2017

Novel Pharmacological Action of Clozapine at D2 Dopamine Receptors

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NOVEL PHARMACOLOGICAL ACTION OF CLOZAPINE AT D2 DOPAMINE
RECEPTORS

By

JOSEPH MICHAEL SCHRADER

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

PHARMACEUTICAL SCIENCES

UNIVERSITY OF RHODE ISLAND

2017

DOCTOR OF PHILOSOPHY DISSERTATION

OF

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UNIVERSITY OF RHODE ISLAND
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2017

ABSTRACT

Clozapine is an antipsychotic drug (APD) with a uniquely efficacious therapeutic profile that is preeminent for treatment resistant schizophrenia. A common property of all clinically available APDs is that they bind D2-like dopamine receptors at therapeutic concentrations. However, unique molecular actions for clozapine that are different from all other APDs have not been established. Here we show that treatment with all APDs upregulates cell-surface expression of D2-dopamine receptors (D2R), relative to that produced by clozapine. Multiple experiments, including a pulse-chase assay for surface D2R indicated that the APD-mediated upregulation of D2R is due to enhanced insertion of D2R into the plasma membrane and not differences in receptor occupancy or protein synthesis. The potency of amisulpride, an APD with poor membrane permeability, for upregulating surface D2R was increased by coexpressing OCT1, an amisulpride transporter, suggesting an intracellular site of action for APDs. Competition experiments and experiments with a non-APD-binding D2R mutant indicated that APD upregulation of surface D2R is a consequence of direct receptor binding. Enhancement by APDs of surface D2R, relative to that produced by clozapine, was magnified in both a glycosylation-deficient and a misfolding D2R mutant. Moreover, every APD, except clozapine, enhanced the detergent solubility of intracellular D2R. These and other data suggest that APDs act intracellularly as chemical chaperones to stabilize a conformation of D2R with enhanced detergent solubility and transport to the cell surface. These results display a unique molecular event of clozapine at D2R, as it possesses the lowest efficacy with respect to these actions of APDs. Results displaying unique binding of clozapine to D2R harbor intriguing implications for APD discovery and development, and provokes novel speculative thought and questions on the nature of ligand-receptor interaction.

ACKNOWLEDGEMENT

I would like to give my sincerest thanks to my adviser Dr. Abraham Kovoov. I cannot express the gratitude I have for the opportunity to learn and discover that he has given me. He has taught me to not be satisfied with the status quo of data collection, and passed on a true passion for solving creation's mysteries through Science. Thank you for your patience, guidance, and instruction. Thank you for sharing with me your passion for discovery. Thank you for the intellectual and financial support that fueled my development as a researcher, and that provided me with the ability to complete this work.

I would also like to thank my committee: Dr. Robert Rodgers, Dr. Brenton DeBoef, Dr. Niall Howlett, and Dr. James Agostinucci. Thank you for the guidance, discussion, the questions, and high standards to push me further.

I also thank my lab mates, both past and present. To Dr. Jeremy Cerver, Dr. Meenakshi Sharma, and Dr. Chris Octeau, thanks for teaching me many of the techniques I now rely on every day. To Craig Irving, thanks for the support, and the contributions made to this project. Thanks to the College of Pharmacy for providing an environment to learn, to grow, to collaborate, and explore the natural world.

I would also like to thank my wife, Alexandra. Thank you for your patience, love, and support that has allowed me to persevere through the ups and downs of bench Science. I know that I would have not completed this research without you. Finally, I give thanks to my Heavenly Father, and Lord Jesus Christ. All that I have you have given me. You are the source. How I love studying your creation.

PREFACE

This dissertation is organized in manuscript format, as it is being prepared for submission for publication. The manuscript is not yet published, but is in the process of submission to the Proceeding of the National Academy of Science. The dissertation is original, and independent work by the first author, Joseph Schrader, unless otherwise specified. Data presented in Fig.s 4A and Fig. 9A are the results of experiments performed by C. Irving.

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MANUSCRIPT 1

Title:

Unique Actions of Clozapine at D2-Dopamine Receptors

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STATUS: Prepared for submission to Proceedings of the National Academy of Science

ABSTRACT

Clozapine is an antipsychotic drug (APD) with a uniquely efficacious therapeutic profile that is preeminent for treatment resistant schizophrenia. A common property of all clinically available APDs is that they bind D2-like dopamine receptors at therapeutic concentrations. However, unique molecular actions for clozapine that are different from all other APDs have not been established. Here we show that treatment with all APDs upregulates cell-surface expression of D2-dopamine receptors (D2R), relative to that produced by clozapine. Multiple experiments, including a pulse-chase assay for surface D2R indicated that the APD-mediated upregulation of D2R is due to enhanced insertion of D2R into the plasma membrane and not differences in receptor occupancy or protein synthesis. The potency of amisulpride, an APD with poor membrane permeability, for upregulating surface D2R was increased by coexpressing OCT1, an amisulpride transporter, suggesting an intracellular site of action for APDs. Competition experiments and experiments with a non-APD-binding D2R mutant indicated that APD upregulation of surface D2R is a consequence of direct receptor binding. Enhancement by APDs of surface D2R, relative to that produced by clozapine, was magnified in both a glycosylation-deficient and a misfolding D2R mutant. Moreover, every APD, except clozapine, enhanced the detergent solubility of intracellular D2R. These and other data suggest that APDs act intracellularly as chemical chaperones to stabilize a conformation of D2R with enhanced detergent solubility and transport to the cell surface. These results display a unique molecular event of clozapine at D2R, as it possesses the lowest efficacy with respect to these actions of APDs.

INTRODUCTION

The development of antipsychotic drugs (APDs) beginning more than 70 years ago revolutionized the treatment of schizophrenia and related psychotic disorders (Tandon, 2011a). A common action of all clinically available APDs is that they bind D2-like dopamine receptors (D2-like DR) at therapeutic concentrations (Kapur and Mamo, 2003a; Kapur and Remington, 2001; Seeman, 2006; Urs et al., 2017).

D2-like DR are members of the seven transmembrane domain G protein coupled receptor (GPCR) superfamily and, of the three D2-like DR (D2, D3 and D4) (Beaulieu et al., 2015; Vallone et al., 2000), the D2-dopamine receptor has the most widespread distribution in the brain (Beaulieu and Gainetdinov, 2011a; Missale et al., 1998).

Alternative splicing produces two D2-dopamine receptor isoforms, D2 short (D2Rsh), and a longer form (D2R) which contains 29 additional amino acids inserted in the 3rd cytoplasmic loop (Beaulieu et al., 2015; Missale et al., 1998; Vallone et al., 2000).

D2-like DR expressed in certain striatal neurons are important for the control of movement, and blockade of these receptors by APDs can produce both acute and “tardive” movement disorders collectively called extrapyramidal symptoms (EPS) (Dayalu and Chou, 2008; Meltzer, 2013a; Tandon, 2011a). Acute symptoms include Parkinsonism, and chronic treatment may produce a potentially irreversible and debilitating movement disorder called tardive dyskinesia (TD).

Schizophrenia symptoms may be organized into three groups: 1) positive symptoms such as hallucinations and delusions; 2) negative symptoms such as apathy and severely impaired social functioning; and 3) cognitive symptoms such as disorganized thought and inability to concentrate (Freedman, 2003a). The early APDs, while effective at

suppressing positive symptoms, had limited efficacy against negative and cognitive symptoms (Meltzer, 2013a; Tandon, 2011a).

The introduction of clozapine in the 1970s was a major milestone in the pharmacotherapy of schizophrenia because clozapine appeared to demonstrate superior efficacy against both positive and negative symptoms, with significantly minimized risk of acute EPS and TD relative to the older drugs (Casey, 1989a; Essali et al., 2009; Iqbal et al., 2003; Wenthur and Lindsley, 2013a). However, clozapine-induced agranulocytosis and metabolic side effects that increase risk of cardiovascular morbidity have restricted the use of this highly effective APD (Iqbal et al., 2003; Naheed and Green, 2001; Opgen-Rhein and Dettling, 2008). The unique therapeutic profile of clozapine catalyzed efforts to develop safer drugs that replicate clozapine's unique efficacy and produce fewer and less severe side effects. Several 2nd-generation APDs were developed by searching for molecules that shared two pharmacological properties of clozapine which were unique relative to those of 1st-generation drugs, namely 1) reduced binding affinity for D2-like DR, and 2) enhanced binding affinities for other brain receptor systems, particularly 5-HT_{2A} serotonin receptors (Jibson and Tandon, 1998a; Kuroki et al., 2008a; Meltzer and Huang, 2008; Seeman, 2002).

Nevertheless, evidence from both clinical trials and practice, reviewed in numerous manuscripts (Agid et al., 2008, 2010a; Kang and Simpson, 2010; Lewis and Lieberman, 2008a; Manschreck and Boshes, 2007; Meltzer, 2012a; Tandon et al., 2008), indicate that clozapine outperforms all other APDs, both atypical 2nd generation and typical 1st generation APDs, with respect to nearly all aspects of schizophrenia pharmacotherapy. Further, clozapine treatment produces the greatest suppression of mortality in schizophrenia (Agid et al., 2010a; Kerwin and Bolonna, 2004a; Meltzer, 2012a) and is

the first choice for treatment-resistant patients (Foussias and Remington, 2010; Meltzer, 2012a, 2013a; Siskind et al., 2016). Clozapine also has a uniquely favorable motor side-effect profile as it is the least likely of the 2nd generation drugs to cause TD (Margoless et al., 2005) and is the only APD that has “level A” evidence to support its use in suppressing psychoses in Parkinson’s disease patients (Friedman, 2013). A review by Meltzer states that “the advantages of clozapine which have emerged from controlled clinical trials have been borne out in clinical practice and in the often remarkable improvement noted in patients who have failed previous attempts at treatment” (Meltzer, 2012a). Accordingly, clozapine has been referred to as the “gold standard” drug for the treatment of schizophrenia (Kang and Simpson, 2010; Meltzer, 2012a), but its adverse effects have restricted its use.

The dopamine-serotonin antagonism theory (Meltzer et al., 2003) does not entirely explain the unique efficacy of clozapine, since this pharmacological property is shared with other 2nd generation APDs. Multiple alternative molecular actions for clozapine have been proposed to explain its distinctive efficacy, and these have been reviewed (Seeman, 2014; Wenthur and Lindsley, 2013a). A partial list includes binding to subtypes of muscarinic acetylcholine receptors, adrenergic receptors, and glutamate receptors, “fast-off” binding, and low or transient occupancy of D2R at therapeutic concentrations (Seeman, 2014; Wenthur and Lindsley, 2013a). However, each of these actions cannot explain the singularity of clozapine in schizophrenia because some or all are common to other APDs. For example, muscarinic and adrenergic receptor antagonism (Jibson and Tandon, 1998a), low D2R occupancy, and “fast off” D2R binding kinetics are properties shared by multiple 2nd generation APDs (Seeman, 2006). It is conceivable that it is the set of quantified actions at all of these targets that is different between clozapine and all of the

other APDs, but a set unique to clozapine has yet not been clearly defined. Alternatively, there may yet be undiscovered molecular actions of clozapine, which are clearly different from those of all other APDs, which could contribute to its unique efficacy.

