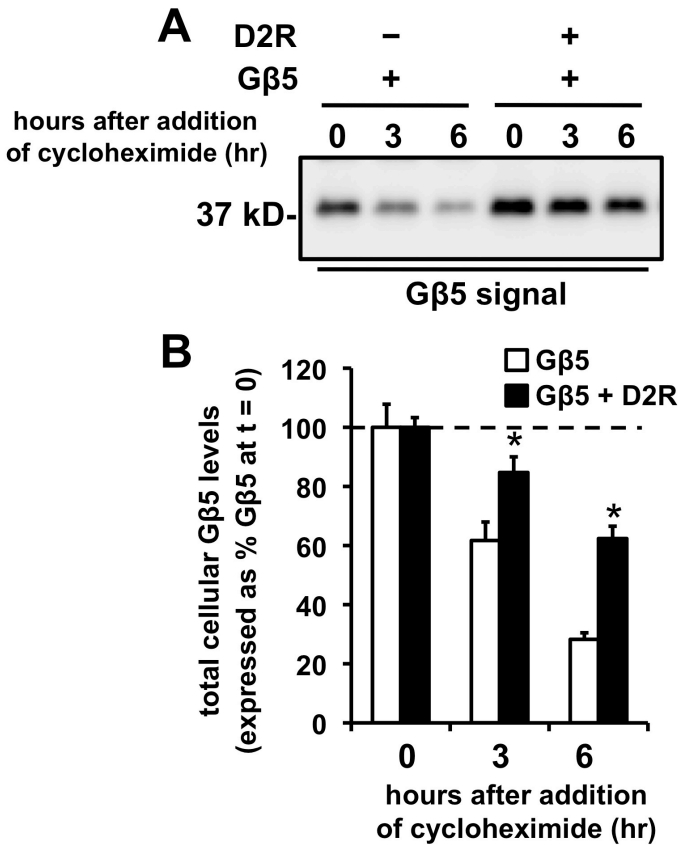

A
D2R
–
+
Gβ5
+
+
hours after addition
of cycloheximide (hr)
0 3 6 0 3 6
37 kD-
Gβ5 signal
B
Gβ5
Gβ5 + D2R
total cellular Gβ5 levels
(expressed as % Gβ5 at t = 0)
120
100
80
60
40
20
0
*
*
0 3 6
hours after addition
of cycloheximide (hr)

Fig. 4. Interactions of Gβ5 with TX100 soluble (S) and insoluble (I) D2R populations as assessed by an in-cell "proximity biotin transfer assay."

A. Schematic of the Gβ5-BL construct, a fusion of Gβ5 with BirA, an *E. coli* derived biotin ligase (BL) enzyme that specifically biotinylates a unique acceptor peptide (AP) sequence (top panel). N and C refer to the N- and C-terminus, respectively, of the fusion protein. Schematic of KRAS-BL, a fusion of the BirA biotin ligase (BL) enzyme with the plasma membrane targeting peptide motif from KRAS (center panel). Schematic of the D2R-AP fusion construct where the AP sequence that is specifically biotinylated by BirA was inserted into a region of the 3rd cytoplasmic loop of FLAG-tagged D2R (bottom panel).

B. Representative image of a Western blot depicting the segregation, into TX100-soluble (S) and insoluble (I) HEK293 cellular fractions, of the D2R-AP construct that was biotinylated by either co-expressed Gβ5-BL or KRAS-BL. The left and center panels represent samples prepared from HEK293 cells transiently coexpressing D2R and Gβ5-BL. Samples in the left panel were from cells not treated with biotin (biotin −). Samples depicted in the center panel were from cells transiently coexpressing D2R and KRAS-BL and samples depicted in the right panel were from cells transiently coexpressing D2R and Gβ-BL. Cells were cultured in biotin-depleted medium and biotinylation of the D2R-AP construct by either Gβ5-BL or KRAS-BL was initiated in intact cells by treatment with 10 μM biotin for 2 min (biotin +). Biotinylated D2R-AP segregating into TX100-soluble (S) and insoluble (I) fractions was detected by probing the blots with streptavidin.

C. Quantification of the percent of the biotinylated D2R-AP segregating into TX100-insoluble (I) and soluble (S) fractions after biotinylation by either KRAS-BL or Gβ5-BL (mean ± SEM; n = 4, *p<0.05, t-test, compared to the segregation after biotinylation by KRAS-BL).

D. Schematic of an explanation for the data presented in **B** and **C**. The majority of the D2R-AP construct is expressed in plasma membrane domains that are TX100-insoluble (black filled-in region of the plasma membrane) but some D2R-AP is also expressed in a TX100-soluble form (white open region of the plasma membrane). Gβ5-BL is able to access, interact with and biotinylate both forms with equal efficacy (lower panel). The interactions of KRAS-BL, on the other hand, are largely restricted to the TX100-soluble form of D2R-AP indicated that it is compartmentalized away from the TX100-insoluble D2R-AP (upper panel).

Figure 4.

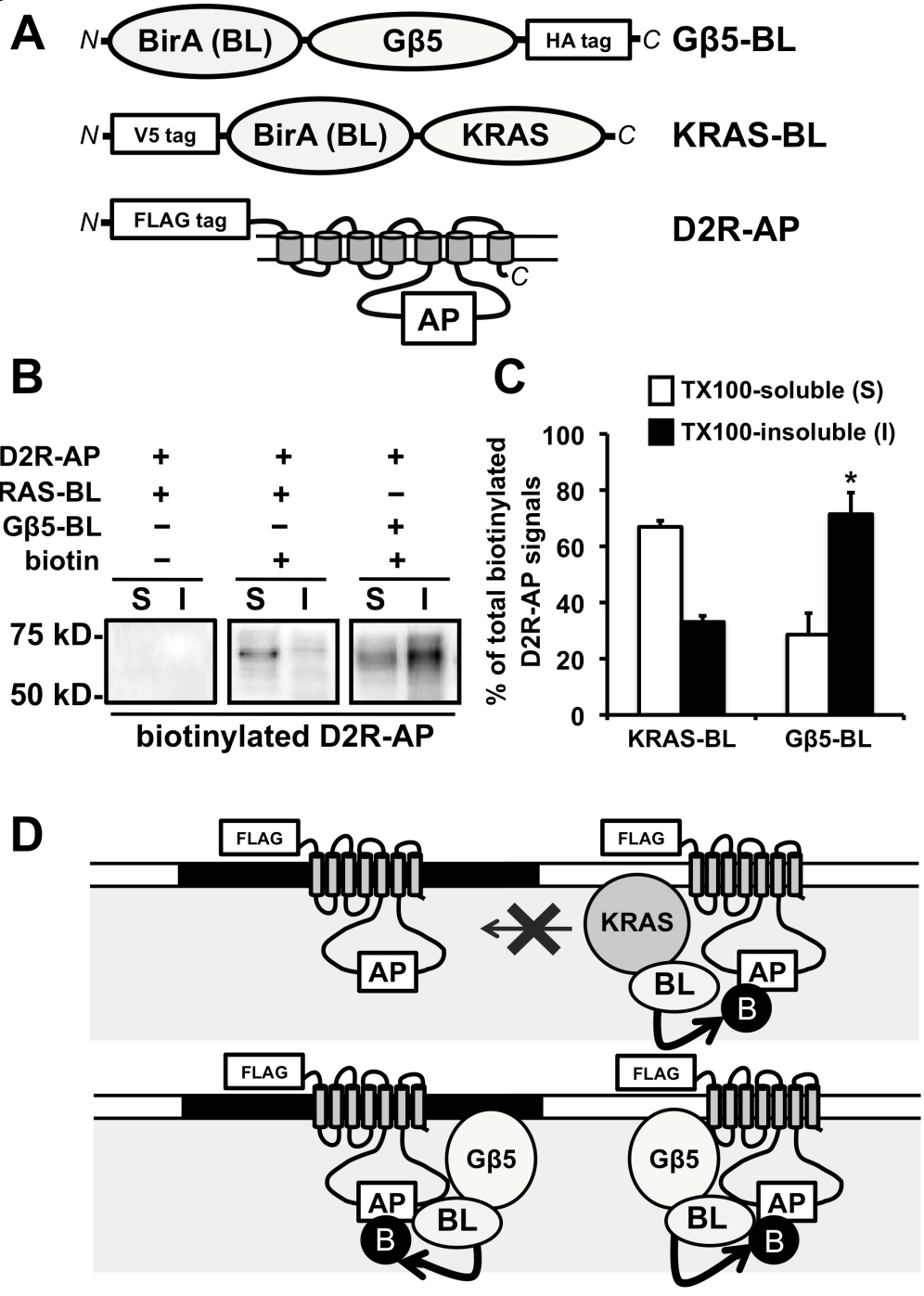

A
N
BirA (BL)
Gβ5
HA tag
C
Gβ5-BL
N
V5 tag
BirA (BL)
KRAS
C
KRAS-BL
N
FLAG tag
C
AP
D2R-AP
B
D2R-AP + + +
KRAS-BL + + –
Gβ5-BL – – +
biotin – + +
S I S I S I
75 kD-
50 kD-
biotinylated D2R-AP
C
TX100-soluble (S)
TX100-insoluble (I)
% of total biotinylated D2R-AP signals
100
80
60
40
20
0
*
KRAS-BL
Gβ5-BL
D
FLAG
AP
KRAS
BL
B
Gβ5

Fig. 5. Effect of Gβ5 on dopamine-mediated activation of D2R-coupled G protein signaling as measured by a fast kinetic BRET assay.

A. Averaged traces (± SEM) of changes in the BRET signal (ΔBRET or the BRET response) over time obtained from HEK293 cells transfected with cDNA for D2R, Gαo, Venus-Gβγ, masGRK3ct-NanoLuc and treated sequentially with dopamine (10 nM) and haloperidol (100 μM). D2R stimulation by dopamine application leads to the dissociation of the G protein heterotrimer into Gβγ-Venus and GTP-bound Gαo subunits. Free Gβγ-Venus interacts with masGRK3ct-NanoLuc to produce a BRET signal. The black trace is from HEK293 cells that did not coexpress Gβ5 and the dark and light grey traces are from HEK293 cells transiently coexpressing two different levels (l, for low and h, for high) of Gβ5 (mean ± SEM; n = 4).

B. Quantification of the amplitude of the BRET signal elicited by the application of sub-saturating dopamine concentration (10 nM) in the cells described above.

C. Quantification of the deactivation kinetics of the dopamine-elicited BRET response after application of the D2R antagonist, haloperidol (100 μM).

Figure 5.

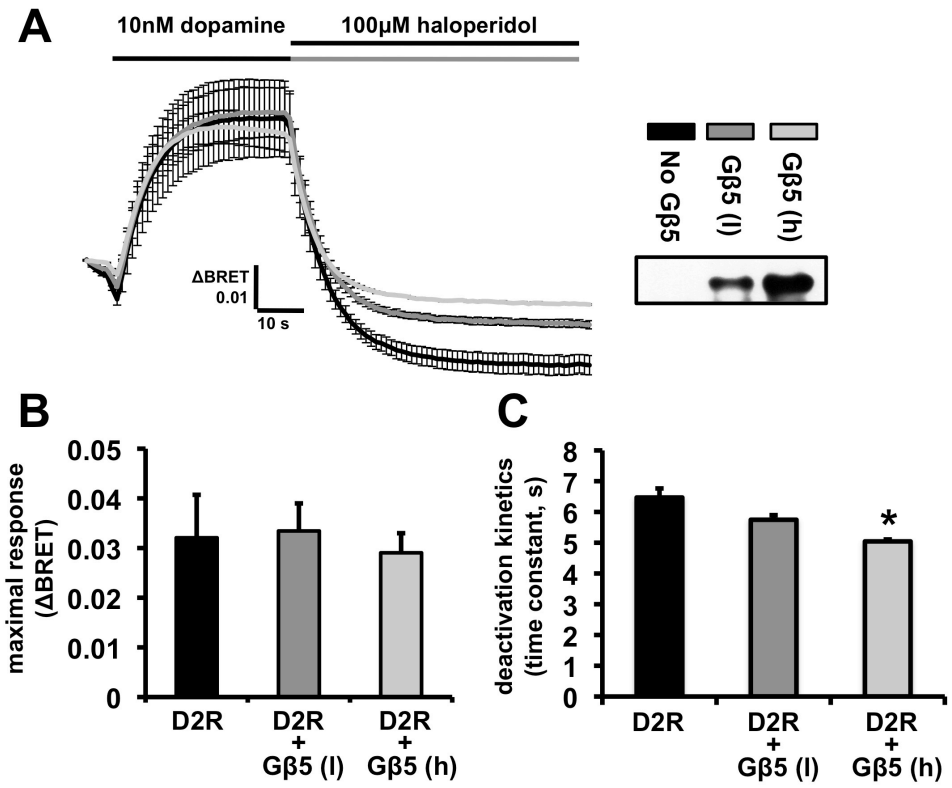

A
10nM dopamine
100μM haloperidol
ΔBRET
0.01
10 s
No Gβ5
Gβ5 (l)
Gβ5 (h)
B
maximal response (ΔBRET)
0.05
0.04
0.03
0.02
0.01
0
D2R
D2R + Gβ5 (l)
D2R + Gβ5 (h)
C
deactivation kinetics (time constant, s)
8
7
6
5
4
3
2
1
0
*
D2R
D2R + Gβ5 (l)
D2R + Gβ5 (h)

Fig. 6. Effects of Gβ5 and Gβ1 coexpression on levels of cell surface D2R and MOR and on agonist-induced receptor internalization.

A. Quantification of the relative levels of cell surface D2R in HEK293 cells transiently transfected with a fixed amount of D2R cDNA and with cDNA for either Gβ1 or Gβ5. The cell surface D2R signal is expressed as a percent of the signal measured in cells transfected with the only the fixed amount of D2R cDNA. The levels of D2R specifically at the cell surface was evaluated by probing intact, non-permeabilized cells with anti-FLAG antibody targeting the D2R-fused extracellular N-terminal FLAG tag (mean ± SEM; n = 8-16, *p<0.01, Tukey's post-hoc test, compared to cells expressing D2R alone).

B. Quantification of the relative levels of cell surface MOR in HEK293 cells transiently transfected with a fixed amount of MOR cDNA and with cDNA for Gβ5. The cell surface MOR is expressed as a percent of the signal measured in cells transfected with only the fixed amount of MOR cDNA. The levels of MOR specifically at the cell surface were evaluated by probing intact, non-permeabilized cells with anti-FLAG antibody targeting the MOR-fused extracellular N-terminal FLAG tag (mean ± SEM; n = 11-12).

C. Quantification of the relative levels of transiently expressed cell surface D2R in HEK293 cells expressing D2R alone, D2R and Gβ1 or D2R and Gβ5, after treatment of with dopamine (50 μM for 45 min). The cell surface D2R signal is expressed as a percent of the signal measured in corresponding cells that were not treated with dopamine (mean ± SEM; n = 8, *p<0.01, t-test, compared to cell surface D2R signal from corresponding cells not treated with dopamine).

D. Quantification of the relative levels of transiently expressed cell surface MOR in HEK293 cells expressing MOR alone and MOR with Gβ5, after treatment of DAMGO (50 μM for 45 min). The cell surface MOR signal is expressed as a percent of the signal measured in corresponding cells that were not treated with DAMGO (mean ± SEM; n = 11-12, *$p<0.001$, t-test, compared to cell surface MOR signal from corresponding cells not treated with DAMGO).

Figure 6.

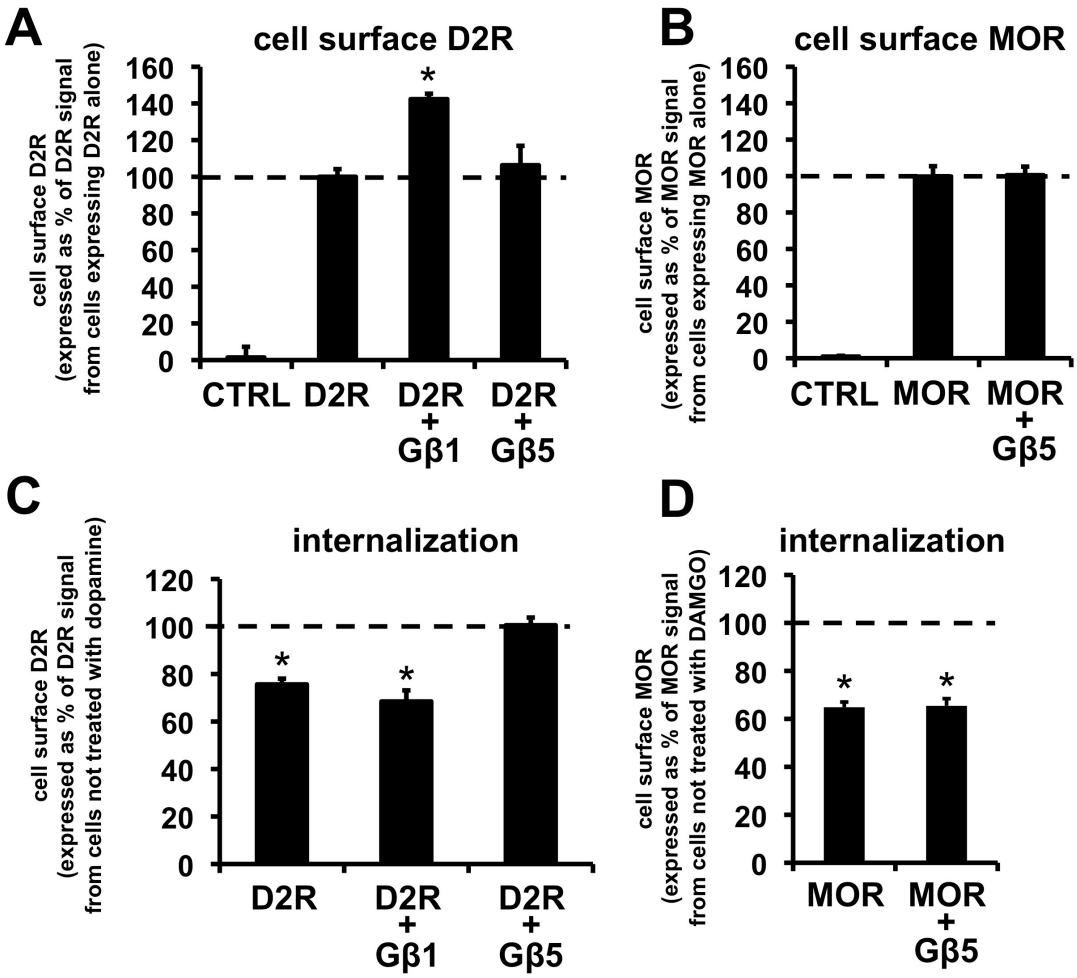

A
cell surface D2R
cell surface D2R (expressed as % of D2R signal from cells expressing D2R alone)
160
140
120
100
80
60
40
20
0
*
CTRL
D2R
D2R + Gβ1
D2R + Gβ5
B
cell surface MOR
cell surface MOR (expressed as % of MOR signal from cells expressing MOR alone)
160
140
120
100
80
60
40
20
0
CTRL
MOR
MOR + Gβ5
C
internalization
cell surface D2R (expressed as % of D2R signal from cells not treated with dopamine)
120
100
80
60
40
20
0
*
*
D2R
D2R + Gβ1
D2R + Gβ5
D
internalization
cell surface MOR (expressed as % of MOR signal from cells not treated with DAMGO)
120
100
80
60
40
20
0
*
*
MOR
MOR + Gβ5

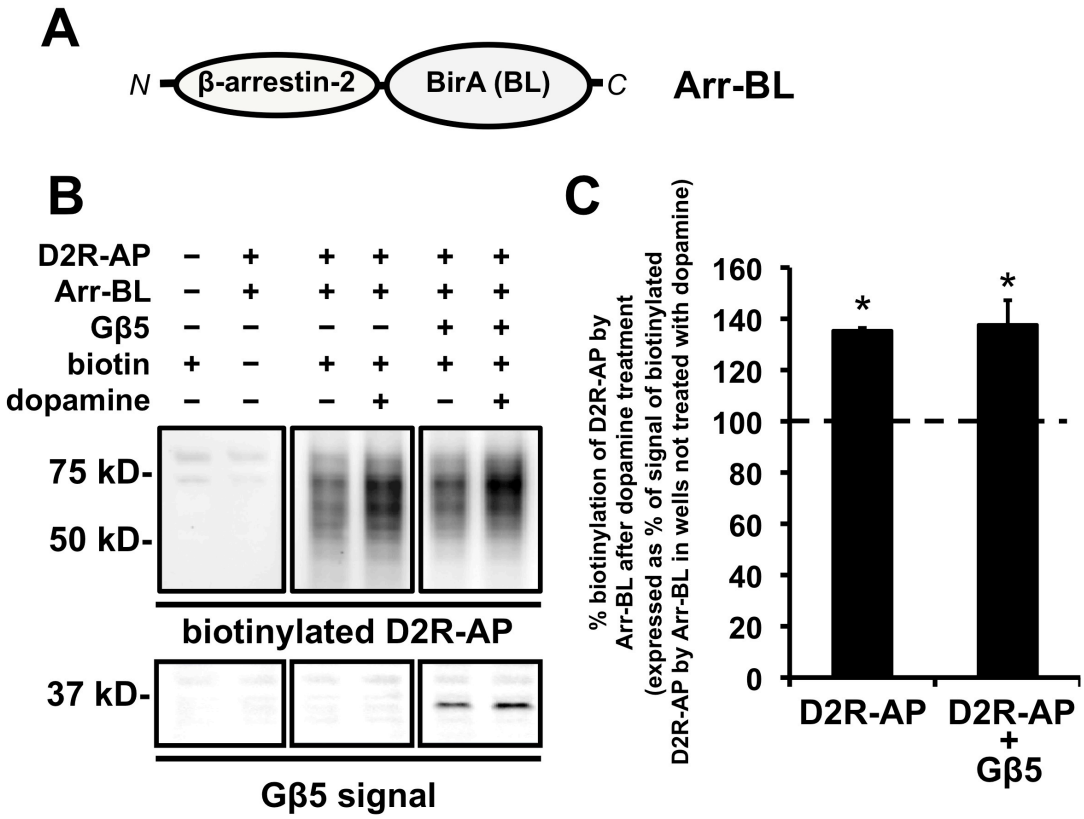
Fig. 7. Effect of Gβ5 coexpression on arrestin recruitment to D2R-AP upon dopamine treatment as assessed by an in-cell "proximity biotin transfer assay."

A. Schematic of the Arr-BL construct, a fusion of arrestin and BirA, an *E. coli* derived biotin ligase (BL) enzyme that specifically biotinylates a unique acceptor peptide (AP) sequence. N and C refer to the N- and C-terminus, respectively, of the fusion protein.

B. Representative images from a Western blot depicting total cellular biotinylated D2R-AP by coexpressed Arr-BL (top panels) and coexpressed Gβ5 (bottom panels). The top left panel represents samples prepared from cells which were untransfected with either D2R-AP (−) or Arr-BL (−) and treated with 10 μM biotin for 2 min (biotin +, left) or samples that were transfected with both D2R-AP (+) and Arr-BL (+) and not treated with biotin (biotin −, right). The top center panel represents samples prepared from cells that were transfected with both D2R-AP (+) and Arr-BL (+) and treated with vehicle (dopamine −, left) or 10 μM dopamine for 30 minutes (dopamine +, right) and treated with biotin. The top right panel represents samples prepared from cells that were transfected with both D2R-AP (+), Arr-BL (+), and Gβ5 (+) and treated with vehicle (dopamine −, left) or 10 μM dopamine for 30 minutes (dopamine +, right) and treated with biotin (+). D2R-AP biotinylated by Arr-BL was detected by probing the blots with streptavidin. The bottom panels represent corresponding western blots from samples in the upper panel probed for Gβ5.

C. Quantification of the relative levels of D2R-AP biotinylated by Arr-BL in response to dopamine treatment (10 μM for 30 min) in cells expressing only D2R-AP and Arr-BL or cells expressing D2R-AP, Arr-BL, and Gβ5. The biotinylated D2R-AP signal is expressed as a percentage of biotinylated D2R-AP signal from cells that were not treated with dopamine (mean ± SEM; n = 4, *$p<0.05$, t-test, compared to biotinylated D2R-AP signal from corresponding cells not treated with dopamine).

Figure 7.


A
N
β-arrestin-2
BirA (BL)
C
Arr-BL
B
D2R-AP
Arr-BL
Gβ5
biotin
dopamine
75 kD-
50 kD-
biotinylated D2R-AP
37 kD-
Gβ5 signal
C
% biotinylation of D2R-AP by Arr-BL after dopamine treatment (expressed as % of signal of biotinylated D2R-AP by Arr-BL in wells not treated with dopamine)
160
140
120
100
80
60
40
20
0
*
*
D2R-AP
D2R-AP + Gβ5

MANUSCRIPT II

Effect of antipsychotic drugs on plasma membrane compartmentalization of D2-dopamine receptors

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(prepared for submission to the Journal of Biological Chemistry)

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ABSTRACT

It is thought that all available antipsychotic drugs can reduce psychotic symptoms by blocking D2R at therapeutic concentrations; however it is unclear how the blockade of D2R is sufficient to produce these effects. Previously we have demonstrated that D2R exists predominantly in a detergent insoluble and compartmentalized biochemical fraction and that insoluble D2R at the plasma membrane is internalized in response to dopamine. To determine if antipsychotics produced changes to the receptor's compartmentalization characteristics we examined the effects of numerous antipsychotic drugs on the biochemical properties of D2R in vitro. We found that all antipsychotics tested significantly enhanced the surface localization, as well as enhancing the solubility of D2R, without producing changes on the overall expression of the receptor in our system. The one drug that did not produce changes in the solubility of D2R was clozapine, an antipsychotic agent thought to be particularly effective in treatment-resistant schizophrenia. We found that these changes in surface receptor expression levels resulted in enhanced accessibility of D2R, as measured by a proximity dependent, biotin-transfer assay, in both a dose and time-dependent fashion. Among the drugs we tested, haloperidol uniquely enhanced the accessibility of detergent insoluble D2R and this effect was blocked by saturating concentrations of clozapine.