We recently reported (Sharma et al., 2013a) that D2R are functionally compartmentalized in the plasma membrane. On the basis of that observation, here we compared APDs with respect to their potential influences on plasma membrane D2R expression. We report here for the first time unique molecular actions of clozapine, compared to all other APDs tested, on D2R functional expression in HEK293 cells, a widely used experimental model of D2R function (Min et al., 2015; Namkung and Sibley, 2004a; Octeau et al., 2014; Sharma et al., 2013a; Thibault et al., 2011).

RESULTS

APD treatment enhanced cell-surface expression of D2R. Concentration-response curves for APD-enhancement of cell-surface D2R expression were generated after 24 hr of treatment with a 1st-generation APD, haloperidol, a second-generation APD, olanzapine, and clozapine (Fig. 1). As described in the Methods section, relative cell-surface levels of D2R, transiently expressed in HEK293T cells, were detected using an ELISA-based assay, which measured binding of the intact cells to an antibody targeted against the D2R construct. The relative levels of surface D2R after APD treatment are reported as the percent increase in surface D2R expression over vehicle. Treatment with all the three APDs significantly enhanced cell-surface D2R expression (Fig. 1A).

Relative to the actions of clozapine, all APDs except aripiprazole significantly enhanced cell-surface expression of D2R. We then performed a screen that compared the actions of a wider selection of APDs to that of clozapine on cell-surface D2R expression (Fig. 1B).

The maximal enhancement of cell-surface D2R expression produced by clozapine was achieved at a concentration of 10 μ M. This is also the concentration that produces close to saturation D2R-binding for all of the tested APDs, according to previously summarized affinity measurements for the cloned receptor construct in intact cells (Richtand et al., 2007; Sahlholm et al., 2016). Accordingly, all APDs were tested at 10 μ M.

Treatment with all APDs except aripiprazole produced significantly more D2R surface expression than the maximum that could be produced with clozapine treatment (Fig. 1B). Hence, we can conclude that all tested APDs, except for aripiprazole, are more efficacious than clozapine for enhancing cell-surface D2R expression.

The greater enhancement of surface D2R expression produced by other APDs, relative to clozapine, could not be attributed to relatively greater enhancement of total cellular D2R expression. A subset of the APDs, including haloperidol and clozapine, were tested for actions on expression levels of total cellular D2R (Fig. 1C). The relative levels of total cellular D2R were measured using an ELISA assay, similar to that utilized above to specifically measure cell-surface receptor expression. However, to measure relative levels of total cellular receptors instead, cells were fixed in methanol, so that both surface and intracellular pools of D2R were accessible to the D2R-detecting antibody. Treatment with all tested APDs, including clozapine, but not aripiprazole, significantly enhanced total cellular D2R expression levels by at least 250%. Aripiprazole only enhanced D2R expression levels by about 125%. All other APD treatments were not significantly different from each other.

Enhancement of surface D2R expression by haloperidol is a result of long-term APD treatment. All previous APD treatments in this study were done over a 24 hr period. To

test whether enhancement of cell surface D2R expression was truly an effect of long-term APD treatment, we measured relative levels of surface D2R expression following 6, 12, and 24 hr treatment, using haloperidol as a representative because it had displayed the most pronounced effect on D2R surface expression. Haloperidol-mediated enhancement of surface D2R expression increased from almost no effect after 6 hr to an increase of about 300% after 12 hr, to about 500% after 24 hr (Fig. 1D). Thus, APD-mediated enhancement of surface D2R expression appears to be a function of time; the longer the treatment the greater the effect.

Compared to clozapine, both haloperidol and aripiprazole produced significantly greater enhancements of the rate of surface D2R insertion into the plasma membrane. Loss of GPCRs from the cell surface may occur via sequestration (Rajagopal and Shenoy, 2017) or via *in situ* proteolysis and degradation. Therefore, the amount of receptor at the cell-surface is the difference between the rate of insertion and the rate of loss of the receptor from the plasma membrane. Hence, by measuring the relative effect of APD treatments on the specific removal of D2R from the cell surface we attempted to discern whether the greater APD-induced enhancement of D2R surface expression, relative to that produced by clozapine, was a consequence of increased receptor insertion, decreased removal, or both.

In order to evaluate the relative effects of APDs specifically on the removal of cell-surface D2R, we utilized a previously validated pulse-chase protocol (Kumagai et al., 2015), as described in the Methods section. The protocol monitored the loss of a pre-biotinylated pool of HaloTag D2R, a D2R construct with an extracellular N-terminal insertion of HaloTag, from the cell surface. The HaloTag insertion allows for the specific biotinylation of cell surface-expressed HaloTag D2R using a membrane-impermeable

HaloTag-PEG-Biotin reagent. After pre-biotinylation of cell surface-expressed HaloTag D2R, cells were treated with haloperidol, aripiprazole, or clozapine (10 μ M, 24 hr). The relative levels of HaloTag-PEG-Biotin-labeled D2R remaining at the cell surface after APD treatment were quantified by probing intact cells with HRP-conjugated streptavidin. Under these conditions, receptors that were newly inserted into cell surface during APD treatment were not detected and only pre-biotinylated receptors that remained at the cell surface during the course of APD treatment is measured.

The rates of loss of surface D2R during haloperidol and clozapine treatment were similar, because levels of biotin-labeled D2R remaining at the cell surface were not significantly different between the haloperidol- and clozapine-treated cells (Fig. 2A). Hence, it may be concluded that haloperidol's enhancement of cell-surface D2R expression (Fig. 1B), relative to that of clozapine, was a consequence of an enhanced rate of insertion of D2R into the plasma membrane, and not to a retarded rate of removal.

Compared to either haloperidol or clozapine treatment, aripiprazole treatment significantly lowered levels of pre-biotinylated D2R that remained at the cell surface (Fig. 2A). The observed enhanced sequestration or loss of D2R from the plasma membrane produced by aripiprazole was not surprising, since aripiprazole is a partial agonist at D2R with respect to recruitment of beta-arrestin, and may thus be expected to promote receptor endocytosis (Allen et al., 2011; Beaulieu and Gainetdinov, 2011a; Namkung and Sibley, 2004a). However, even though aripiprazole promoted a greater loss of cell-surface D2R than clozapine did (Fig. 2A), it did not produce lower cell-surface D2R levels (Fig. 1A). Therefore we can conclude that, of the two, aripiprazole must have produced a greater enhancement of the rate of insertion of D2R into the plasma membrane.

The ability of the pulse-chase biotinylation assay to reveal aripiprazole's enhancement of sequestration of cell-surface HaloTag D2R (Fig. 2A) - even when total surface D2R levels are elevated (Fig. 1B) - supports the validity of utilizing the assay for comparing the specific loss of D2R from the cell surface. It also suggests that the HaloTag D2R and wild-type D2R function similarly.

Nevertheless, the validity of utilizing the combination of the HaloTag D2R construct and the HaloTag-PEG-Biotin reagent to investigate the relative actions of APDs, specifically on D2R sequestration was further investigated in two control experiments. Dopamine-induced internalization is a well characterized phenomenon associated with D2R (Clayton et al., 2014; Gurevich et al., 2016). Here, we showed that dopamine treatment resulted in a loss of cell-surface HaloTag D2Rs (Fig. 2B). This result added support to the view that HaloTag D2R and wild-type D2R respond to dopamine in a functionally similar manner.

For the second control experiment, we investigated if the differential actions of the APDs, haloperidol and clozapine, on enhancing cell-surface D2R expression could be replicated in an assay that utilized the membrane impermeable HaloTag-PEG-Biotin reagent to measure the relative levels of cell-surface HaloTag D2R. This experiment and subsequent investigations into the mechanisms of APD-induced upregulation of cell-surface D2R were limited to haloperidol and clozapine because they produced the greatest and least upregulation of cell-surface D2R, respectively, and thus served to model the full range of this action of APDs.

HEK293T cells transiently expressing HaloTag D2R were first treated with either clozapine or haloperidol (10 μ M, 24 hr). The relative levels of cell-surface HaloTag D2R were then evaluated as described in the Methods section via biotinylation with HaloTag-

PEG-Biotin reagent. The pattern of action of haloperidol and clozapine treatment with respect to enhancement of surface D2R levels was similar to that obtained with the ELISA-based assay. Compared to clozapine treatment, haloperidol treatment resulted in a greater enhancement of cell-surface HaloTag D2R levels (Fig. 2C).

APD-mediated cell-surface D2R upregulation is specific and consequent to D2R binding.

We carried out further control experiments to determine the specificity of the response. In one, we found that neither haloperidol, nor clozapine produced surface upregulation of the β 2-adrenergic receptor (β 2AR) (Fig. 3A) or the mu-opioid receptor (MOR) (Fig. 3B).

Previously, it has been shown that the mutant D2R, D2R_{D114A}, does not bind agonists or antagonists (Han et al., 2009a; Xu et al., 1999). In a second control experiment, we found that while D2R_{D114A} expression was detected at the cell surface, neither cell-surface (Fig. 3C) nor total cellular levels (Fig. 3D) of the mutant receptor was upregulated by treatment with either haloperidol or clozapine. These data support the view that APD-induced upregulation of cell-surface D2R is a consequence of specific binding to D2R.

Treatment with haloperidol, but not clozapine, alters cellular co-localization of D2R with an ER marker. Multiple studies from different laboratories have reported that the majority of D2R is atypically localized in intracellular perinuclear compartments, whether they are expressed across different cell lines, including HEK293, COS, HeLa, CHO, and NG108 (Fishburn et al., 1995a; Kovoov et al., 2005a; Prou et al., 2001a; Sharma et al., 2013a), or endogenously expressed in neurons (Kim et al., 2008a; Kovoov et al., 2005a; Tirotta et al., 2008a). Furthermore, one study showed that these compartments were labeled by an endoplasmic reticulum (ER) marker (Prou et al., 2001a). The molecular basis for the anomalous cellular localization of D2R was recently identified (Kubale et al.,

2016a). A di-arginine motif can function as an ER retention signal, and it was shown that a conserved arginine cluster in the 3rd cytoplasmic loop of D2R (residues 267, 268 and 269) functions as such a signal for D2R (Kubale et al., 2016a).