Keywords: Antipsychotics, Detergent resistant membrane compartments, G protein coupled receptor, D2-dopamine receptor, Plasma membrane compartmentalization, Mechanism of action

Abbreviations: ANOVA, analysis of variance; AP, attenuated acceptor peptide with the sequence GLNDIFEAQKIE; βMCD, β-methylcyclodextrin; cDNA, complementary DNA; DRM, detergent resistant membrane; EPS, extrapyramidal symptoms; GPCR, G-protein coupled receptor; HEK293, human embryonic kidney cells stably expressing the SV40 T-antigen; HRP, horseradish peroxidase; KRAS-BL, BirA biotin-ligase fusion construct with the membrane-targeting domain with the sequence KKKKKKSKTKCVIM from the protein KRAS; PET, positron emission tomography; TCA, trichloroacetic acid; TX100, Triton X-100; V5, V5 epitope tag with the amino acid sequence GKPIPNPLLGLDST;

INTRODUCTION

Schizophrenia is an intractable and debilitating chronic mental disorder known to affect approximately 1% of the population. Antagonists of the D2-like family of dopamine receptors (antipsychotics) are used in the treatment of the hallucinations and delusions that are the so-called the “positive” symptoms of schizophrenia. Specifically the D2 dopamine receptor (D2R) is a clinically important G coupled receptor (GPCR), as it serves as the major target of drugs used to treat a variety of mental disorders such as Parkinson’s disease, depression and schizophrenia (1, 2).

A common property of all available antipsychotic drugs is that they specifically block the D2 dopamine receptor (D2R) at therapeutic concentrations (3). Also, it has been demonstrated that the clinical potency of the first generation or “typical” antipsychotics is directly correlated with their relative affinities for D2-like dopamine receptors (Hartman, 1996). From these data it is evident that antipsychotic drugs necessarily block D2R to produce reduction of psychotic symptoms, however it is still unclear how antipsychotic induced blockade of D2R is sufficient to produce this effect.

In this report we demonstrate that all antipsychotics tested in this study can decrease the segregation of D2R into detergent resistant membrane structures (DRMs) and enhance in the accessibility of DRM localized D2R to other cellular proteins, as measured by a novel biotinylation assay. It is this consistent alteration between antipsychotics that represents a model for antipsychotic drug actions and a potential target for the development of antipsychotic drugs that are

more directly effective in accomplishing a complete reduction of positive schizophrenic symptoms.

In several large, multi-site randomized controlled-trial studies of the effectiveness of antipsychotic drugs it was determined that there existed little or no differences between the newer atypical and conventional typical antipsychotic drugs(16, 17). Unique among these antipsychotic agents, was the drug clozapine that was found to be efficacious for applications in treatment resistant schizophrenia. Fundamental to our understanding of the classical mechanism of antipsychotic drug action is that antipsychotics block of the native ligand dopamine from binding to D2R that leads to the prevention of downstream signaling. However, with notable few exceptions, complete occupancy of D2R, by antipsychotics in the brain is sufficient to produce serious extrapyramidal side (EPS) effects and therefore demonstrates the need to titrate doses accordingly to produce a consistent reduction in schizophrenic symptoms (7). Interestingly even when sufficiently high doses of the atypical agent quetiapine were used to produce clinically significant reduction of schizophrenic symptoms, receptor occupancy was significantly lower than the “therapeutic window” (8). It therefore seems unlikely that simple measures of receptor occupancy are conclusive in determining antipsychotic efficacy.

The difference in effectiveness of suppression of psychotic symptoms in schizophrenia seen with the newer atypical antipsychotics might be due to their decreased binding affinity of D2R (9, 10). Differences between the binding affinities of atypical and typical antipsychotics are almost entirely (~99%) due to

the relative differences in the dissociation constant. The enhanced dissociation of antipsychotic from receptor is proposed to enable endogenous dopamine to continue to signal via D2Rs, despite receptor blockade, and may be involved in the reduction of extrapyramidal symptoms in the atypical antipsychotics. Yet, the difference between the relative binding affinities of typical and atypical drugs does not explain the unique effectiveness of clozapine in producing a reduction in treatment-resistant schizophrenic symptoms.

Previous studies on the antipsychotic interaction with D2R have measured ligand-binding properties using receptor radioactive assay or PET imaging (7, 8, 9). Although these types of studies can describe quantitative changes in receptor-ligand affinities, these techniques do not specifically describe the underlying molecular consequences of the interaction between antagonists and D2R on the ability of the receptor to signal. Most notably, neither of these methods can ask how different biochemical properties of specific pools of D2R within the same cell can contribute to signaling.

We have previously demonstrated that compared to other GPCRs, D2 dopamine receptors are preferentially localized in detergent resistant membrane structures (DRMs) in the plasma membrane and coexpression of D2R can alter the detergent solubility of important members of the D2 signaling cascade such as RGS9-2 (11). Additionally we have identified that the compartmentalization of D2Rs within these detergent resistant plasma membrane structures functionally limits the accessibility of D2R to other plasma membrane associated proteins (13). Together these discoveries suggest that not only does the majority of

cellular exist in a membrane fraction that is resistant to detergent solubilization, but also that this fraction specifically allows for the compartmentalization of D2R and its associated signal transducers. Therefore, drugs that are able to alter the relative compartmentalization of D2R into or out of DRMs could potentially alter its signaling characteristics.

Here we demonstrate a unique effect of antipsychotics on not only the distribution of D2Rs between detergent resistant membranes and detergent soluble partitions, but also a change in the potential signaling characteristics of both fractions of the receptor. Initially we show that D2Rs enhance the relative amount of D2R found in the detergent soluble fraction. Additionally, we used a previously described biotin ligase mediated in-cell biotin transfer assay, to demonstrate that antipsychotic enhances the accessibility of D2R in both detergent soluble and insoluble fractions to membrane bound proteins. Furthermore, by correcting for the relative protein found within each membrane fraction we found that haloperidol preferentially enhanced the efficacy of biotinylation of detergent insoluble targeted D2Rs. Critically these effects represent novel effects of drug on receptors and their function and therefore add a significant dimension to the understanding of antipsychotic drugs mechanisms of action at D2R.

MATERIALS AND METHODS

Chemicals

Ziprasidone hydrochloride monohydrate, olanzapine, quetiapine hemifumarate salt, fluphenazine dihydrochloride, resperidone, (S)-(-) sulpiride, and droperidol

were acquired from Sigma- Aldrich (St. Louis, MO, USA). Haloperidol and clozapine were acquired from MP Biomedicals LLC (Solon, OH, USA). Aripiprazole was acquired from AK Scientific (Union City, CA, USA). All other chemicals and reagents were purchased from Fisher Scientific (Pittsburg, PA, USA) or from other suppliers specifically identified below.

Cell Culture and Transfection

HEK293 cells(American Type Culture Collection, Manassas, VA, USA) were maintained and transfected in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (company) plus penicillin/streptomycin. The cells were grown at 370C and 5%CO2. Transfection of HEK293T cells was carried out using Lipofectamine LTX(Invitrogen) according to manufacturer's instructions. Total transfected DNA was kept constant between groups using the empty vector pcDNA 3.1+/zeo (Invitrogen)

cDNA Constructs

All plasmid constructs utilized below were designed and made using standard techniques in molecular biology. The N-terminal FLAG-tagged version of the human D2-dopamine receptor (D2R) (12), the FLAG-tagged D2R construct with the biotin ligase acceptor peptide insertion into the 3rd cytoplasmic loop (D2R-AP), the BirA biotin ligase fusion construct with the plasma membrane targeting domain of Lyn kinase (LYN-BL), and the BirA biotin-ligase fusion construct with the membrane targeting domain of KRAS (KRAS-BL) (13) have previously been described. The D2R-AP construct consists of the FLAG-tagged D2R into which an attenuated acceptor peptide sequence (GLNDIFEAQKIE) is

inserted between amino acids at position 305 and 306 in the 3rd cytoplasmic loop. LYN-BL consisted of the following fused peptide sequences, in order, from the N to the C-terminus: a membrane targeting peptide sequence (MGCIKSKRKDNLNDDE) from Lyn kinase, the GSGSG linker, BirA and the V5 epitope-tag. KRAS-BL consisted of the following peptide sequences in order from the N to the C-terminus: the V5 epitope-tag, BirA biotin ligase enzyme (BL), a GSGSG linker and a membrane targeting peptide sequence (KKKKKKSKTKCVIM) from the protein KRAS.

TX100 Biochemical Fractionation of Proteins

The method for the Triton X-100 (TX100) biochemical fractionation of proteins has been adapted from previous publications (11, 13). Briefly, 48 hr post transfection cells were lysed in phosphate-buffered saline (PBS, in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 2 KH_2PO_4, pH 7.4) containing 2% v/v of the non-ionic detergent, TX100 and a 1× concentration of SigmaFast Protease inhibitor (Sigma-Aldrich) for 1 hr at 4 ºC. The samples were centrifuged (10,000 g, 10 min at 4 ºC) to pellet the insoluble proteins. Supernatant proteins (i.e. the TX100-soluble fraction) were precipitated by the addition of trichloroacetic acid (TCA, final concentration 10% v/v). Supernatant proteins were washed 3× with ice-cold 95% v/v acetone (4 °C). Both the TX100-soluble and the insoluble proteins were re-suspended in equal volumes of SDS sample buffer (2% w/v SDS, 0.01% w/v bromophenol blue, 8 M urea, 20 mM dithiothreitol, 50 mM Tris-HCl, pH 6.8). Samples were sonicated, on ice, 25× for approximately 0.5 seconds at power setting of 10 for ~0.5 s to reduce

sample viscosity prior to loading using a sonicator (XL-2000, qSonica). Equal volumes of the samples were then resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the relative levels of protein expression were then compared by Western blotting.

In-Cell Biotin Transfer Assay

We utilized an in cell biotin transfer assay to detect whether or not KRAS-BL interacted with detergent soluble or insoluble forms of D2R. On day one approximately 2.5×10^5 cells were transfected in a 2:1 ratio with plasmid DNA containing KRAS-BL and D2R-AP Lipofectamine transfection reagent (Invitrogen) according to manufacturer's instructions, in biotin free media. 24 hours post transfection cells were incubated with an appropriate concentration of an antipsychotic drug. At 48 hours post-transfection or 24 hours post drug application, cells were treated with a 40 μM biotin pulse for 5 minutes and placed back into the incubator. Cells were then harvested from the plate using a micropipette and washed 3 times with cold 1x PBS. Cell samples were solubilized in ice-cold phosphate buffered saline containing 2% TX-100 and protease inhibitors for an hour. After an hour samples were centrifuged at 10,000 g for 10 minutes. Supernatant was decanted by micropipette into a separate tube and precipitated by the addition of 10% Trichloroacetic acid. Soluble and insoluble proteins were then harvested using the method as described in the "Triton X-100 Biochemical Fractionation of Proteins" section.

Samples were then resolved by SDS PAGE. The proteins were then transferred to methanol-wetted polyvinylidene fluoride (PVDF) membranes and probed with

streptavidin conjugated to horseradish peroxidase (HRP). These procedures and reagents are described in the “Protein Immunoblotting” section below.

Detection of Total Surface Expression of D2R-AP

To determine the effect of antipsychotic drugs on receptor surface expression we used an ELISA-based assay to determine the amount of receptor present at the plasma membrane. Day 1, 5 × 104 HEK293 cells were transfected with appropriate cDNA plasmids containing D2R-AP in a 96-well plate. 36 hours post-transfection cells were treated with a saturating concentration (10 μM) of the indicated antipsychotic drug for 24 hours. The media was then aspirated, and cells were gently washed 3 times with cold (4 °C) PBS. Cells were then fixed with 4% v/v formaldehyde in PBS, and then washed 3 times with PBS. Wells were blocked for 30 minutes with 5% nonfat milk dissolved in PBS. Surface receptor was then probed for using HRP-conjugated mouse monoclonal anti-FLAG M2 antibody (1:5,000 dilution in 5% nonfat milk in PBS, Sigma) for 1 hour at 37 °C and then washed 3 × with PBS. Supersignal West Femto chemiluminescent substrate (Pierce-Thermo Fisher Scientific) was then applied to each well and signals were detected and quantified using a multi-well plate compatible luminometer (Glomax) and attached computer.

Indirect Detection of TX100 Soluble and Insoluble Cell Surface D2R-AP

HEK293T cells were transiently transfected with cDNA for FLAG tagged D2R-AP or with the empty vector. The intact cells were then incubated in cell-culture medium with antibody (Sigma Aldrich; clone M2, catalog no. F1804, 1 : 500 dilution) directed against the extracellular FLAG-tag for 1 h at 37°C to label the

respective surface receptors. The cells were then washed three times in ice-cold PBS (5 min, 4°C, incubation in PBS for each wash). TX100-soluble and -insoluble proteins were isolated, resolved on SDS–PAGE and western-blotted as described below. The anti-FLAG antibody light chain that labeled the surface receptors and co-fractionated into the TX100-soluble and -insoluble compartments was visualized by detection using a anti-murine HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories).