As predicted by previous reports, we found in our lab, using confocal microscopy that the vast majority of D2R co-localized with the general ER marker, mCh-Sec61 beta (Fig. 4A). Treatment with haloperidol, but notably not clozapine, resulted in: 1) the emergence of a ring of D2R signal that coincided with the cell boundaries and, 2) significantly decreased D2R-mCh-Sec61 beta co-localization.

The particular D2R tri-arginine ER retention signal identified above, D2R_{R267, 268, 269}, is not present in the shorter alternatively spliced, D2Rsh isoform, and previously it has been shown that D2Rsh is retained in the ER to a lesser degree than D2R (Prou et al., 2001a). Therefore, we compared the actions of haloperidol and clozapine treatment on cell-surface levels of D2Rsh. The aim was to determine whether the two APDs act differently to up-regulate cell-surface receptors with greater and lesser ER retention. Indeed, treatment with haloperidol, but not clozapine increased cell-surface D2Rsh levels and, to a lesser extent, levels of D2R (Fig. 5A). This is not unexpected, because D2Rsh is less encumbered in trafficking to the cell surface, and should display higher surface expression in vehicle-treated cells. Nevertheless, haloperidol did produce some upregulation of D2Rsh. We next tested whether these differences between the effects of haloperidol and clozapine were not a consequence of differences in the processes of total cellular D2Rsh upregulation. We compared the effects of haloperidol and clozapine on total D2Rsh in the same manner as we did on levels of D2R. Both haloperidol and clozapine similarly increased total D2Rsh expression, relative to vehicle, by about 50% (Fig. 5B).

Increased surface D2R expression induced by amisulpride, but not tiapride, is enhanced by OCT1. We then asked whether the key interaction between the APDs and D2R that results in the upregulation of surface receptor occurs at the cell membrane or intracellularly. Amisulpride is an APD with poor membrane permeability, and is a substrate for the membrane transporter OCT1 (Dos Santos Pereira et al., 2014). As previously mentioned, direct binding of APDs to D2R is required for APD-induced increases in surface D2R expression. If this key specific interaction occurs intracellularly, then amisulpride's potency for inducing increased expression of surface D2R should be enhanced by co-expression of OCT1, because more surface OCT1 should result in more amisulpride's entering the cell. To answer this question, we generated concentration-response curves for amisulpride's ability to increase surface D2R expression in the presence and absence of OCT1. HEK293T cells expressing D2R with and without OCT1 co-expression were treated with increasing concentrations (10-10,000 nM) of amisulpride. We found that OCT1 co-expression greatly enhanced amisulpride's effect, reducing its EC50 from 663 nM to 10 nM, with the maximum effect attained at an amisulpride concentration 100X lower than that of OCT1-deficient cells (Figure 6A). This shift to the left in amisulpride's dose-response curve indicates that transport into the cell is necessary for its effect, and provides evidence that the key interaction leading to APD-induced enhanced surface D2R expression occurs intracellularly.

In a control study, we repeated the experiment with tiapride, another APD with poor membrane permeability that *is not* a substrate for OCT1. Here we saw no change in tiapride's effect with OCT1 co-expression (Figure 6B). Therefore, these data suggest that it was specific transport of amisulpride into the cell by OCT1 that enhanced its effect on

D2R surface expression. Thus, entrance into the cell is critical for APD-mediated enhancement of D2R surface expression, and the key interaction is intracellular.

Haloperidol rescues misfolded receptor for export to the cellular membrane. Several studies have reported on the ability of ligands to overcome ER-retention motifs, and rescue misfolded receptors, to promote their export from intracellular membranes to the cell surface in a process known as pharmacochaperoning (Lan et al., 2012; Lester et al., 2012; Wüller et al., 2004). Since we have shown that APDs can overcome D2R's tri-arginine ER retention motif to enhance their cell surface expression of D2R, we then asked whether APDs could rescue misfolded protein for export to the plasma membrane. Previous reports have utilized misfolding GPCR constructs, made by mutating a single conserved tryptophan residue, to investigate ligand pharmacochaperone capabilities and their proficiency in rescuing misfolded receptor for export to the plasma membrane (Lan et al., 2012). We utilized a D2R_{W160A} mutant, with a point mutation at the conserved tryptophan residue that misfolds, and displays no significant membrane receptor expression, to assess the ability over APDs to rescue misfolded D2R for export to the cell surface. Cells treated with haloperidol displayed enhanced surface D2R_{W160A} 2000% relative to vehicle, while clozapine was barely able to raise the signal above background (Fig 7A). Vehicle treated cells displayed no surface signal above background. To control for differences in total D2R_{W160A} signal, we then assessed effects of clozapine and haloperidol treatment on total D2R_{W160A} expression as described in previous experiments. While all cells transfected with D2R_{W160A} cDNA displayed significant total D2R_{W160A} expression over background, neither clozapine nor haloperidol treatment significantly increased total receptor expression relative (Fig 7B). Thus, haloperidol is capable of

rescuing misfolded D2R for insertion into the plasma membrane and displays strong pharmacochaperone capabilities, while clozapine does not.

Haloperidol enhances membrane insertion of a glycosylation-deficient D2R mutant. It is well established that post-translational modification (PTM) of GPCRs along the excretory pathway greatly contributes to proper membrane expression (Dong et al., 2007). A PTM of particular importance for membrane expression of D2R is N-linked glycosylation at the N-terminus (Min et al., 2015). We determined whether APD treatment could enhance the membrane insertion an N-linked glycosylation-deficient D2R, a D2R construct even further restricted from cell surface expression. This mutant, D2R_{N5,N17,N23→Q} was created by causing three point mutations (N 5, 17,23 →Q) to remove N-linked glycosylation. We assessed the effect of APD treatment on the surface expression of D2R_{N5,N17,N23→Q} in the same manner as described above. Both haloperidol and clonidine substantially increased the total expressions of D2R_{N158A}(Figure 7C) and D2R_{N5,N17,N23→Q} (Figure 7E), but only haloperidol increased the cell-surface levels of D2R_{N5,N17,N23→Q} (Figure 7D). Thus, the efficacy of haloperidol and lack of efficacy of clozapine as pharmacochaperones of D2R is highlighted by D2R mutants that are further restricted from membrane insertion.

Haloperidol treatment increases detergent solubility of total D2R. We previously reported that D2R segregates predominantly into Triton X-100 insoluble biochemical fractions (Octeau et al., 2014; Sharma et al., 2013a). We also reported that dopamine treatment reduced the amount of Triton X-100 insoluble D2R expressed at the plasma membrane (Sharma et al., 2013a). Our lab has previously shown that D2R detergent solubility can be altered by APD treatment (Octeau, 2014). Therefore we asked what effect specifically haloperidol and clozapine treatment could have on the biochemical profile of D2R.

HEK293T cells expressing D2R were treated with receptor-saturating concentrations of the indicated APDs, and the TX-100-soluble and insoluble fractions of D2R were isolated and receptor expressed in each was measured as described in the Materials and Methods. Treatment with haloperidol significantly increased the detergent solubility of D2R, while clozapine did not (Fig 8B). In fact, clozapine *decreased* the detergent solubility of D2R with respect to vehicle. Thus we conclude that the APDs, except clozapine, efficaciously increase the detergent solubility of D2R, while clozapine is the only drug that decreases the detergent solubility.

Intracellular D2R restricted from interacting with ER marker PTP1B evidenced by BRET ratios. Previous studies have reported that detergent-insolubility can be characteristic of aggregated proteins (Basso et al., 2009; Schlager et al., 2012; Shaw et al., 2008). Because clozapine increases the detergent insolubility of intracellular D2R, we further characterized this receptor pool and investigated the possibility that ER-targeted D2R is aggregated. Based on the aforementioned PCCs and confocal images in ICC, we know that D2R predominantly co-localizes with ER marker Sec61 (Fig 4). However, though it may display co-localization, aggregated protein may not be able to interact functionally with other nearby proteins. To assess D2R's ability to interact with other ER targeted proteins, we utilized bioluminescent resonance energy transfer (BRET). We compared the ability of D2R fused with Renilla-luciferase (D2R-Rluc8) to participate in BRET with ER marker PTP1B with other cell surface and ER targeted GPCRs. Previously, the trafficking β 2-adrenergic receptor (β 2-AR) was compared with a misfolding β 2-AR mutant, β 2-AR_{W158A} via BRET with the PTP1B (Lan et al., 2012). It was reported that, based on the calculated BRET ratios occurring between each receptor construct and PTP1B, wild type β 2-AR displayed much greater membrane expression, while β 2-AR_{W158A} was indeed

retained in the ER (Lan et al., 2012). We performed ICC and obtained confocal images of the cellular localization β 2-AR and β 2-AR_{W158A}. Here we show dramatic cell surface expression of β 2-AR, while β 2-AR_{W158A} displays predominant co-localization with the ER-marker Sec61 (Fig 9A); this pattern is similar to that of D2R. This provided an opportunity to compare BRET efficiencies of two heavily membrane-targeted and ER-retained GPCRs belonging to different classes. As described in the Materials and Methods section, we measured BRET ratios occurring between PTP1B-Venus and either D2R-Rluc8, β 2-AR-Rluc8, or β 2-AR_{W158A}Rluc8. BRET ratios calculated between PTP1B-Venus and β 2-AR-Rluc8 represent the level of BRET occurring between an ER targeted marker, and a membrane targeted receptor with almost no ER co-localization. BRET ratios calculated for each of the three receptors were expressed as a percentage of the calculated BRET ratio for β 2-AR (Fig 9B). β 2-AR_{W158A} displayed a much higher BRET ratio than β 2-AR (Fig 9B), which is expected because it also exhibits much higher ER localization. Interestingly, D2R resulted in the lowest BRET ratio with PTP1B, despite displaying high ER localization (Fig 9B). In other words, D2Rs and β 2-ARs exhibit contrasting distributional and interactive characteristics. D2Rs are localized predominantly in the ER while β 2-AR are found mainly in the plasma membrane, but D2Rs interact with ER-targeted proteins less than β 2-ARs do. This suggests that D2R located in the ER may not functionally interact with other ER targeted proteins, thus and may be non-functional aggregated protein in the ER. Furthermore, since clozapine increases D2R total expression and detergent insolubility - without altering the solubility and only slightly affecting the expression of total surface D2Rs - it could actively promote the accumulation of insoluble, aggregated, non-interacting D2Rs in the ER. None of the other APDs seem to display this ability.

PTX co-expression lowers APD-mediated enhancement of surface D2R expression. We then explored the effect of G-protein inhibition on APD-induced enhancement of surface D2R expression. HEK293T cells were co-transfected with cDNAs for D2R and PTX, and treated with receptor-saturating concentrations of haloperidol, clozapine, or vehicle for 24 hr. Both haloperidol and clozapine were able to increase surface expression of D2R, and haloperidol-treated cells exhibited significantly higher surface D2R expression than those treated with clozapine (Fig 10A). Interestingly, the effect of haloperidol was dramatically reduced in cells co-expressing PTX relative to those expressing D2R alone, suggesting that G-protein inhibition reduces the ability of APDs to enhance surface expression of D2R.

Enhancement of surface D2R expression effect on G-protein signaling. In order to investigate the effect of increased surface D2R expression on G-protein activation, we utilized BRET to measure agonist-induced recruitment of G-protein receptor kinase (GRK) following 24 hr APD treatment as described in Materials and Methods. Measuring G-protein activation in this manner has been previously described and validated (Donthamsetti et al., 2015). BRET can only occur between the G $\beta\gamma$ subunit and GRK3 once the receptor has been activated by the agonist and G-proteins dissociate. HEK293T cells transiently expressing D2R, Gai, Venus 155-239 G β 1, Venus 155 G γ 2, and the masGRK3ct NanoLuc were treated with receptor-saturating concentrations of haloperidol, clozapine, or appropriate vehicle for 24 hr. To wash away APDs following treatment, cells were incubated with drug-free medium for 2 hr, and then washed 3x in 1XPBS. Net BRET between activated G-proteins and recruited GRK3 was calculated by subtracting the measured BRET ratio before the addition of agonist to the measured BRET ratio

following the addition of agonist. A dose-response curve for dopamine-induced activation of D2R-coupled G-proteins was generated, reaching maximum responses at ~100 nM dopamine (Fig 10B). Unfortunately, we were unable to obtain a dopamine response in APD treated cells even at dopamine concentrations of 10 μ M. Thus, we conclude that an inability to wash out the APDs following prolonged treatment prevents us from measuring agonist-induced G-protein activation by this method.

DISCUSSION

APDs act as active pharmacochaperones of D2R. Several studies have reported on the ability of ligands to act as pharmacochaperones for cell surface receptors (Lan et al., 2012; Lester et al., 2012; Wüller et al., 2004) by facilitating cellular receptor inter-compartmental translocation. These pharmacochaperones can serve to both rescue misfolded receptors, and to overcome coded ER retention signals to produce enhanced export trafficking of GPCRs (Lan et al., 2012; Lester et al., 2012; Wüller et al., 2004). It is well established that prolonged treatment with antagonists in animals can result in GPCR upregulation (Millan et al., 1988; Unterwald et al., 1995). We have conclusively shown here that APD treatment can actively enhance membrane insertion of D2R in the *absence of agonist*. With the exception of partial agonists like aripiprazole (Burris et al., 2002; Frankel and Schwartz, 2017), APDs are considered antagonists of D2R, and are thus assumed to have no intrinsic activity (Boyd and Mailman, 2012; Meltzer, 2013b; Sahlholm et al., 2014). Here we also present the first evidence that APD binding of D2R activates a cellular signal. The data presented in figure 1A-B clearly show that APD treatment enhances surface D2R signal relative to vehicle-treated cells, with clozapine treatment producing significantly less surface D2R expression than the other APDs. As shown in figure 1C, total D2R expression was similarly influenced by all tested APDs

(except aripiprazole), indicating that enhanced surface expression was not simply a result of increased total D2R expression. Results of the Halo-D2R experiments show that surface D2R degradation rates were not different in APD- and vehicle-treated cells. Thus, differences in surface receptor levels produced by APDs are a result of increased membrane insertion and not reduction in surface receptor degradation.

APD-induced membrane insertion of D2R is a result of direct, specific binding to the receptor, because APD-mediated effects on both surface and total D2R are diminished in the ligand-binding-deficient D2R_{D114A} mutant (fig 3C-D). Confocal images and PCCs derived from ICC, as depicted in figure 4, display haloperidol's ability to cause a redistribution of D2R from the ER to the plasma membrane, overcoming D2R's naturally coded ER retention. Surface expression of D2Rsh, which lacks the tri-arginine ER retention motif of D2R, is less affected by haloperidol treatment because they are already more extensively distributed on the cell surface. This further suggests that haloperidol is able to overcome ER retention signals of D2R. The shift in the dose-response curve of poorly membrane soluble amisulpride, in the presence of OCT1, indicates that the location of the key interaction for APD-enhanced membrane insertion occurs intracellularly (fig 6). Haloperidol also displays the ability to rescue the misfolded D2R_{W160A} mutant and mediate its membrane insertion, which is otherwise completely absent (fig 7). As protein folding and handling of misfolded protein, largely occur in the ER, this is further indication that the site of key interaction is not only intracellular, but also takes place in the ER. From this data, we reasonably conclude that in addition to blocking D2R-mediate G-protein signaling at the cell surface, APDs bind D2R intracellularly and overcome receptor misfolding and natural ER retention, to actively enhance membrane insertion of

D2R. Thus, APDs have intrinsic activity as active pharmacochaperones of D2R, and should be considered agonists for this effect.

Clozapine binding stabilizes D2R in a unique conformation, and displays unique molecular action at D2R. As previously mentioned, it has been extensively reported that clozapine, in practice and in numerous clinical studies, out performs all other APDs in effectively treating both the positive and negative symptoms of schizophrenia (Agid et al., 2010b; Lewis and Lieberman, 2008b; Meltzer, 2012b; Tandon R et al., 2007).

Hypothesized explanations for clozapine's superior efficacy arising from action at receptors other than D2R, such as muscarinic or adrenergic receptors, have fallen short, as these binding characteristics are shared by many of the other APDs (Jibson and Tandon, 1998b). As a result, many researchers are returning to the belief that action at D2R is essential for APD efficacy (Kapur and Mamo, 2003b). Here we present the first evidence of unique molecular actions of clozapine at D2R. The dose-response curves depicted in figure 1A clearly show clozapine's pharmacochaperone effects on D2R saturating at higher concentrations and only producing 35% of haloperidol's efficacy. Comparison with a wide array of other APDs revealed that long-term treatment with clozapine produced significantly less surface D2R expression than any of the other tested APDs except aripiprazole (Figure 1B). Evaluation of total D2R expression among indicated APD treated groups (Figure 1C) revealed similar increases in total D2R expression after treatment and other APDs, including haloperidol, indicating that the observed lower surface D2R expression in clozapine-treated cells was not a function of lower total receptor signal in those cells. As previously discussed, we have shown that the APD-induced enhancement of surface D2R is a result of enhanced membrane insertion of the receptor. Replacing with the ligand-binding deficient D2R_{D114A} mutant, diminishes effects

of both clozapine and haloperidol, indicating a like binding site for clozapine and the other APDs (fig 3C-D). Although, aripiprazole or clozapine treatment results in similar levels of surface D2R, aripiprazole-treated cells display dramatically less total D2R (fig 1C) and higher rates of surface D2R degradation (fig 2A) when compared with clozapine. Thus, for aripiprazole treatment to result in similar levels of surface D2R expression to that of clozapine, it must induce a higher rate of membrane insertion of D2R relative to that of clozapine. Therefore, we have clearly shown that in the absence of agonist, all tested APDs, including aripiprazole, enhance membrane insertion of D2R relative to clozapine. Confocal images and PCCs derived from ICC experiments reveal that, unlike haloperidol, clozapine does not cause redistribution of D2R from the ER to the cell membrane. Furthermore, co-treatment with clozapine resulted in blockade of haloperidol-mediated increases in surface D2R, indicating that while they are binding in the same location, clozapine and haloperidol are producing, in the absence of agonist, distinct effects at D2R. Clozapine was unable to reproduce haloperidol's ability to rescue misfolded D2R_{W160A} for efficient surface expression, despite resulting in similar total receptor signal. Thus, haloperidol can stabilize the misfolded D2R in a conformation suitable for membrane insertion while clozapine cannot, further highlighting the existence of distinct binding confirmations for the two drugs. We have previously shown that treatment with all tested APDs, except for clozapine, increases the detergent solubility of D2R (Octeau, 2014). Here, again, we show that while haloperidol treatment dramatically increases the detergent solubility relative to vehicle, clozapine treatment does not. Further, the data suggests that clozapine may in fact increase the detergent insolubility of D2R, but the effect is not statistically significant. Binding by all tested APDs, except for clozapine, significantly alters the biochemical properties of D2R. Thus we have unequivocally shown that although all the tested APDs share a common D2R binding site, only clozapine induces a

completely unique binding conformation of D2R, as evidenced by its distinct actions at the receptor. This is the first evidence of unique molecular actions of clozapine at D2R.

D2R ER retention and drug effective doses highlight physiological relevance. A common criticism of studies such as this is that drug doses used exceed physiologically relevant concentrations. It seems that such concerns do not apply here, because APD concentrations have been measured in the 1-5 μM range in both human and rat brains (Baldessarini et al., 1993; Froemming et al., 1989; Kornhuber et al., 2006). Further, the in the dose response curves shown in figure 1B, response maxima are achieved at concentrations in the 0.1 – 3 μM range, comparable to the in-brain measured concentrations.

It seems unlikely that either the observed intracellular distribution of D2R or possible protein misfolding is an artifact of overexpression in HEK293 cells. However, as previously stated, this pattern of distribution is typical of D2R in a variety of models and cell types (Fishburn et al., 1995b; Kovoov et al., 2005b; Prou et al., 2001b; Sharma et al., 2013b). Additionally, the tri-arginine motif found in the 3rd intracellular loop of D2R has been reported to target D2R to the ER. Hence, the intracellular accumulation of D2R observed here would be expected, as it appears naturally coded to do so. As previously stated, D2Rsh is a splicing variant of D2R, lacking 29 amino acids within the 3rd cytoplasmic loop, including the A267-269 tri-arginine ER retention motif in D2R long isoform. Since the intracellular localization of D2R has been shown to be heavily influenced by the A267-269 ER retention motif, it is not surprising that D2sh, which lacks this motif, displays much greater surface expression (Prou et al., 2001b). It has also been reported that actions of pharmacochaperones include overcoming ER retention signals to

promote export of transmembrane receptors to the cell surface (Wüller et al., 2004). If, as we propose, APDs are acting as pharmacochaperones, and, as has been reported, D2R contains a strong ER retention motif that D2Rsh lacks, then APD treatment should have a much greater effect on the surface expression of D2R long isoform than D2Rsh. This is exactly what we observed. The haloperidol-induced increase of surface D2Rsh was only half that of its effect on D2R. While clozapine increased surface expression of D2R by 60%, it did not alter surface D2Rsh expression. Therefore, we conclude that our observed intracellular, perinuclear expression of D2R is not an artifact of overexpression. Moreover, this highlights the physiological relevance of our finding, as D2R is endogenously coded for ER targeting, and APD treatment drastically alters that distribution.