Protein Immunoblotting

Protein samples, prepared as described above, were resolved by SDS-PAGE and transferred, using wet electrophoretic elution buffer (25mM Tris-base, 192mM glycine, 20% v/v methanol, 0.1% w/v SDS, pH~8.3), and subsequently transferred onto methanol-wetted polyvinylidene fluoride (PVDF) membranes. After blocking remaining protein-binding sites with 5% w/v nonfat milk powder in PBS, the proteins of interest were detected by probing the blots sequentially with the appropriate primary and HRP conjugated secondary antibodies, diluted in 5% w/v nonfat milk and PBS. For Streptavidin blotting, the membrane was incubated with streptavidin-HRP (Sigma, 1:5000 in 3% BSA, PBS) for 1 hr, followed by four 10 min washes in PBS. For anti-V5 blotting, the membrane was incubated with Anti-V5 HRP (Invitrogen, 1:5000 in 3% BSA, PBS) for 1hour, then washed by four 10 min washes in PBS. In order to detect Flag-D2R or D2R-AP signal , the membrane was incubated with anti-FLAG antibody (Sigma-Aldrich) for 1 hr, followed by three 10 min washing with PBS. For the detection of total cellular protein segregating into TX100 soluble and insoluble biochemical fractions, after

HRP-based detection, PVDF membranes were incubated in Coomassie stain (40% methanol, 1% acetic acid, 0.1% brilliant blue R-250 dye) and washed 3× for 10 minutes each with Coomassie wash buffer (50% methanol, 1% acetic acid). For HRP conjugated antibodies, blots were developed with Supersignal West Femto substrate (Pierce), and images were taken on the Chemiluminescence setting on Bio Rad Gel Doc XRS. The intensity of each band was quantified using Image J software. Images were taken using exposure settings that did not saturate any of the charge-coupled device camera pixels.

Data and Statistical Analysis

Signals from the target protein bands were quantified using the free image processing and analysis software ImageJ (National Institutes of Health, Bethesda, MD, http://rsbweb.nih.gov/ij/). Statistical analyses were performed using Microsoft Excel or GraphPad Prism 4 software (GraphPad Software, Inc.). The signals resulting from detergent-soluble and insoluble preparations of a protein, respectively, were expressed as a fraction of the total signal per sample of cells or in cases specifically indicated as a fraction of vehicle in the corresponding (T100 soluble or insoluble) sample. When testing the significance of means for more than 2 experimental groups, one-way ANOVA was used to first determine group statistical significance and only followed by either Dunnett's post-hoc test, for determination of mean difference from vehicle treated controls or Tukey's post-hoc analysis if comparing between multiple different treatment conditions. Post-hoc analyses were performed only if the results of the initial

ANOVA were determined to be significant ($p<0.05$).

RESULTS

All antipsychotic drugs tested enhanced the detergent solubility of total cellular D2R-AP without enhancing expression of D2R-AP, with the exception of clozapine.

Previously we have shown that D2R expressed in the brain or exogenously in HEK293 cells exists predominantly within a fraction of the plasma membrane that is insoluble in nonionic detergent and D2R retargets proteins to these biochemical fractions (11, 13). Furthermore we have demonstrated that these insoluble biochemical fractions respond to dopamine treatment by reducing available cell surface D2R within this fraction, likely through internalization (13). Therefore we asked whether or not alternative D2R binding ligands produce changes in the accessibility of either (soluble or insoluble) pool of D2R.

24 hour, saturating concentrations of antipsychotics appeared to specifically enhance the pool of soluble D2R-AP (Fig. 1B). The exception to this statement was the antipsychotic drug, clozapine, which showed no difference from vehicle treated cells, in terms of solubility of all cellular D2R-AP. Furthermore, antipsychotics did not uniformly enhance the expression of total D2R-AP under these treatment conditions (Fig. 1C). Rather, it appeared that two drugs, resperidone and aripiprazole, decreased the overall expression of the D2R-AP construct.

We have previously demonstrated that the insoluble pool of receptor is functionally segregated at the plasma membrane (13). Therefore it may be that

antipsychotics that enhance the soluble fraction of cellular D2R thereby disrupt targeting to insoluble biochemical fraction. It is likely that through an alteration in the targeting of D2R to different compartments in the plasma membrane alters the ability of D2R to signal once it has reached this compartment.

Antipsychotic drugs enhanced surface translocation of D2R-AP as measured by modified ELISA assay

To compare the relative abilities of these antipsychotics to enhance the surface receptor concentrations we transiently expressed only D2R-AP in 96-well plates and treated these cells with a 10μM concentration of antipsychotic for 24 hours. We found that after this treatment, total surface receptors were significantly increased from 1.7 to approximately 5 fold after the drug treatment (Fig. 2).

Haloperidol and clozapine enhance surface translocation of D2R-AP, but do not increase detergent solubility of surface D2R-AP.

In order to examine whether or not the effect of antipsychotics on the solubility of total cellular D2R-AP was also conferred to surface receptors we examined the solubility of surface receptors after antipsychotic treatment using an indirect detection method summarized in figure 3A. Although haloperidol and clozapine both robustly enhanced the total amounts of cell surface receptors (Fig. 3D), we found that the relative solubility of the surface receptors was unchanged compared to vehicle treated cells (Fig. 3C). A summary of this effect is provided in figure 9A.

Certain antipsychotic drugs enhance the accessibility of D2R-AP to interactions occurring at the plasma membrane.

To assess the potential for changes in the cell signaling of D2R upon antipsychotic drug treatment we used an in-cell, proximity-dependent, biotin transfer assay that involves the *E. coli* biotin ligase (BL). Biotinylation of D2R-AP, which occurs within 5 minutes prior to cell lysis, is further evidence of an interaction that has occurred in living cells (14, 15).

To assess if antipsychotics could disrupt the compartmentalization of D2R-AP at the plasma membrane, we used an in-cell proximity dependent biotinylation, a diagram of the proximity dependent biotinylation assay is provided in figure 4. The effect of antipsychotics on the accessibility of cell surface D2R is likely due to the increased receptors levels that were observed in figure 2.

Several antipsychotics enhance the accessibility of cell surface D2R-AP as measured by in-cell proximity dependent biotinylation assay, however haloperidol most robustly enhances accessibility of insoluble pools of receptor (fig. 5B).

Haloperidol treatment alters the biochemical fractionation of biotinylated D2R-AP, by enhancing soluble fraction of biotinylated D2R-AP (Fig. 5C). Additionally, haloperidol most robustly enhanced insoluble biotinylated D2R-AP (Fig 5D) and robustly enhanced the biotinylation of D2R-AP only after 4 hours of treatment (Fig 7B). However, there was no effect observed in the biotinylation of soluble D2R-AP at 4 hours (Fig. 7C).

Biotinylation in the insoluble pool of receptor increased more dramatically with haloperidol treatment, approximately 8 fold, than those of soluble receptors, which differed from vehicle by only ~3 fold (Fig. 6C and D).

Haloperidol's effect on insoluble D2R-AP compartmentalization is blocked by the drug clozapine.

To determine if the effect of haloperidol was specific to the interaction of haloperidol with the receptor and not through a non-specific interaction, we treated D2R-AP expressing cells with a 1μM concentration of haloperidol and found an increase in biotinylation of D2R-AP in both soluble and insoluble fractions (Fig. 8B) which was consistent with our previously observed results in figures 6C and D. A similar strategy was originally used in the discover of D2-dopamine receptors, which used other antipsychotic drugs could block tritiated haloperidol from interaction with brain tissue (16, 17). A diagrammatic representation of the results obtained of decreased insoluble D2R-AP compartmentalization is provided in figure 9B.

DISCUSSION

Unique responses of plasma membrane and intracellular D2R to antipsychotic treatment

Understanding the way D2R functions under antipsychotic blockade is crucial to the future development of safer and more effective of antipsychotic drugs. Generally, greater than 50% of receptors are bound by antipsychotic drugs for therapeutically effective concentration of these agents (18). Because of a wide

difference in the relative affinity of antipsychotics for D2R (e.g. approximately 225 fold difference in binding constant of droperidol relative to quetiapine) we used saturating (10μM) concentrations of all antipsychotics to study their function on the receptor (30).

Previously we demonstrated that both detergent insoluble form and soluble forms of D2R exist within the plasma membrane (13). Insoluble D2R has recently been shown to exhibit the property of plasma membrane compartmentalization in HEK293 cells (13). Whereas, the detergent soluble form of D2R is likely to originate from a fluid region of the cell membrane that does not functionally restrict the access of D2R to other signaling molecules (19). We found that 1) all antipsychotics enhanced the accessibility of cell surface receptors and 2) the majority of antipsychotics enhanced the solubility of total cellular receptors.

Importantly, the total solubility of D2R is not representative of surface D2R solubility, as evidenced by the observation that when we examined the solubility of cell surface receptors, we found that haloperidol did not enhance the fraction of soluble receptors compared to vehicle. It is possible that D2R exhibits undiscovered intracellular roles in addition to the signaling, as there are significant pools of intracellular D2R in striatal neurons and in transfected cell lines (20).

Previously it has been shown that alternative signaling via arrestin may occur in endosomal targeted pools of adrenoreceptors (21). Furthermore, internalization processes mediated through arrestin may activate a number of non-G-protein mediated signal transduction mechanisms that may yet be undescribed (22).

Therefore it is unlikely that the only physiologically important forms of the receptor are those that are targeted to the cellular plasma membrane.

Changes in receptor compartmentalization mediated by antipsychotics is unlikely to be mediated through to lipid-drug interactions.

It is a common assumption that the therapeutic actions of antipsychotic drugs results form the antagonistic activity of the drug in binding and blocking D2R from interacting with dopamine. It has been demonstrated that haloperidol can produce changes in model membranes of lipid bilayers (23). Therefore it was hypothesized that the actions of antipsychotics within biological membranes may be to enhance the fluidity of the cell membrane. Our finding that haloperidol seems to disrupt the compartmentalization of insoluble forms of D2R-AP, seems to fit with this hypothesis.

This hypothesis was recently revisited and demonstrated that antipsychotics can produce different changes in lipid-ordered domains in model membranes, through disruption of proteins within lipid raft structures; there is no evidence that D2R currently exists in such a cholesterol dependent membrane domain (13, 19–21). If disruption of the plasma membrane compartments was simply a function of drug concentration in the bilayer, then we should see an additive effect of antipsychotics in enhancing plasma membrane fluidity, as measured with our proximity biotinylation assay. Our experiments showing that 1μM haloperidol, which exhibits high enhancement of D2R-AP fluidity, can be effectively competed off of it's target by 100μM clozapine, which exhibits significantly lower enhancement of D2R-AP fluidity.

There are chemical agents that have been used to disrupt theoretical plasma membrane compartments, such as β-methylcyclodextrin (βMCD). βMCD is a widely used to chelate cholesterol that are thought to disrupts plasma membrane lipid raft compartments, through sequestration of cholesterol (24). However haloperidol's disruption of insoluble D2R compartments, is unlikely to occur through a non-specific disruption of detergent insoluble pools because 1) surface D2R is not made more soluble upon administration of the antipsychotic drug haloperidol and the 2) effect of increasing biotinylation of soluble D2R-AP is blocked through the co-administration of the drug clozapine.

Altered D2R compartmentalization likely produces changes in receptor signaling

In general, the effect of antipsychotic drugs in increasing the total biotinylation of D2R-AP was likely mediated through a similar process as the total enhancement of cell surface D2R-AP because these two results tended to correlate well. However, we found haloperidol uniquely disrupts the plasma membrane compartmentalization of insoluble D2R-AP, at the cell surface.

Signaling mediated by the D2R-dopamine complex involves the G protein activation that suppresses the formation of cAMP, though $g\alpha_i$-mediated inhibition of adenylate cyclase. As an enzymatic reaction, the rate of activation of G proteins is dependent on both those molecules' concentration and proximity. Spatial restriction limits the local concentration of signaling molecules and can enhance the local cellular concentration of signaling molecules that could be diminished in their global cellular concentration. Therefore, by enhancing the accessibility of insoluble D2R to plasma membrane molecules, the unique

capacity of restricted-access, plasma membrane targeted D2R to limit signaling to certain molecules has been disrupted.

On the other hand, most antipsychotics do not change the accessible fraction of cell surface receptor and this change does not appear to alter the ability of receptors at the cell surface to interact with membrane-targeted molecules. Rather, antipsychotic induced plasma membrane targeted receptors exhibit similar potential signaling properties to receptors that were integrated in the plasma membrane prior to antipsychotic treatment; i.e. there was minimal difference between total receptor interactions normalized per unit of receptor between drugs. Therefore, by virtue of enhanced surface expression and altered solubility, antipsychotic binding may produce actions of D2R on yet undefined pathways and not on classically defined interactions with G proteins and ion channels (25).