Repeatability with multiple detection methods confirm results are not artifacts of the detection methodology. Recently, it has been reported that sulfation of N-terminus linked FLAG epitope-tagged D2R greatly reduces binding of Anti-FLAG M2 antibody (Hunter et al., 2016), and that surface D2R expression was underestimated because of increased FLAG epitope sulfation. Thus, some might argue that our observations are simply a function of APDs effecting FLAG-epitope post-translational modification, specifically its sulfation, rather than pharmacochaperone effects on D2R itself. We believe this is not the case for two reasons. First, the experiments revealing the differences in FLAG D2R recognition because of sulfation were done in HEK293 cells stably expressing FLAG D2R (Hunter et al., 2016). Therefore, total D2R expression levels were dramatically lower than in our model, and any differences in receptor recognition observed based on sulfation would be much more dramatic than in our system. Secondly, we have repeated the initial observation - done with Anti-FLAG M2 antibody - with multiple detection

methods. The Halotag experiments (Figure 2) utilize covalent linkage of PEG-biotin reagent, probed for with HRP-conjugated streptavidin, which cannot be explained away by FLAG-epitope sulfation. ICC experiments, completed in our lab, using D2R fused to yellow fluorescent protein display the same punctate, intracellular, and perinuclear distribution of FLAG D2R labeled with fluorophore-conjugated anti-FLAG M2 antibody (data not shown). Thus, we have repeated observations acquired with anti-FLAG M2 antibody with multiple detection methods, suggesting that the pattern of D2R localization we observe is not a function of differences in post-translation modification of the FLAG epitope.

Implications. Perhaps the unique binding interaction of clozapine with D2R that we report here contributes to its superior clinical efficacy. The concept of ligand bias, where ligands bind the same receptor, but produce distinct conformational changes resulting in selective receptor signal activation, has been observed for quite some time (Drake et al., 2008; Erickson et al., 2013; Luttrell, 2014; Makita and Iiri, 2013; Violin and Lefkowitz, 2007). Here we report for the first time that APDs are pharmacochaperone agonists for the promotion of D2R membrane insertion, as well as antagonists of endogenous ligand-induced G-protein signaling. Thus, we have displayed the ability of APDs to activate cellular signals not yet considered. Of all of the APDs that we tested, clozapine displayed the least pronounced intrinsic activity as a pharmacochaperone of D2R, and clearly displays unique binding interactions with the receptor. Perhaps, clozapine's seemingly bias-ligand binding profile, antagonizing D2R-mediated G-protein signaling while avoiding the pharmacochaperone effects of the other APDs is the key to its superior clinical efficacy. It is possible that the unique binding of clozapine to D2R either propagates or preserves cellular signals that the other APDs do not. If so, these findings

harbor great potential for the future of schizophrenia pharmacotherapy. As previously mentioned, because of the fatal agranulocytosis produced by clozapine, its utility and impact in the treatment of schizophrenia is drastically limited (Farooq and Taylor, 2011; Pirmohamed and Park, 1997). However, these results potentially provide a model for APD development, in which investigational compounds can be compared to clozapine to reproduce these observed unique receptor-ligand interactions, while avoiding agranulocytosis. Maybe these results are the basis for the development of safe and tolerable antipsychotics that, like clozapine, effectively treat all aspects of schizophrenia.

MATERIALS AND METHODS

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA) unless otherwise indicated.

Cell Culture. HEK293T cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum supplemented with penicillin and streptomycin, and grown at 37 °C and 5% CO₂. cDNA transfections were performed with Lipofectamine LTX according to the manufacturer's guidelines. Transfected DNA amounts were kept constant between groups using empty pcDNA 3.1 Zeo (+) plasmid vector.

cDNA Constructs. The N-terminal FLAG epitope tagged D2R (FLAG-D2R) long isoform and the N-terminal FLAG epitope tagged mu opioid receptor (FLAG-MOR) (Clever et al., 2012a), the β 2-adrenergic receptor RLuc8 fusion (β 2AR-RLuc8), the β 2AR_{W158A}-RLuc8, and V-PTP1b (Lan et al., 2012), the FLAG-D2R_{T225,S228,S229→A} (Clever et al., 2013), the Venus 155-239 G β 1, Venus 155 G γ 2, and the masGRK3ct NanoLuc (Masuho et al., 2015), the OCT1 transporter (Boxberger et al., 2014), the N-terminal FLAG epitope

tagged β 2AR (Hanyaloglu et al., 2005), the endoplasmic reticulum marker mCh-Sec61 beta (Zurek et al., 2011a), and the catalytic subunit of pertussis toxin-GFP fusion (Celver et al., 2010) have been previously described.

The HaloTag-D2R fusion consisted of the N-terminal influenza hemagglutinin signal sequence (MKTIIALSYIFCLVFA) followed by the FLAG epitope (DYKDDDDA), a two amino acid linker (TV) linker and the HaloTag enzyme (Los et al., 2008a) (Promega, Madison, WI) tethered to the N-terminus of D2R via a 3 amino acid linker (AAG). Thus, the FLAG-HaloTag-D2R construct consisted of, in order from the N to the C terminus, the influenza hemagglutinin signal sequence epitope, FLAG epitope, TV linker, HaloTag, AAG linker, and the human D2R long isoform. The FLAG-D2Rsh fusion was constructed by fusing the influenza hemagglutinin signal sequence and FLAG epitope to the N-terminus of the human D2R short isoform. The FLAG-D2R-RLuc8 construct was made by fusing the Renilla Luciferase mutant, RLuc8, to the C-terminus of the Flag-D2R. For the FLAG-D2R_{N5,N17,N23→Q}, the FLAG-D2R_{D114A} and the FLAG-D2R_{W158A} mutants, the mutations refer to mutations in residues numbered as in the non-tagged wild type human D2R long form.

Measurement of surface and total cellular FLAG-tagged receptor expression by ELISA with anti-FLAG antibody. To evaluate the effect of APD treatment on cell-surface expression of receptor, we employed a previously well characterized ELISA-based protocol that utilized anti-FLAG antibodies to specifically label FLAG-tagged receptors expressed on the surface of fixed non-permeabilized cells (Celver et al., 2010; Daigle et al., 2008; Walker et al., 1999). On day 1, HEK293 cells were seeded in a 96-well plate (5×10^4 cells/well) and transfected with plasmid vectors with cDNAs for the indicated FLAG-tagged receptor. 24 hr post-transfection, cells were treated with the specified

drugs, or corresponding vehicle, at the indicated concentrations, and for the indicated durations (usually 24 hr). After drug-treatment, cells were fixed with 4% v/v methanol-free paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in phosphate-buffered saline (PBS) for 15 min at 4 °C, and then washed 2x with PBS. Wells were blocked for 1 hr at 4 °C with 5% w/v nonfat milk dissolved in PBS. The levels of FLAG-tagged surface receptor were then assessed by probing with horse radish peroxidase (HRP)-conjugated mouse monoclonal anti-FLAG M2 antibody (1:5,000 dilution in 5% w/v nonfat milk dissolved in PBS) for 45 min at 20 °C, and then washed 2x in PBS. Supersignal West Femto chemiluminescent substrate (Pierce-ThermoFisher Scientific) was added to each well, and the luminescence signal was measured using a Glomax luminometer (Promega).

Total FLAG-tagged cellular receptor was evaluated in a similar manner, except that cells were fixed and permeabilized in 100% methanol (10 min, -20 °C) to allow antibody access to intracellular receptor. Subsequent washing, blocking, and antibody probing steps were identical to those described above.

Dose response curves for clozapine, haloperidol, olanzapine, amisulpride or tiapride-induced enhancement of surface D2R were generated by measuring surface receptor as described above. Effects of OCT1 co-expression on dose-response curves were tested by co-transfecting cDNA for the OCT1 transporter with that for FLAG-D2R.

Pulse-chase assay for specifically monitoring loss of cell-surface D2R. This assay has been previously validated and used to track endocytosis of G protein coupled receptors (GPCR) via pulse-chase biotinylation specifically of the cell-surface expressed GPCRs (Kumagai et al., 2015). HEK293T cells were seeded in 10 cm diameter culture

dishes, 2×10^6 cells per dish, transfected with HaloTag-D2R cDNA. The HaloTag D2R fusion construct is described above and contains HaloTag inserted at the extracellular N-terminus of D2R. HaloTag is a modified bacterial enzyme designed so that it may be specifically and covalently tagged by a wide variety of synthetic probes (Los et al., 2008a). 24 hr post-transfection, cell-surface HaloTag D2R was specifically biotinylated by incubating with the membrane-impermeable Halotag-PEG-Biotin reagent (100 nM in complete DMEM, 1 hr, 37 °C). Cells were then washed 2x with 1X PBS, to remove unreacted Halotag-PEG-Biotin reagent, seeded in a 96-well plate at a cell number of 160,000 cells/well and treated with 10 μ M haloperidol, clozapine, aripiprazole, or vehicle. Following 24 hr of APD treatment, cells were fixed in 4% methanol-free paraformaldehyde (v/v in 1X PBS, 15 min, 4 °C) and then washed 2x in 1X PBS. Wells were blocked with 3% BSA (w/v in 1X PBS) for 45 min at 20 °C. Biotinylated HaloTag receptor remaining at the cell-surface receptor was detected using horseradish-peroxidase (HRP)-conjugated streptavidin (1:10,000 dilution in 3% w/v BSA dissolved in 1X PBS). Supersignal West Femto chemiluminescent substrate was added to each well, and the HRP-catalyzed luminescent signal was detected and quantified using the Glomax luminometer.

Measurement of the effect of APD treatment on total cell-surface HaloTag D2R with HaloTag-PEG-Biotin reagent. Day 1 HEK293 cells were seeded in a 96 well plate at a density of 5×10^4 , and transfected with Halotag D2R cDNA. 24 hr post-transfection, cells were treated with 10 μ M haloperidol, clozapine, or vehicle. Following 24 hr of APD treatment, and prior to fixation, total cell-surface receptor was specifically biotinylated by incubating with the membrane-impermeable Halotag-PEG-Biotin reagent (100 nM in complete DMEM, 1 hr, 37 °C). Unreacted reagent was removed by washing 2X with

complete DMEM. Cells were then fixed in 4% methanol-free paraformaldehyde (v/v in 1X PBS, 15 min, 4 °C) and then washed 2x in 1X PBS. Wells were blocked with 3% BSA (w/v in 1X PBS) for 45 min at 20 °C. Biotinylated HaloTag receptor at the cell-surface was detected using horseradish-peroxidase (HRP)-conjugated streptavidin (1:10,000 dilution in 3% w/v BSA dissolved in 1X PBS). Supersignal West Femto chemiluminescent substrate was added to each well, and the HRP-catalyzed luminescent signal was detected and quantified using the Glomax luminometer.

Measurement of agonist-induced Halotag D2R internalization. To assess its functionality, we tested the ability of the Halotag D2R fusion to internalize using a variation of the same ELISA-based technique in a manner previously described and validated (Cerver et al., 2010; Oceau et al., 2014). HEK293 cells seeded in a 96-well plate at a density of 5×10^4 cells/well were transfected with Halotag D2R cDNA. 48 hr post-transfection cells were treated with dopamine (10 μ M from 10mM stock that contained 5mM ascorbic acid to avoid dopamine oxidation) or vehicle for 45 min at 37 °C. Following dopamine treatment, cells were fixed in 4% methanol-free paraformaldehyde (v/v in 1X PBS, 15 min, 4 °C) and then washed 2x in 1X PBS. Wells were blocked for 1 hr at 4 °C with 5% w/v nonfat milk dissolved in 1X PBS. The levels of FLAG-tagged surface receptor were then assessed by probing with horse radish peroxidase (HRP)-conjugated mouse monoclonal anti-FLAG M2 antibody (1:5,000 dilution in 5% w/v nonfat milk dissolved in PBS) for 45 min at 20 °C, and then washed 2x in 1X PBS. Supersignal West Femto chemiluminescent substrate was added to each well, and the HRP-catalyzed luminescent signal was detected and quantified using the Glomax luminometer.

Measurement of G-protein activation with BRET. We monitored dopamine-induced D2R-mediated G-protein activation via bioluminescence resonance energy transfer (BRET)

measuring recruitment of G-protein coupled receptor kinase. Utilization of BRET in this manner has been well validated (Kamal et al., 2009; Masuho et al., 2015). HEK293T cells were seeded in 6cm diameter culture dishes at a density of 1×10^6 cells/dish, and transfected with D2R, G α i, Venus 155-239 G β 1, Venus 155 G γ 2, and the masGRK3ct NanoLuc cDNA. 24 hr post transfection, media was replaced with fresh completed DMEM. 48 hr post-transfection, cells were washed 2x in 1X PBS, harvested by trituration, and seeded in opaque white-bottomed 96-well plates. Cells were then treated with specified concentrations of dopamine or vehicle, and luminescence measurements were made using the POLARstar OPTIMA multidetection microplate reader. Raw BRET signals were calculated as the emission intensity at 520-545 nm (acceptor) divided by the emission intensity at 475-495 (donor). Net BRET was found by subtracting the average raw BRET ratio before the addition of drug from the average raw BRET ratio after the addition of drug. Dopamine response was calculated by subtracted the net BRET in dopamine treated cells from that observed in vehicle treated cells.

G-protein coupled receptor ER co-localization measured with BRET. To assess the cellular localization of the indicated GPCRs, we employed a previously validated BRET based protocol (Lan et al., 2012). HEK293T cells were seeded in 6cm diameter culture dishes at a density of 1×10^6 cells/dish, and transfected with appropriate amounts of V-PTP1b, FLAG-D2R-RLuc8, β 2AR-RLuc8, or β 2AR W158A-RLuc8. 24 hr post transfection, media was replaced with fresh completed DMEM. 48 hr post-transfection, cells were washed 2x in 1X PBS, harvested by trituration, and seeded in opaque black and white 96-well plates. V-PTP1b fluorescent signal intensity was measured at 520-545 nm in the black-bottomed 96 well plate, following excitation at 490 nM using the POLARstar OPTIMA multidetection microplate reader. Coelenterazine h (5 μ M) was added to all

wells of the white-bottomed plate immediately prior to measurement, and luminescence measurements were made with the POLARstar OPTIMA multidetection microplate reader. V-PTP1b emissions measured at 520-545 nM were normalized against the measured fluorescence intensity for each transfection group. BRET ratios were then calculated by dividing the normalized 520-545 nM emission intensity by that measured at 475-495 nM.

Immunocytochemistry. HEK293T cells were cultured on cover slips, coated with poly-D-lysine, in a 12-well plate at a density of 1×10^5 cells/well, and transfected with indicated cDNA constructs. 24 hr post transfection, cells were treated with indicated antipsychotics or vehicle. 48 hr post-transfection, cells were fixed and permeabilized by incubation with ice cold methanol for 10 min on ice or at 4 °C. Cells were washed 3x in 1X PBS, and blocked in 5% (w/v in 1X PBS) nonfat milk for 1 hr at 20 °C. FLAG-tagged receptor was probed for with Anti-FLAG M2 Antibody (1:1000 dilution in 5% nonfat milk) overnight at 4 °C. Cells were then washed 2x with PBS for 15 min at 20 °C, and then probed with fluorophore-conjugated anti-mouse secondary antibody (1:1,000 dilution in 5% w/v Nonfat milk) for imaging under fluorescent microscopy. Images were taken with a Zeiss Confocal Microscope, and Pearson co-localization coefficients (PCCs) with indicated cellular markers determined using the Zeiss imaging software.

Triton X-100 Biochemical Fractionation of D2R. To assess APD effect on the detergent solubility of D2R, we employed a similar method of fractionation to those described in our previous publications (Octeau et al., 2014; Sharma et al., 2013b). Briefly, HEK293T cells expressing D2R were treated with indicated APDs at the specified concentrations, or vehicle for 24 hr. 48 hr post-transfection cells were lysed in TX100 lysis buffer consisting of 2% Triton X-100 (v/v in 1X PBS) and 1X SigmaFast Protease Inhibitor (made

according to manufacturer's guidelines, Sigma-Aldrich) 1 hr at 4 °C. To pellet TX100-insoluble proteins, cells were centrifuged at 10,000 g for 10 min at 4 °C. Supernatant fractions were separated, and the TX100-insoluble pellet was washed 2x in ice cold 1X PBS. Supernatant proteins (TX100-soluble) were precipitated by the addition of trichloroacetic acid (final concentration 10% v/v). Supernatant protein pellets were then washed 3x in ice cold 95% v/v acetone at 4 °C. Both TX100-soluble and –insoluble fractions were resuspended in equal volumes of SDS/urea sample buffer (2% w/v SDS, 0.01% w/v bromophenol blue, 8 M urea, 20 mM dithiothreitol, 50mM Tris-HCl, pH 6.8). Samples were heated for 10 min at 65 °C and then sonicated 20x at a power setting of 5 for approximately 0.5 sec to reduce viscosity. Equal volumes of the samples were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and relative protein expression was compared via Western blotting.

Assessment of APD effect on surface D2R expression via Western blot. HEK293T cells expressing D2R were treated with the indicated APDs and the specified concentrations or vehicle, approximately 24 hr post-transfection. 48 hr post transfection, total cell surface receptor was labeled by incubation with Anti-FLAG M2 antibody (1:5000 dilution in complete DMEM) for 1 hr at 37 °C. Unbound excess antibody was removed by washing 2x in 1X PBS, and TX100-soluble and –insoluble fractions were isolated as described above. Proteins were resolved sing SDS-PAGE, and relative protein expression was compared via Western Blotting. Relative expression of surface D2R was detected by probing blots with anti-IgG antibody against the M2 antibody.

Western Blotting. Proteins resolved by gel electrophoresis were transferred to methanol wetted PVDF membranes (Imobilon-FL, EMD Millipore) by Western blotting apparatus (IBI Scientific) in 1X Transfer buffer (25 mM Tris base, 192 mM glycine, 20% v/v

methanol). Membranes were blocked by incubation with 5% (w/v in 1X PBS) nonfat milk for 1 hr at 20 °C. FLAG epitope tagged proteins were detected by incubating the membranes with HRP-conjugated anti-FLAG M2 antibody (Sigma-Aldrich 1:5000 dilution in 5% w/v nonfat milk in 1X PBS). Following primary antibody incubation, membranes were washed 2x with 1X PBS for 15 min at 20 °C. When necessary, blots were then probed with HRP-conjugated secondary antibody, and washed again 2x in 1XPBS for 15 min at 20 °C. Supersignal West Femto chemiluminescent substrate was then added to each blot, and the chemiluminescent signal was detected using a Chemidoc XRS Molecular Imager (Bio-Rad Laboratories). When comparing TX100-soluble and –insoluble fractions of a cell sample, proteins in both fractions were loaded onto a single gel, and transferred to a single PVDF membrane.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: JS JCO AK. Performed the experiments: JS CI.

Analyzed the data: JS AK. Contributed reagents/ material/ analysis tools: JS JC TA DK.

Contributed to the writing of the manuscript: JS AK.

Fig. 1. Effect of antipsychotic drug (APD) treatments on cellular levels of D2 dopamine receptor constructs.

Fig. 2. APD modulation specifically of cell-surface D2R removal.

Fig. 3. Specificity of APD-mediated upregulation of cell surface receptors.

Fig. 4. Effect of APD treatments on the cellular localization of D2R.

Fig. 5. Effect of APD treatments on cellular levels of D2 dopamine receptor short form construct

Fig. 6. Cellular site for the action of APDs in enhancing cell surface D2R expression.

Fig. 7. Effect of APD treatment on the surface expression of misfolding and glycosylation D2R mutants.

Fig. 8. APD modulation of D2R detergent solubility.

Fig. 9. Interactions of $\beta 2AR$, the misfolding $\beta 2AR$ mutant, $\beta 2AR_{W158A}$ with the ER markers, Sec61 and PTP1b.

Fig. 10. G protein signaling and APD-mediated enhancement of D2R surface expression.

FIGURE LEGENDS

Fig. 1. Effect of antipsychotic drug (APD) treatments on cellular levels of D2 dopamine receptor constructs.

APD treatments were performed on HEK293 cells transiently expressing the indicated receptor constructs.

A. Dose-response curves of APD-induced enhancement of D2R surface expression and effect of clozapine (10 μ M) co-treatment on haloperidol dose-response curve. Cells were treated for 24 hr with receptor-saturating (10 μ M) concentrations of the indicated APDs. The levels of cell-surface D2R were then quantified by probing the intact, non-permeabilized cells with anti-FLAG antibody and are reported as a percent of haloperidol's maximum effect (percentage increase in surface receptor relative to vehicle). Treatments are labeled as: haloperidol alone (solid circles), olanzapine (diamonds), clozapine alone (solid squares), haloperidol and 10 μ M clozapine (open circles) Receptor levels after treatment with haloperidol only were significantly greater than all other treatments, receptor levels after treatment with olanzapine were significantly greater than those obtained after clozapine treatment, and receptor levels after treatment with haloperidol only were significantly different from those obtained after clozapine co-treatment (mean $\pm$ SEM, n = 7, Student's t-test, p < 0.05).