Although a number of authors have suggested moieties within D2R that reduce the relative affinity of haloperidol or other antipsychotic drugs for D2R, at the time of publication, no mutation construct has been shown to demonstrate selectivity of drug-receptor interaction (26–29). Furthermore, by significantly disrupting the largely hydrophobic binding pocket of D2R, this process may produce receptors that lack proper folding or membrane targeting.

Antipsychotic actions of clozapine may be related an ability to maintain a large intracellular pool of insoluble D2R.

Although binding of the dopamine D2 receptor has been shown to be directly correlated to the therapeutic action of antipsychotic drugs, it is unclear how

antagonistic blockade of D2R relates to the therapeutic effectiveness (16, 30). We have shown here that antipsychotic drugs all seem to enhance the surface translocation of D2R-AP, through a mechanism independent of total expression of receptor. This result fits with the well-documented ability of antipsychotic agents to antagonize D2R arrestin recruitment and internalization (31–33). However, it appears that most of the antipsychotics we tested also demonstrate an ability to enhance the soluble fraction of D2R, through an undocumented process. The exception to this finding was the drug clozapine, an agent that is uniquely efficacious at reducing the psychotic symptoms of treatment resistant schizophrenia (34).

Previously Seeman et al. demonstrated that antipsychotic drug potencies directly correlated with their relative binding affinities to D2R. Interestingly, clozapine, a drug with lowered incidence of movement disorder side effects also fits this correlation. Therefore it is likely that clozapine mediates its antipsychotic therapeutic action through binding to D2R (16, 17). The reason why certain the atypical antipsychotics possess this lower liability might be due to a rapid dissociation of the drug from the D2R. However recently it has been shown that, at least in electrophysiology experiments with the Xenopus laevis oocyte system, that most antipsychotic do not fully dissociate from their target receptor, even after extended washing (35, 36).

The data we present here implicates the solubility of total cellular receptor as a potential therapeutic target for the development of antipsychotics that exhibit similar properties to clozapine.

Conclusions

D2R is a receptor with numerous physiological functions that is implicated in the pharmacotherapy of a number of diseases, notably Parkinson's disease and schizophrenia. Although it is more than 60 years since the discovery of the first antipsychotic agent, their exact mechanism of action remains to be elucidated. Here we show that antipsychotics can produce changes in the biochemical characteristics of D2R and that these changes are likely mediated through direct binding to D2R. It may be that antipsychotics produce changes in cell signaling through directly blocking the interaction of D2R and dopamine, but the effects of antipsychotics on receptor compartmentalization remain an intriguing possibility for the future development of more efficacious agents. Critical to our understanding of D2R function is the further elucidation of compartmentalized, non-compartmentalized, and intracellular forms of D2R-AP.

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Fig. 1. TX100 solubility of total cellular D2R under various antipsychotic drug treatments.

A. Representative image of a western blot depicting the distribution of D2R-AP protein in TX100-soluble (S) and insoluble (I) biochemical fractions. Fractions correspond to proteins extracted from transiently transfected HEK293 cells, treated with the indicated antipsychotic drug.

B. Quantification of the relative levels of D2R-AP protein segregating into TX100 soluble and insoluble biochemical fractions prepared from transiently transfected HEK293 cells. Bars represent average soluble D2R-AP, expressed as a percentage of total cellular D2R-AP in each sample (mean ± SEM, n= 4-13; † indicates $p<0.01$ significantly different from vehicle soluble D2R-AP; ANOVA followed by Dunnett's post-hoc test).

C. Quantification of the total cellular levels of D2R-AP expressed in transiently transfected HEK293 cells that were treated with the indicated antipsychotics. The total cellular D2R-AP is expressed as a percentage of the D2R-AP specific signal that was measured in corresponding wells that were treated with vehicle (mean ± SEM, n= 4-13; † indicates $p<0.01$ significantly different from vehicle total D2R-AP; ANOVA followed by Dunnett's post-hoc test).

Figure 1

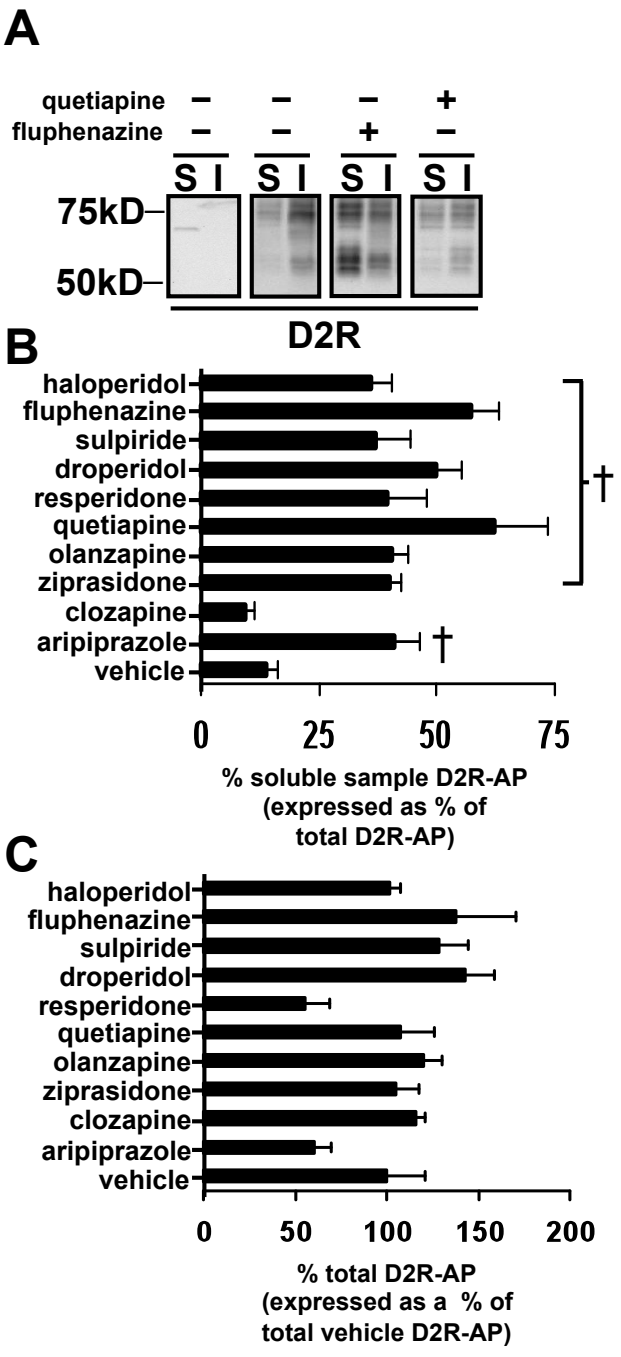

A
quetiapine – – – +
fluphenazine – – + –
S I S I S I S I
75kD
50kD
D2R
B
haloperidol
fluphenazine
sulpiride
droperidol
resperidone
quetiapine
olanzapine
ziprasidone
clozapine
aripiprazole
vehicle
†
†
0 25 50 75
% soluble sample D2R-AP
(expressed as % of
total D2R-AP)
C
haloperidol
fluphenazine
sulpiride
droperidol
resperidone
quetiapine
olanzapine
ziprasidone
clozapine
aripiprazole
vehicle
0 50 100 150 200
% total D2R-AP
(expressed as a % of
total vehicle D2R-AP)

Fig. 2. Antipsychotic drug treatment enhances total surface expression of D2R as measured by modified ELISA.

HEK293 cells were transiently transfected with plasmid containing the cDNA for D2R. 36 hours post-transfection cells were treated with a10μM concentration of the antipsychotic drug indicated. 24 hours post-drug treatment cells were then fixed and cell surface receptors were detected using an HRP-conjugated antibody directed against a FLAG epitope at the N-terminus of D2R. Bars represent average receptor signal from wells treated with the indicated antipsychotic drug expressed as a percentage of signal from vehicle treated wells (mean ± SEM, n= 15-16; * indicates $p<0.05$, † indicates $p<0.01$ significantly different from vehicle treated cells; ANOVA followed by Dunnett's post-hoc test).

Figure 2

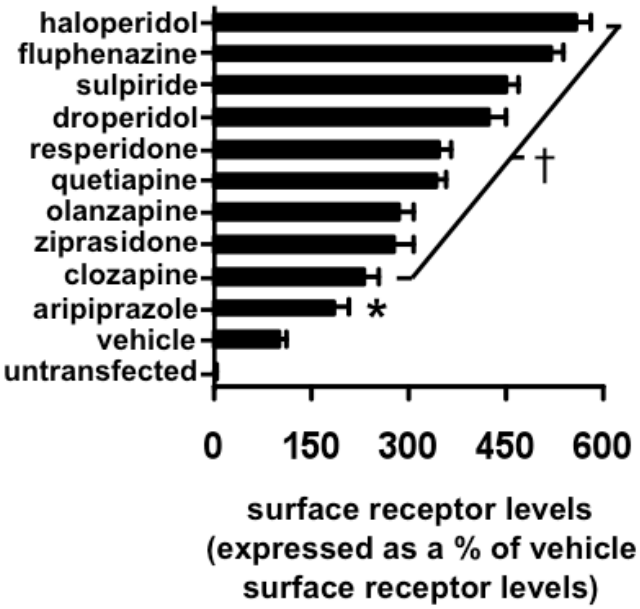

haloperidol
fluphenazine
sulpiride
droperidol
resperidone
quetiapine
olanzapine
ziprasidone
clozapine
aripiprazole
vehicle
untransfected
†
*
0
150
300
450
600
surface receptor levels
(expressed as a % of vehicle
surface receptor levels)

Fig. 3. TX100 solubility of cell surface D2R is unchanged with haloperidol or clozapine, as measured by indirect detection of D2R.

A. Diagrammatic representation of the receptor detected by co-immunoprecipitation with the monoclonal anti-FLAG antibody. HEK293 cells transiently expressing D2R-AP were pre-treated with haloperidol, clozapine or vehicle. After drug treatment, cells were incubated with anti-FLAG antibody and then lysed in buffer containing TX100. After lysis, soluble and insoluble biochemical fractions were resolved by SDS-PAGE and an anti-murine antibody was used to detect anti-FLAG antibody light chain.

B. *Upper panels*: Representative images of a western blot depicting the distribution of anti-FLAG antibody light chain in TX100 soluble (S) and insoluble (I) biochemical fractions, from cells transiently transfected with D2R-AP and treated with the indicated antipsychotic drug or vehicle. CTRL treated cells were transfected with plasmid containing cDNAs for D2R-AP but not treated with the anti-FLAG antibody. *Lower panels*: Representative images of a western blot depicting the distribution of total cellular proteins. Lanes in the lower panel are treated in the same manner as those in the corresponding lanes of the images of the upper panel.

C. Quantification of the relative levels of surface D2R-AP segregating into TX100 soluble (S) and insoluble (I) biochemical fractions. Bars represent average percentage of the surface D2R-AP per sample of treated cells as determined by indirect detection of cell surface receptor (mean ± SEM, n= 5-8).

D. Quantification of the total levels of anti-FLAG antibody from transiently transfected HEK293 cells treated with the indicated antipsychotic or vehicle. Bars represent total surface receptor detected by the anti-FLAG antibody expressed as a percentage of vehicle treated cells (mean ± SEM, n= 4-7; * indicates $p<0.05$, † indicates $p<0.01$ significant difference between the bars indicated; ANOVA followed by Tukey's post-hoc test).