B. Effect of antipsychotic drug (APD) treatments on surface expression of D2R. The levels of cell-surface D2R transiently expressed in HEK293 cells were quantified, as in "A", after treatment with receptor-saturating (10 μ M) concentrations of the indicated APDs. [†]All APDs, except for clozapine and aripiprazole produced significantly enhanced surface D2R expression compared to vehicle and [‡]D2R surface expression after clozapine treatment was significantly less than after treatment with all APDs, except aripiprazole (mean $\pm$ SEM, n = 31-32, Tukey's HSD, p < 0.05).

C. Effect of APD treatments on total cellular expression of D2R, assessed by ELISA. Cells were treated, as in "A", with the indicated APDs (10 μ M, 24 hr), and permeabilized with the detergent, triton-X100 (TX100). Total cellular D2R was then assessed by probing with anti-FLAG antibody and levels are reported as a percent of the signal from vehicle-treated cells. [†]Aripiprazole displayed significantly less total cellular D2R signal than all other tested APD treatments (mean $\pm$ SEM, n = 6-16 Tukey's HSD, p < .05).

D. Time course of haloperidol effect on surface expression of D2R. Levels of cell-surface D2 were evaluated after treatment with Haloperidol (10 μ M) for 6, 12, and 24 hr, as in “A”, and are reported as a percentage of haloperidol’s effect following 24 hr treatment. Surface D2R expression levels after each time point were significantly different from each other (mean $\pm$ SEM, n = 6, Tukey’s HSD, p < 0.05).

Fig. 2. APD modulation specifically of cell-surface D2R removal.

A. Effect of antipsychotic drug (APD) treatment on the specific removal of FLAG-HaloTag D2R from the plasma membrane examined via pulse-chase labeling with biotin. HaloTag PEG Biotin reagent was used to specially biotinylate the cell surface pool of an N-terminal FLAG-HaloTag D2R construct transiently expressed in HEK293 cells. After washing-off unreacted HaloTag PEG Biotin reagent, cells were treated with the indicated APDs (10 μ M, 24 hr), and the biotin-tagged HaloTag D2R remaining at the surface after APD treatment was quantified by probing the intact, non-permeabilized cells with horseradish peroxidase (HRP)-conjugated streptavidin. [†]The levels of biotinylated receptor that remained at the surface after aripiprazole treatment was significantly lower than that observed after vehicle, haloperidol or clozapine treatment (mean $\pm$ SEM, n = 8, Tukey’s HSD, p < 0.05).

B. Effect of dopamine treatment on the internalization of the HaloTag D2R construct. HEK293 cells transiently expressing the extracellular N-terminal HaloTag D2R construct were treated with either vehicle or dopamine (10 μ M) for 45 min. The N-terminal HaloTag D2R also contains an extracellular FLAG epitope and the levels of the receptor construct remaining at the cell surface after dopamine treatment were quantified by probing the intact, non-permeabilized cells with anti-FLAG antibody and are reported as a percent of the signal from the vehicle-treated cells. [†]Surface receptor levels were significantly less after dopamine treatment (mean $\pm$ SEM, n = 14-16, Student’s t-test, p < 0.05).

C. Effect of antipsychotic drug (APD) treatment on surface expression of the HaloTag D2R construct. HEK293 cells transiently expressing the extracellular N-terminal HaloTag D2R construct were treated with the indicated APDs (10 μ M, 24 hr) following which the surface receptor was specifically biotinylated by treating the intact cells with HaloTag

PEG Biotin ligand. The levels of biotinylated receptor were then quantified by probing the intact, cells with HRP-conjugated streptavidin and are reported as a percent of the signal from vehicle-treated cells. [†]The levels of cell-surface receptor after either haloperidol or clozapine treatment was significantly higher than with vehicle and significantly different from each other (mean $\pm$ SEM, n = 14-16, Tukey's HSD, p < 0.05).

Fig. 3. Specificity of APD-mediated upregulation of cell surface receptors.

A. Effect of antipsychotic drug (APD) treatment on the surface expression of the β 2-adrenergic receptor (β 2AR). HEK293 cells transiently expressing an extracellular N-terminal FLAG-tagged β 2-AR construct were treated with the indicated APDs (10 μ M, 24 hr). Following APD treatment, the levels of cell-surface β 2AR were quantified by probing the intact, non-permeabilized cells with anti-FLAG antibody and are reported as a percent of the signal from vehicle-treated cells (mean $\pm$ SEM, n = 12).

B. Effect of antipsychotic drug (APD) treatment on the surface expression of the mu-opioid receptor (MOR). The levels of cell-surface MOR transiently expressed in HEK293 cells were evaluated after treatment with the indicated APDs (10 μ M, 24 hr) as in "A" and are reported as a percent of the signal from vehicle-treated cells (mean $\pm$ SEM, n = 12).

C. Effect of antipsychotic drug (APD) treatment on the surface expression of the non-binding D2R mutant, D2R D114A. The levels of cell-surface receptor in HEK293 cells transiently transfected expressing an extracellular N-terminal FLAG-tagged D2R D114A construct were evaluated after treatment with the indicated APDs (10 μ M, 24 hr) as in "A", and are reported as a percent of the signal from vehicle-treated cells (mean $\pm$ SEM, n = 16).

D. Effect of antipsychotic drug (APD) treatment on the total cellular expression of the non-binding D2R mutant, D2R D114A. The levels of total receptor in HEK293 cells transiently expressing the extracellular N-terminal FLAG-tagged D2R D114A construct were evaluated after treatment with the indicated APDs (10 μ M, 24 hr) by probing the triton-X100 (TX100) permeabilized cells with anti-FLAG antibody and are reported as a percent of the signal from vehicle-treated cells (mean $\pm$ SEM, n = 16).

Fig. 4. Effect of APD treatments on the cellular localization of D2R.

A. Confocal microscopy images of cellular distribution of D2R after APD treatments.

HEK293 cells transiently co-expressing the extracellular N-terminal FLAG-tagged D2R construct and a Sec61-RFP fusion, a marker for the endoplasmic reticulum (ER) were treated with the indicated APDs (10 μ M, 24 hr). Representative images of D2R cellular distribution (green, left column), Sec61 cellular distribution (red, central column) and the level of D2R-Sec61 co-localization (yellow, right column) following treatment with vehicle (top row), haloperidol (central row) and clozapine (bottom row) are depicted. Each row represents confocal microscope scans of the same field.

B. Quantification of the extent of colocalization between D2R and Sec61-RFP. The graph depicts the colocalization coefficient, quantified in cells coexpressing D2R and Sec61-RFP, and defined as the number of pixels positive for both D2R and Sec61, expressed as a percent of pixels positive for D2R, after APDs treatment. [†]The correlation coefficient of D2R colocalized with Sec61 was significantly reduced after haloperidol treatment when compared to either vehicle or clozapine treatment (mean $\pm$ SEM, n = 25-46 cells, Tukey's HSD, p < 0.05).

Fig. 5. Effect of APD treatments on cellular levels of D2 dopamine receptor short form construct

A. Effect of APD treatments on surface expression of the D2-dopamine receptor short form (D2Rsh) construct.

Cells were treated for 24 hr with receptor-saturating (10 μ M) concentrations of the indicated APDs. The levels of cell-surface D2Rsh were then quantified by probing the intact, non-permeabilized cells with anti-FLAG antibody and are reported as a percent of the signal from vehicle-treated cells. [†]The levels of cell-surface D2Rsh measured after haloperidol treatment was significantly greater than after clozapine or vehicle treatment (mean $\pm$ SEM, n = 12, Tukey's HSD, p < 0.05).

B. Effect of APD treatments on total cellular expression of D2Rsh assessed by ELISA.

Total cellular levels of D2Rsh were evaluated after treatment with the indicated APDs (10 μ M) as in "A" and are reported as a percent of the signal from vehicle-treated cells (mean $\pm$ SEM, n = 16).

Fig. 6. Cellular site for the action of APDs in enhancing cell surface D2R expression.

A. Effect of coexpression of the OCT1 transporter on the dose-response curves for amisulpride-mediated enhancement of cell-surface D2R expression. HEK293 cells transiently expressing the extracellular N-terminal FLAG-tagged D2R construct alone (circles) or coexpressing the OCT1 transporter (squares) were treated (24 hr) with the indicated concentrations of amisulpride. The amisulpride-mediated enhancement (over vehicle) of cell-surface D2R was quantified by probing the intact cells with anti-FLAG antibody and is plotted as a percent of the maximum response (mean $\pm$ SEM, $n = 15$). The EC_{50} that was modeled for amisulpride enhancement of cell-surface D2R expression was significantly less in OCT1 cDNA cotransfected cells (10 nM and 663 nM, respectively). The 95% confidence interval for the EC_{50} ratio ranged from 0.003 to 0.03.

B. Effect of coexpression of the OCT1 transporter on the dose-response curves for tiapride-mediated enhancement of cell-surface D2R expression. Dose-response curves for tiapride-mediated enhancement of cell-surface D2R expression were generated, plotted and modeled as in “A” (mean $\pm$ SEM, $n = 15-16$).

Fig. 7. Effect of APD treatment on the surface expression of misfolding and glycosylation D2R mutants.

A. Effect of antipsychotic drug (APD) treatment on the surface expression of the misfolding D2R mutant, D2R_{W158A}. HEK293 cells transiently expressing an extracellular N-terminal FLAG-tagged D2R_{W158A} mutant construct were treated with the indicated APDs (10 μ M, 24 hr). The relative levels of cell-surface receptor were then quantified by probing intact cells with anti-FLAG antibody and are reported as a percent of the signal from vehicle-treated cells. [†]The signal for cell-surface receptor after haloperidol treatment was significantly higher than in untransfected cells, or cells treated with either vehicle or clozapine (mean $\pm$ SEM, $n = 7-14$, Tukey’s HSD, $p < 0.05$).

B. Effect of antipsychotic drug (APD) treatment on the total cellular expression of the misfolding D2R mutant, D2R_{W158A}. Representative Western blot depicting the effect of

treatment with the indicated APDs (10 μ M, 24 hr) on total cellular expression of misfolding D2R mutant, D2R_{W158A}.

C. Quantification of total cellular expression of the misfolding D2R mutant, D2R_{W158A}.

HEK293 cells transiently expressing the extracellular N-terminal FLAG-tagged D2R_{W158A} mutant construct were treated with the indicated APDs (10 μ M, 24 hr) as in “A”. After APD treatment, Western blots of the cell lysates subjected to SDS PAGE were probed with either anti-FLAG antibody or an anti-actin antibody and total cellular receptor signal was normalized to the actin signal and is reported as a percent of the signal from vehicle-treated cells (*right panel*) (mean $\pm$ SEM, n = 3).