Figure 3

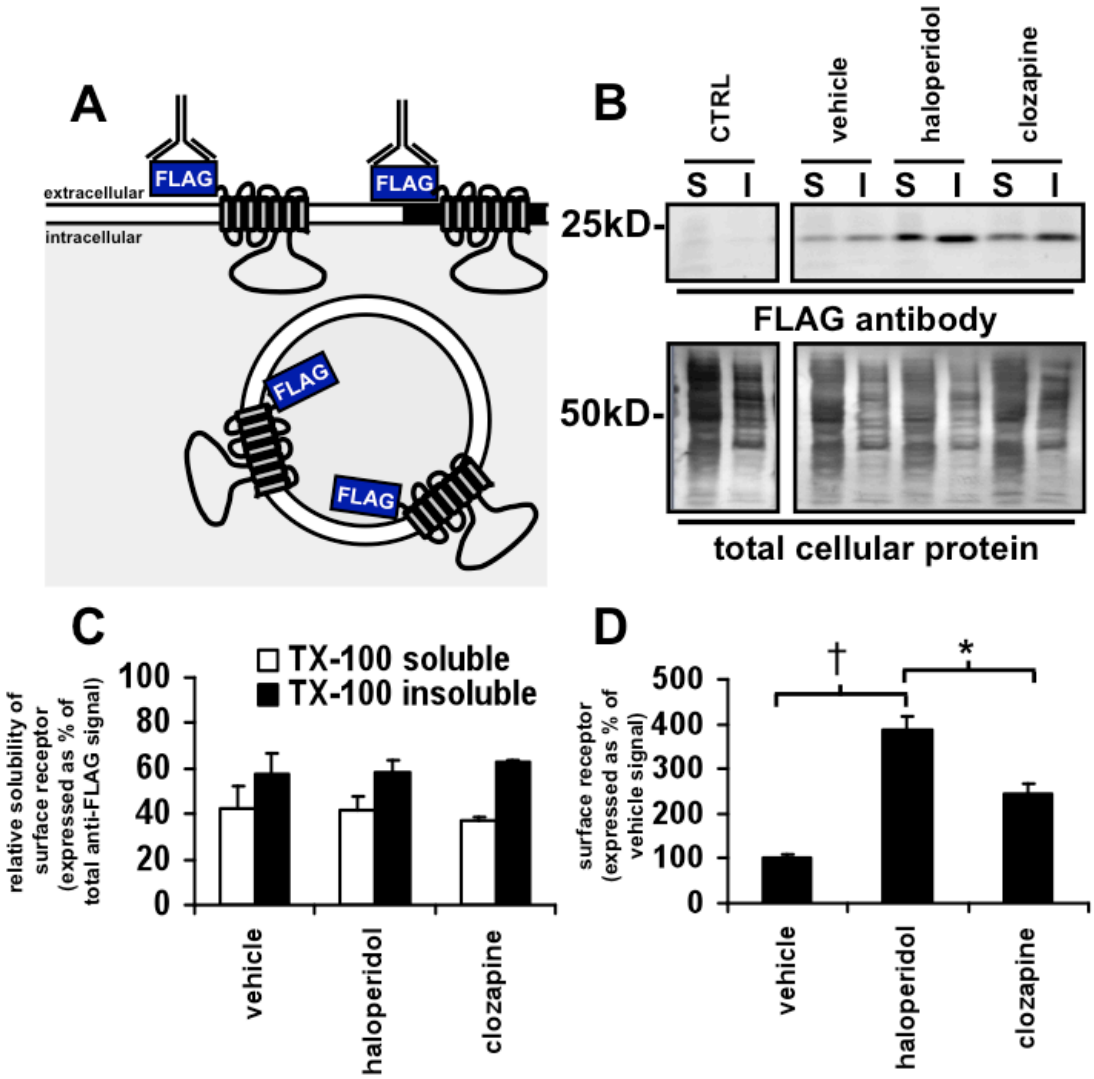

A
extracellular
intracellular
FLAG
FLAG
FLAG
FLAG
B
CTRL
vehicle
haloperidol
clozapine
S I S I S I S I
25kD-
FLAG antibody
50kD-
total cellular protein
C
TX-100 soluble
TX-100 insoluble
relative solubility of surface receptor (expressed as % of total anti-FLAG signal)
100
80
60
40
20
0
vehicle
haloperidol
clozapine
D
†
*
surface receptor (expressed as % of vehicle signal)
500
400
300
200
100
0
vehicle
haloperidol
clozapine

Fig. 4. Representative diagram of D2R-AP and KRAS-BL constructs in in-cell "proximity biotin transfer assay."

An in-cell proximity biotin transfer or biotinylation assay was utilized to detect D2R-AP, a D2-dopamine receptor construct with an acceptor peptide in the third intracellular loop, which interacted with KRAS-BL, a BirA, biotin ligase enzyme fused with the membrane targeting peptide from the protein KRAS. Upon cotransfection of plasmid containing cDNAs that code for each of these proteins some fraction of these transiently expressed proteins are inserted into the plasma membrane (top panel). Upon treatment of cells containing these proteins, KRAS-BL is able to covalently transfer a biotin to the AP region of D2R-AP, but predominantly soluble D2R-AP (white shaded region) and lesser of the insoluble D2R-AP, in the black shaded region (middle panel). Pre-treatment of cells containing these proteins with certain antipsychotic agents may cause changes in the accessibility of the plasma membrane targeted receptors to biotinylation (bottom panel).

Figure 4

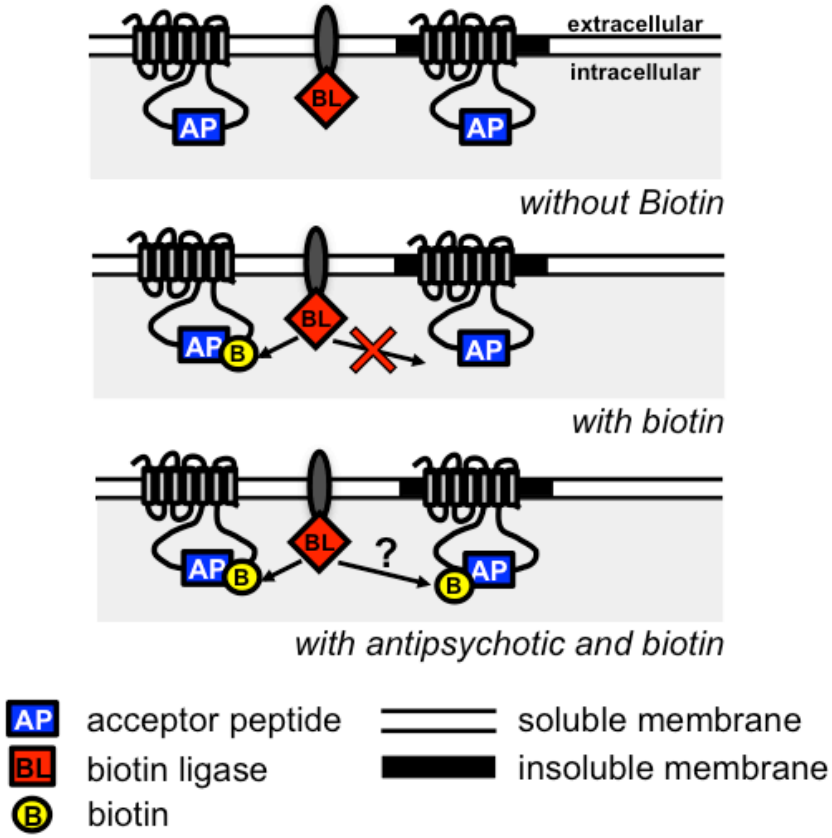

extracellular
intracellular
BL
AP
AP
without Biotin
BL
AP
B
AP
with biotin
BL
?
AP
B
B
AP
with antipsychotic and biotin
AP
acceptor peptide
BL
biotin ligase
B
biotin
soluble membrane
insoluble membrane

Fig. 5. Effect of antipsychotic drugs on plasma membrane accessibility of D2R-AP as assessed by an in-cell "proximity biotin transfer assay."

A. Representative images of a western blot depicting the distribution of biotinylated D2R-AP into TX100 soluble (S) and insoluble (I) biochemical fractions. A plus sign (+) indicates that these cells were treated with the indicated agent, whereas a minus sign (−) indicates that these cells were not treated with the indicated agent. Samples in the far left lane were transfected with plasmids containing cDNAs for both D2R-AP and KRAS-BL, but not treated with biotin (biotin −).

B. Quantification of the relative levels of total biotinylated D2R-AP from cells treated with various antipsychotic drugs. Bars represent an average of the total biotinylated D2R-AP from cells treated with the indicated antipsychotic drug expressed as a fraction of the vehicle treated biotinylated D2R-AP (mean ± SEM, n= 4-9; * indicates $p<0.05$, † indicates $p<0.01$, compared to total biotinylated D2R-AP from vehicle treated cells; ANOVA followed by Dunnett's post-hoc test).

C. Quantification of the relative levels of biotinylated D2R-AP segregating into TX100 soluble and insoluble biochemical fractions from cells treated with various antipsychotic drugs. Bars represent an average of the soluble (white bars) or insoluble (black bars) biotinylated D2R-AP from cells treated with the indicated antipsychotic drug expressed as a fraction of the total sample biotinylated D2R-AP (mean ± SEM, n= 4-9; * indicates $p<0.05$; comparing antipsychotic drug treated cells percent of total biotinylated D2R-AP to percent soluble biotinylated D2R-AP from vehicle treated cells; ANOVA followed by Dunnett's post-hoc test).

D. Quantification of the total levels of biotinylated D2R-AP segregating into TX100 soluble and insoluble biochemical fractions from cells treated with various antipsychotic drugs. Bars represent an average of the soluble (white bars) or insoluble (black bars) biotinylated D2R-AP from cells treated with the indicated antipsychotic drug expressed as a fraction of the soluble biotinylated D2R-AP from vehicle treated cells (mean ± SEM, n= 4-9; * indicates $p<0.05$; compared to soluble biotinylated D2R-AP from vehicle treated cells; ANOVA followed by Dunnett's post-hoc test).

Figure 5

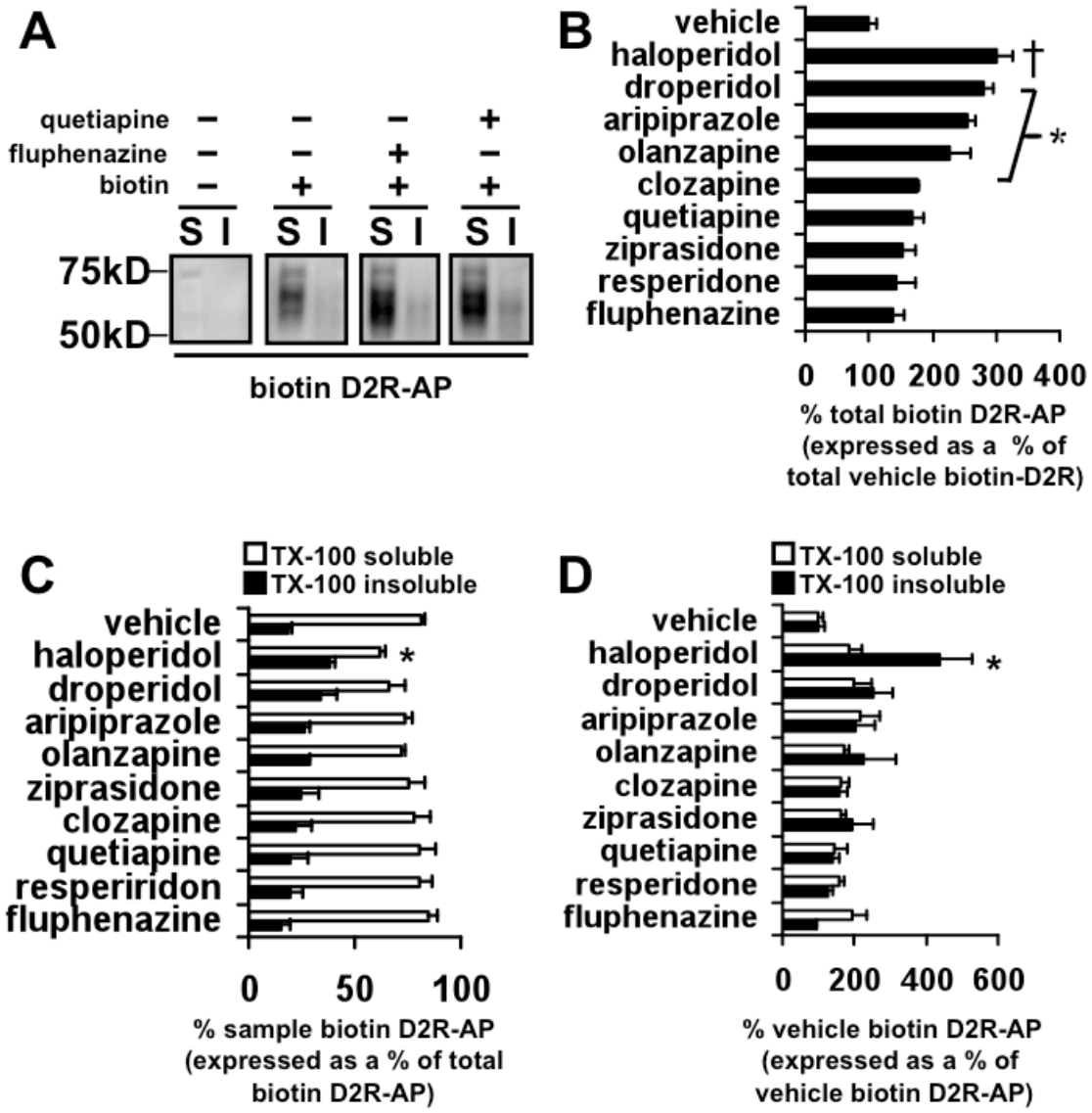

A
quetiapine – – – +
fluphenazine – – + –
biotin – + + +
S I S I S I S I
75kD
50kD
biotin D2R-AP
B
vehicle
haloperidol
droperidol
aripiprazole
olanzapine
clozapine
quetiapine
ziprasidone
resperidone
fluphenazine
†
*
0 100 200 300 400
% total biotin D2R-AP
(expressed as a % of
total vehicle biotin-D2R)
C
TX-100 soluble
TX-100 insoluble
vehicle
haloperidol
droperidol
aripiprazole
olanzapine
ziprasidone
clozapine
quetiapine
resperiridon
fluphenazine
*
0 50 100
% sample biotin D2R-AP
(expressed as a % of total
biotin D2R-AP)
D
TX-100 soluble
TX-100 insoluble
vehicle
haloperidol
droperidol
aripiprazole
olanzapine
clozapine
ziprasidone
quetiapine
resperidone
fluphenazine
*
0 200 400 600
% vehicle biotin D2R-AP
(expressed as a % of
vehicle biotin D2R-AP)

Fig. 6. Haloperidol's effect on plasma membrane accessibility of D2R-AP as assessed by an in-cell "proximity-biotin transfer assay" occurs in a dose-dependent manner.

A. *Upper Panels:* Representative images of a western blot depicting the distribution of biotinylated D2R-AP into TX100 soluble (S) and insoluble (I) biochemical fractions. A numerical concentration indicates that these cells were treated with haloperidol at the indicated concentration; a plus sign (+) indicates that these cells were treated with biotin, and a minus sign (−) indicates that these cells were not treated with haloperidol or biotin. Samples in the far left lane originated from cells that were transfected with plasmids containing cDNAs for both D2R-AP and KRAS-BL, but not treated with biotin; whereas samples in the second image from the left were treated with biotin (+) and only vehicle (−). *Lower Panels*: Representative images of a western blot depicting the total cellular protein from cells that were treated identically to those lanes located in the images directly above (upper panels).

B. Quantification of the relative levels of total biotinylated D2R-AP from cells treated with various concentrations of the antipsychotic drug, haloperidol. Bars represent an average of the total biotinylated D2R-AP from cells treated with the indicated concentration of haloperidol expressed as a fraction of the vehicle treated biotinylated D2R-AP (mean ± SEM, n=4-6; † indicates $p<0.01$ compared to total biotinylated D2R-AP from vehicle treated cells; ANOVA followed by Dunnett's post-hoc test).

C. Quantification of the relative levels of biotinylated D2R-AP segregating into TX100 soluble biochemical fractions from cells treated with various concentrations of haloperidol. Bars represent an average of the soluble biotinylated D2R-AP from cells treated with the indicated concentration of haloperidol expressed as a fraction of the vehicle soluble biotinylated D2R-AP (mean ± SEM, n= 4-6; * indicates $p<0.05$, † indicates $p<0.01$ compared to soluble biotinylated D2R-AP from vehicle treated cells; ANOVA followed by Dunnett's post-hoc test).

D. Quantification of the relative levels of biotinylated D2R-AP segregating into TX100 insoluble biochemical fractions from cells treated with various concentrations of haloperidol. Bars represent an average of the insoluble biotinylated D2R-AP from cells treated with the indicated concentration of haloperidol expressed as a fraction of the vehicle insoluble biotinylated D2R-AP (mean ± SEM, n= 4-6; * indicates $p<0.05$, † indicates $p<0.01$; compared soluble biotinylated D2R-AP from vehicle treated cells; ANOVA followed by Dunnett's post-hoc test).

Figure 6

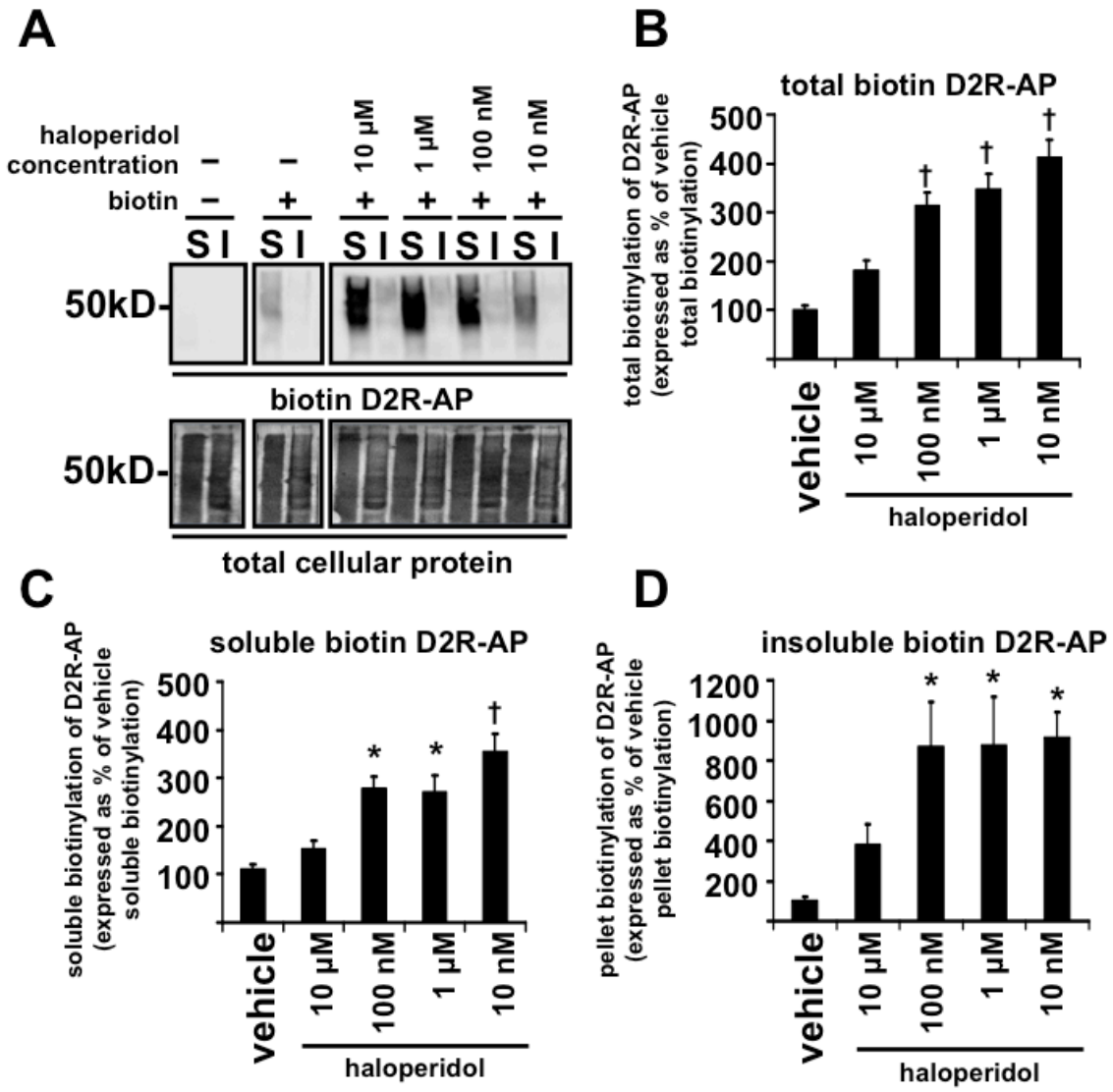

A
haloperidol concentration
biotin
10 µM
1 µM
100 nM
10 nM
S I S I S I S I S I S I
50kD-
biotin D2R-AP
50kD-
total cellular protein
B
total biotin D2R-AP
total biotinylation of D2R-AP (expressed as % of vehicle total biotinylation)
vehicle
10 µM
100 nM
1 µM
10 nM
haloperidol
C
soluble biotin D2R-AP
soluble biotinylation of D2R-AP (expressed as % of vehicle soluble biotinylation)
D
insoluble biotin D2R-AP
pellet biotinylation of D2R-AP (expressed as % of vehicle pellet biotinylation)

Fig 7. Time course of haloperidol's effect on plasma membrane accessibility of D2R-AP as assessed by an in-cell "proximity-biotin transfer assay."

A. *Upper Panels:* Representative images of a western blot depicting the distribution of biotinylated D2R-AP into TX100 soluble (S) and insoluble (I) biochemical fractions. A time value indicates that these cells were treated with haloperidol for the indicated duration, a plus sign (+) indicates that these cells were treated with biotin, and a minus sign (−) indicates that these cells were not treated with haloperidol or biotin. Samples in the far left lane originated from cells that were transfected with plasmids containing cDNAs for both D2R-AP and KRAS-BL, but not treated with biotin; whereas samples in the second image from the left were treated with biotin (+) and only vehicle (−). *Lower Panels*: Representative images of a western blot depicting the total cellular protein from cells that were treated identically to those lanes located in the images directly above (upper panels).

B. Quantification of the relative levels of total biotinylated D2R-AP from cells treated with haloperidol, for the indicated duration. Bars represent an average of the total biotinylated D2R-AP from cells treated with the indicated concentration of haloperidol expressed as a fraction of the vehicle treated biotinylated D2R-AP (mean ± SEM, n=3; † indicates $p<0.01$; compared to total biotinylated D2R-AP from vehicle treated cells; ANOVA followed by Dunnett's post-hoc test).

C. Quantification of the relative levels of biotinylated D2R-AP segregating into TX100 soluble biochemical fractions from cells described above. Bars represent

an average of the soluble biotinylated D2R-AP from cells treated with the indicated concentration of haloperidol expressed as a fraction of the vehicle soluble biotinylated D2R-AP (mean ± SEM, n=3; † indicates $p<0.01$; compared to vehicle treated cells; ANOVA followed by Dunnett's post-hoc test).

D. Quantification of the relative levels of biotinylated D2R-AP segregating into TX100 insoluble biochemical fractions from cells described above. Bars represent an average of the insoluble biotinylated D2R-AP from cells treated with haloperidol for the indicated interval expressed as a fraction of the vehicle insoluble biotinylated D2R-AP ((mean ± SEM, n=3; * indicated $p<0.05$, † indicates $p<0.01$; compared to vehicle treated cells; ANOVA followed by Dunnett's post-hoc test).

Figure 7

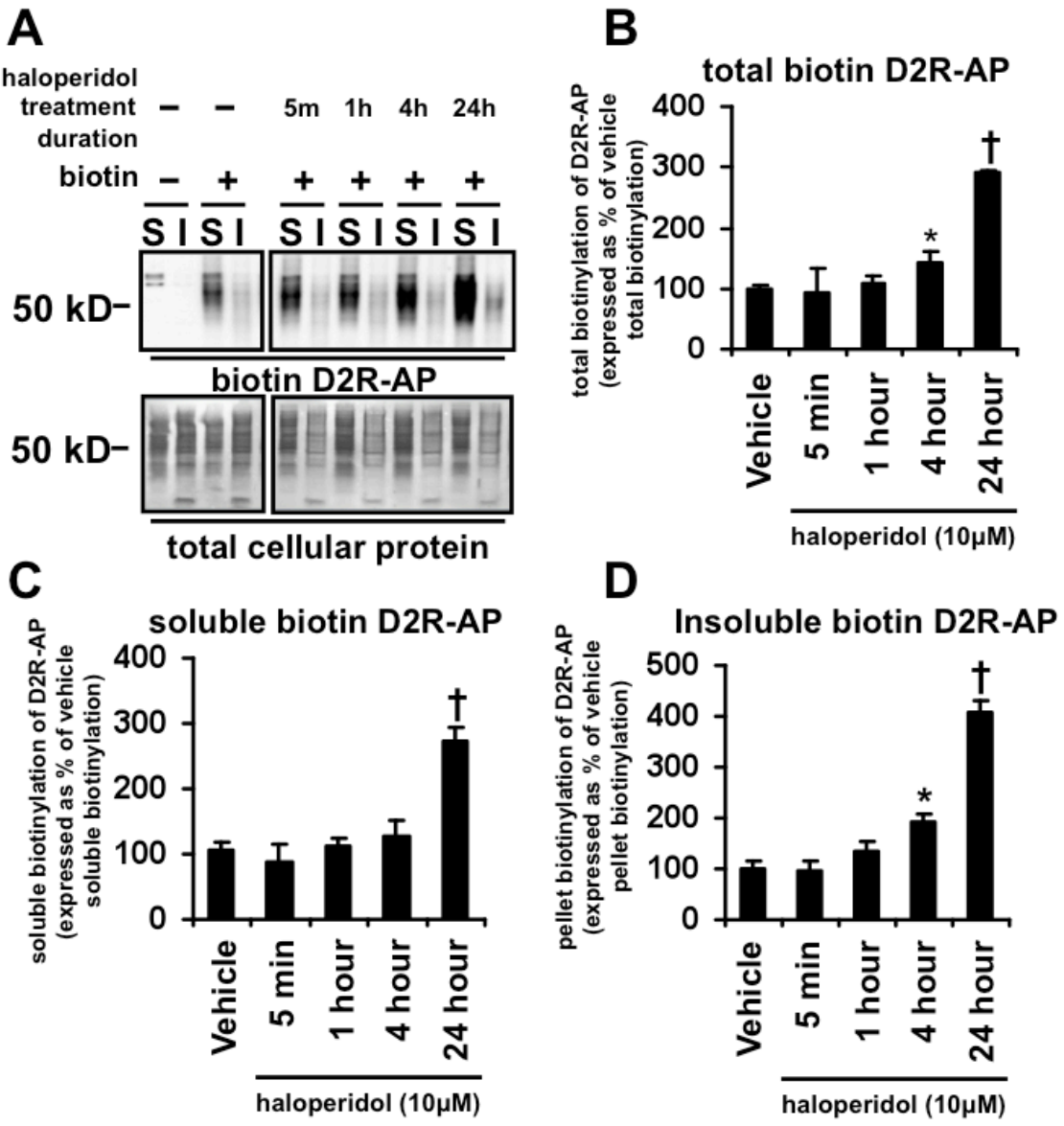

A
haloperidol treatment duration
– – 5m 1h 4h 24h
biotin – + + + + +
S I S I S I S I S I S I
50 kD–
biotin D2R-AP
50 kD–
total cellular protein
B
total biotin D2R-AP
total biotinylation of D2R-AP (expressed as % of vehicle total biotinylation)
400
300
200
100
0
*
†
Vehicle
5 min
1 hour
4 hour
24 hour
haloperidol (10μM)
C
soluble biotin D2R-AP
soluble biotinylation of D2R-AP (expressed as % of vehicle soluble biotinylation)
400
300
200
100
0
†
Vehicle
5 min
1 hour
4 hour
24 hour
haloperidol (10μM)
D
Insoluble biotin D2R-AP
pellet biotinylation of D2R-AP (expressed as % of vehicle pellet biotinylation)
500
400
300
200
100
0
*
†
Vehicle
5 min
1 hour
4 hour
24 hour
haloperidol (10μM)

Fig. 8. High-concentration clozapine treatment D2R-AP prevents haloperidol-induced enhancement of D2R-AP accessibility as measured by an in-cell "proximity biotin transfer assay.

A. *Upper Panels:* Representative images of a western blot depicting the distribution of biotinylated D2R-AP into TX100 soluble (S) and insoluble (I) biochemical fractions. A plus sign (+) indicates that these cells were treated with the indicated reagent, and a minus sign (−) indicates that these cells were not treated with the indicated reagent. Samples in the far left lane originated from cells that were transfected with plasmids containing cDNAs for both D2R-AP and KRAS-BL, but not treated with biotin; whereas samples in the second image from the left were treated with biotin (+) and only vehicle (−). *Lower Panels*: Representative images of a western blot depicting the total cellular protein from cells that were treated identically to those lanes located in the images directly above (upper panels).

B. Quantification of the relative levels of total biotinylated D2R-AP from cells the antipsychotic drug, haloperidol or clozapine, or haloperidol and clozapine, for the indicated duration. Bars represent an average of the total biotinylated D2R-AP from cells treated with the indicated antipsychotic drug(s) expressed as a fraction of the vehicle treated biotinylated D2R-AP (mean ± SEM, n= 3-5; * indicates $p<0.05$; comparing drug treated cells total biotinylated D2R-AP to total biotinylated D2R-AP from vehicle treated cells).

C. Quantification of the relative levels of biotinylated D2R-AP segregating into TX100 soluble biochemical fractions from cells described above. Bars represent

an average of the soluble biotinylated D2R-AP from cells treated with the indicated concentration of haloperidol expressed as a fraction of the vehicle soluble biotinylated D2R-AP (mean ± SEM, n= 3-5; † indicates $p<0.01$; comparing haloperidol treated cells soluble biotinylated D2R-AP to soluble biotinylated D2R-AP from vehicle treated cells).

D. Quantification of the relative levels of biotinylated D2R-AP segregating into TX100 insoluble biochemical fractions from cells described above. Bars represent an average of the insoluble biotinylated D2R-AP from cells treated with haloperidol for the indicated interval expressed as a fraction of the vehicle insoluble biotinylated D2R-AP ((mean ± SEM, n= 3-5; * indicates $p<0.05$, † indicates $p<0.01$; comparing haloperidol treated cells insoluble biotinylated D2R-AP to insoluble biotinylated D2R-AP from vehicle treated cells).

Figure 8

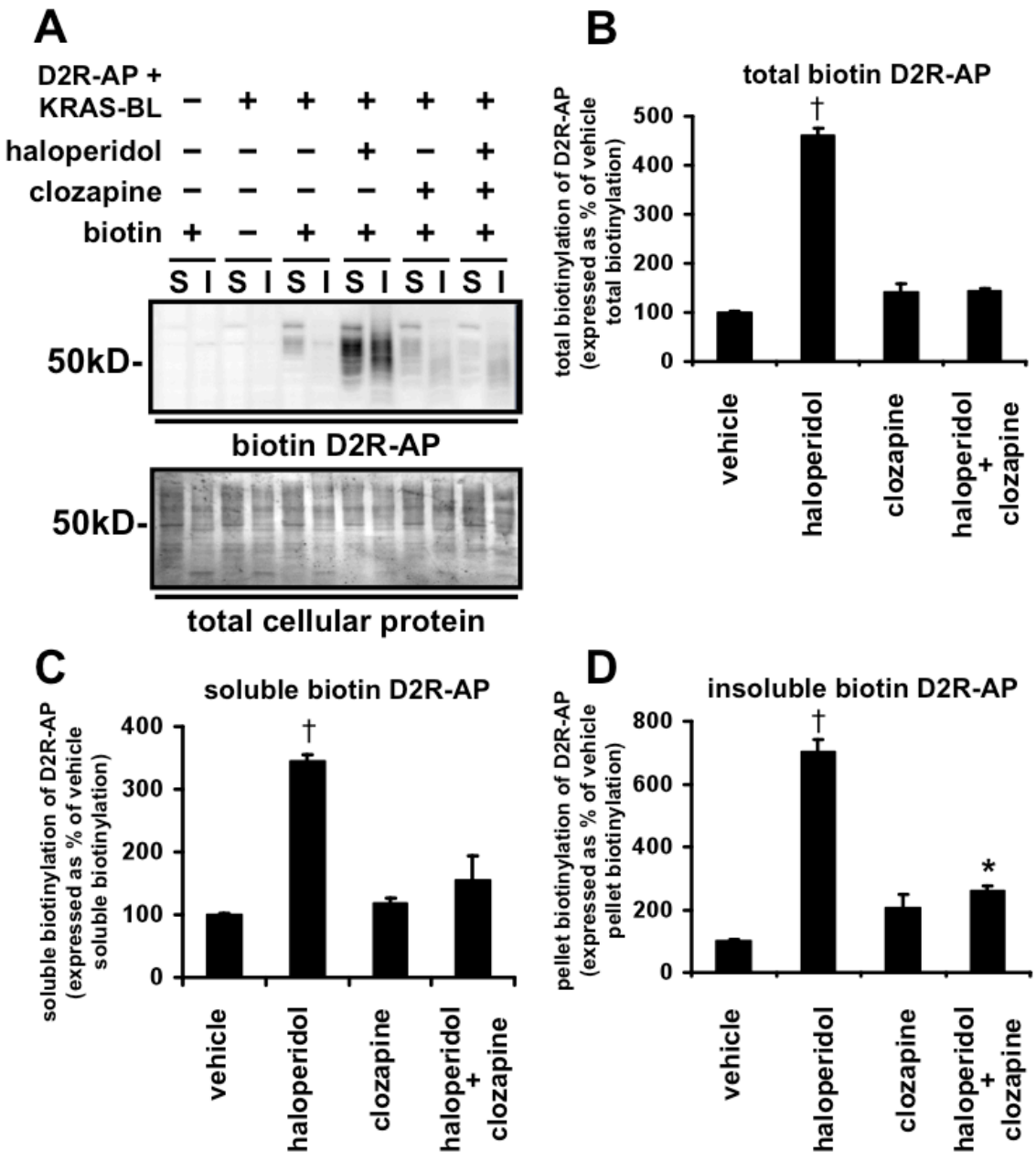
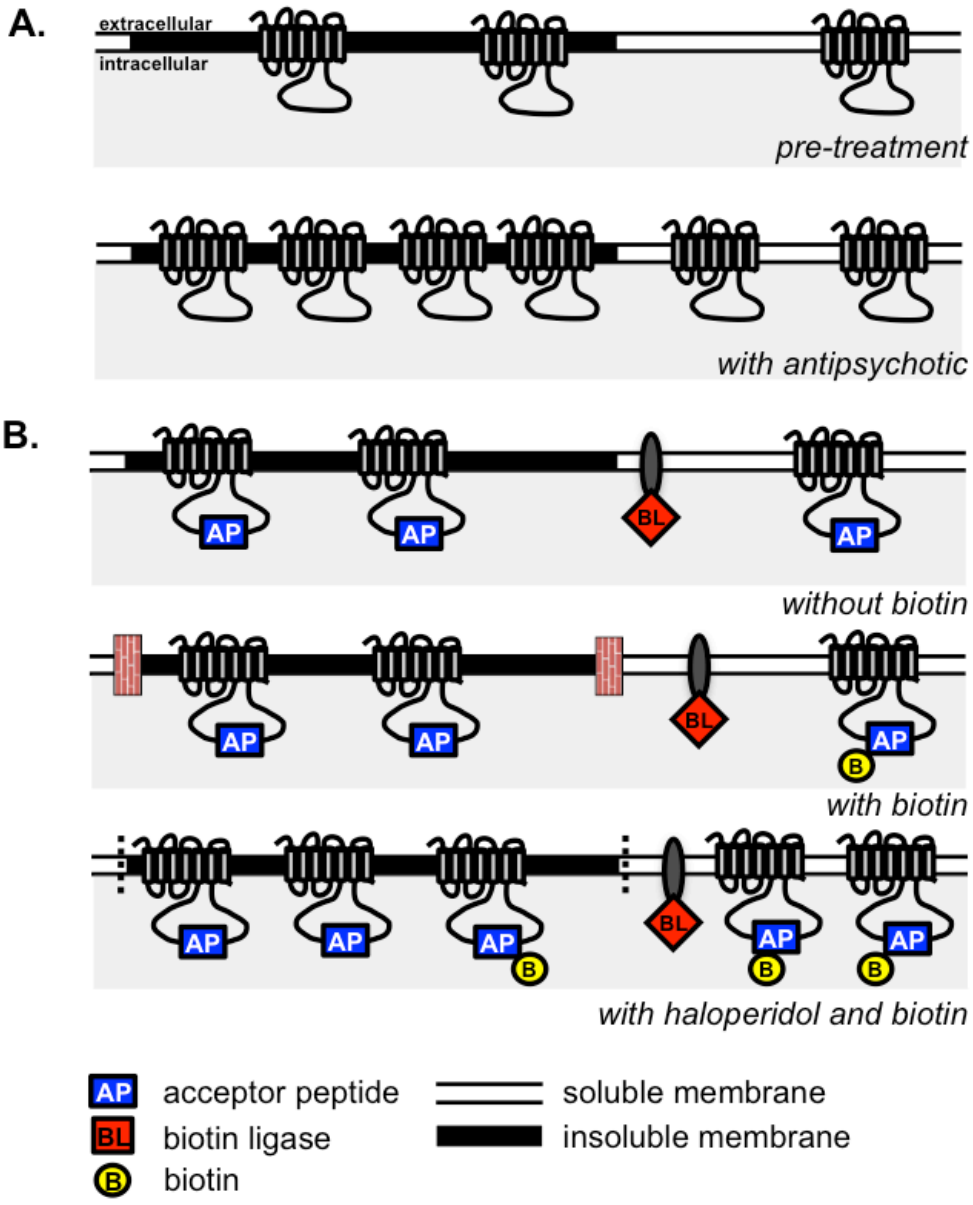

A
D2R-AP + KRAS-BL
haloperidol
clozapine
biotin
S I S I S I S I S I S I
50kD-
biotin D2R-AP
50kD-
total cellular protein
B
total biotin D2R-AP
total biotinylation of D2R-AP (expressed as % of vehicle total biotinylation)
vehicle
haloperidol
clozapine
haloperidol + clozapine
C
soluble biotin D2R-AP
soluble biotinylation of D2R-AP (expressed as % of vehicle soluble biotinylation)
D
insoluble biotin D2R-AP
pellet biotinylation of D2R-AP (expressed as % of vehicle pellet biotinylation)

Fig. 9. Representative diagram of effects of antipsychotic drugs on the surface expression and accessibility of plasma membrane targeted D2R.

A. Diagrammatic representation of the effects of antipsychotic drugs on the plasma membrane targeting of D2R. *Upper panel*: Prior to antipsychotic treatment D2R exists within the plasma membrane in both detergent soluble (white shaded) and detergent insoluble (black shaded) regions of the plasma membrane. *Lower Panel*: Upon treatment with antipsychotic agents the total amount of surface receptors increases, yet the approximate proportion of soluble: insoluble surface receptors are maintained.

B. Diagrammatic representation of the effects of haloperidol on the plasma membrane compartmentalization of D2R-AP. *Upper Panel*: Similar to wild-type D2R, D2R-APs exist both within TX100 soluble and insoluble biochemical fractions of the plasma membrane, whereas the plasma membrane targeted biotin ligase exists predominantly within TX100 soluble biochemical fractions of the plasma membrane. *Middle Panel*: Upon treatment of cells expressing both the D2R-AP and biotin ligase fusion proteins with biotin, the D2R-AP in the TX100 soluble biochemical fraction is labeled with biotin, but D2R-AP is not biotinylated in the TX100 insoluble biochemical fraction of the plasma membrane. *Bottom Panel:* Haloperidol enables a significant pool of the plasma membrane targeted D2R-AP to the TX100 insoluble biochemical fraction to become biotinylated, thereby disrupting the membrane compartmentalization of the insoluble form of D2R-AP

Figure 9


A.
extracellular
intracellular
pre-treatment
with antipsychotic
B.
AP
BL
without biotin
with biotin
with haloperidol and biotin
acceptor peptide
biotin ligase
biotin
soluble membrane
insoluble membrane