D. Effect of antipsychotic drug (APD) treatment on the surface expression of the D2R glycosylation mutant, D2R_{N5,N17,N23→Q}.

HEK293 cells transiently expressing an extracellular N-terminal FLAG-tagged D2R_{N5,N17,N23→Q} mutant construct were treated with the indicated APDs (10 μ M, 24 hr) and the relative levels of cell-surface receptor were quantified as in “A” and are reported as a percent of the signal from vehicle-treated cells. †The signal for cell-surface receptor after haloperidol treatment was significantly higher than in untransfected cells, or cells treated with either vehicle or clozapine (mean $\pm$ SEM, n = 7-14, Tukey’s HSD, p < 0.05).

E. Effect of antipsychotic drug (APD) treatment on the total cellular expression of the D2R glycosylation mutant, D2R_{N5,N17,N23→Q}.

HEK293 cells transiently expressing an extracellular N-terminal FLAG-tagged D2R_{N5,N17,N23→Q} mutant construct were treated with the indicated APDs (10 μ M, 24 hr), as in “C”, and the relative levels of total receptor were quantified by probing triton-X100 (TX100) permeabilized cells with anti-FLAG antibody and are reported as a percent of the signal from vehicle-treated cells (mean $\pm$ SEM, n = 16) .

Fig. 8. APD Modulation of D2R solubility

A. Effect of APD treatment on the TX100 solubility of D2R. Representative Western blot depicting the effect of treatment with the indicated APDs (10 μ M, 24 hr) on the segregation of D2R, transiently expressed in HEK293 cells, into TX100-soluble (S) and insoluble (I) biochemical fractions.

B. Quantification of the TX100 solubility of D2R after APD treatment. Quantification of the percent of total cellular D2R, that segregates into the TX100-soluble fraction, as

shown in “A”, after treatment with the indicated APDs (10 μ M, 24 hr). [†]Treatment with haloperidol, but not clozapine, increased the TX100-solubility of D2R compared to vehicle (mean $\pm$ SEM; n = 4-6, Tukey’s HSD, p < 0.05).

Fig. 9. Interactions of β 2AR, the misfolding β 2AR mutant, β 2AR_{W160A} with the ER markers, Sec61 and PTP1b.

A. Confocal microscopy images of cellular localization of β 2AR and the misfolding β 2AR mutant, β 2AR_{W160A} with respect to the ER marker, Sec61. Representative images of the cellular distribution of the N-terminal FLAG-tagged β 2AR (green, top left, wild-type), the N-terminal FLAG-tagged misfolding β 2AR mutant, β 2AR_{W160A} (green, bottom left) and the ER marker fusion protein, Sec61-RFP (red, central column) and the level of receptor-Sec61-RFP co-localization (yellow, right column) in HEK293 cells transiently expressing the respective constructs.

B. Relative BRET between the ER marker Venus-PTP1b (V-PTP1b,) and β 2AR-RLuc8, β 2AR_{W160A}-RLuc8 or FLAG-D2R-RLuc8. BRET-mediated excitation of V-PTP1b by the indicated receptor-RLuc8 fusions was normalized against expression levels of both V-PTP1b and the receptor-RLuc8 fusion and then reported as a percent of the normalized BRET signal obtained with β 2AR-RLuc. [†]All of the BRET responses are significantly different from each other and [‡]BRET between V-PTP1b and the D2R construct was significantly less than with either the β 2AR or the misfolding β 2AR_{W158A} construct (mean $\pm$ SEM, n = 8 cells, Tukey’s HSD, p < 0.05).

Fig. 10. G protein signaling and APD-mediated enhancement of D2R surface expression.

A. Effect of pertussis-toxin (PTX)-co-expression on APD-mediated enhancement of D2R surface expression. HEK293 cells transiently coexpressing the extracellular N-terminal FLAG-tagged D2R construct and a fusion of the catalytic subunit of PTX with GFP were treated with the indicated APDs (10 μ M, 24 hr) and the relative levels of cell-surface receptor were quantified and reported as a percent of the signal from vehicle-treated cells. [†]The signal for cell-surface receptor after treatment with either clozapine or haloperidol was significantly different and different from that in vehicle-treated cells (mean $\pm$ SEM, n = 16, Tukey’s HSD, p < 0.05).

B. Effect of APD treatment on dopamine and D2R-mediated G protein activation.

HEK293 cells transiently coexpressing constructs for D2R, G α , Venus-G $\beta\gamma$, masGRK3ct-NanoLuc were treated with either vehicle, haloperidol or clozapine (10 μ M, 24 hr) after which the cells were washed and then treated with the indicated concentrations of dopamine. The level of dopamine-elicited G protein activation was measured by evaluating the increase in BRET that occurs between free G $\beta\gamma$ -Venus and the masGRK3ct-NanoLuc construct and are plotted as a function of the maximal response obtained with 10 μ M dopamine (mean $\pm$ SEM, n = 3-6). The EC₅₀ for dopamine in the vehicle-treated cells obtained by nonlinear fit of the data was 24 ± 3.6 nM. No dopamine responses were obtained in the APD-treated cells even after cells were washed for 2 hr.

REFERENCES

- Agid, O., Kapur, S., and Remington, G. (2008). Emerging drugs for schizophrenia. *Expert Opin Emerg Drugs* 13, 479–495.
- Agid, O., Foussias, G., Singh, S., and Remington, G. (2010a). Where to position clozapine: re-examining the evidence. *Can J Psychiatry* 55, 677–684.
- Agid, O., Foussias, G., Singh, S., and Remington, G. (2010b). Where to position clozapine: re-examining the evidence. *Can. J. Psychiatry Rev. Can. Psychiatr.* 55, 677–684.
- Allen, J.A., Yost, J.M., Setola, V., Chen, X., Sassano, M.F., Chen, M., Peterson, S., Yadav, P.N., Huang, X.P., Feng, B., et al. (2011). Discovery of beta-arrestin-biased dopamine D2 ligands for probing signal transduction pathways essential for antipsychotic efficacy. *Proc Natl Acad Sci U S A* 108, 18488–18493.
- Baldessarini, R.J., Centorrino, F., Flood, J.G., Volpicelli, S.A., Huston-Lyons, D., and Cohen, B.M. (1993). Tissue concentrations of clozapine and its metabolites in the rat. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* 9, 117–124.
- Basso, M., Samengo, G., Nardo, G., Massignan, T., D'Alessandro, G., Tartari, S., Cantoni, L., Marino, M., Cheroni, C., Biasi, S.D., et al. (2009). Characterization of Detergent-Insoluble Proteins in ALS Indicates a Causal Link between Nitritative Stress and Aggregation in Pathogenesis. *PLOS ONE* 4, e8130.
- Beaulieu, J.-M., and Gainetdinov, R.R. (2011). The physiology, signaling, and pharmacology of dopamine receptors. *Pharmacol. Rev.* 63, 182–217.
- Beaulieu, J.-M., Espinoza, S., and Gainetdinov, R.R. (2015). Dopamine receptors - IUPHAR Review 13. *Br. J. Pharmacol.* 172, 1–23.

- Boxberger, K.H., Hagenbuch, B., and Lampe, J.N. (2014). Common drugs inhibit human organic cation transporter 1 (OCT1)-mediated neurotransmitter uptake. *Drug Metab. Dispos. Biol. Fate Chem.* *42*, 990–995.
- Boyd, K.N., and Mailman, R.B. (2012). Dopamine receptor signaling and current and future antipsychotic drugs. *Handb. Exp. Pharmacol.* 53–86.
- Burris, K.D., Molski, T.F., Xu, C., Ryan, E., Tottori, K., Kikuchi, T., Yocca, F.D., and Molinoff, P.B. (2002). Aripiprazole, a Novel Antipsychotic, Is a High-Affinity Partial Agonist at Human Dopamine D2 Receptors. *J. Pharmacol. Exp. Ther.* *302*, 381–389.
- Casey, D.E. (1989). Clozapine: neuroleptic-induced EPS and tardive dyskinesia. *Psychopharmacology (Berl.) 99 Suppl.*, S47–53.
- Celver, J., Sharma, M., and Kovoov, A. (2010). RGS9-2 mediates specific inhibition of agonist-induced internalization of D2-dopamine receptors. *J. Neurochem.* *114*, 739–749.
- Celver, J., Sharma, M., and Kovoov, A. (2012). D(2)-Dopamine receptors target regulator of G protein signaling 9-2 to detergent-resistant membrane fractions. *J. Neurochem.* *120*, 56–69.
- Celver, J., Sharma, M., Thanawala, V., Oceau, J.C., and Kovoov, A. (2013). Arrestin-dependent but G-protein coupled receptor kinase-independent uncoupling of D2-dopamine receptors. *J. Neurochem.* *127*, 57–65.
- Clayton, C.C., Donthamsetti, P., Lambert, N.A., Javitch, J.A., and Neve, K.A. (2014). Mutation of three residues in the third intracellular loop of the dopamine D2 receptor creates an internalization-defective receptor. *J. Biol. Chem.* *289*, 33663–33675.
- Daigle, T.L., Kearn, C.S., and Mackie, K. (2008). Rapid CB1 cannabinoid receptor desensitization defines the time course of ERK1/2 MAP kinase signaling. *Neuropharmacology* *54*, 36–44.
- Dayalu, P., and Chou, K.L. (2008). Antipsychotic-induced extrapyramidal symptoms and their management. *Expert Opin. Pharmacother.* *9*, 1451–1462.
- Dong, C., Filipeanu, C.M., Duvernay, M.T., and Wu, G. (2007). Regulation of G protein-coupled receptor export trafficking. *Biochim. Biophys. Acta* *1768*, 853–870.
- Donthamsetti, P., Quejada, J.R., Javitch, J.A., Gurevich, V.V., and Lambert, N.A. (2015). Using bioluminescent resonance energy transfer (BRET) to characterize agonist-induced arrestin recruitment to modified and unmodified G protein-coupled receptors (GPCRs). *Curr. Protoc. Pharmacol. Editor. Board SJ Enna Ed.--Chief AI* *70*, 2.14.1–2.14.14.
- Dos Santos Pereira, J.N., Tadjerpisheh, S., Abu Abed, M., Saadatmand, A.R., Weksler, B., Romero, I.A., Couraud, P.-O., Brockmüller, J., and Tzvetkov, M.V. (2014). The poorly membrane permeable antipsychotic drugs amisulpride and sulpiride are substrates of the organic cation transporters from the SLC22 family. *AAPS J.* *16*, 1247–1258.

- Drake, M.T., Violin, J.D., Whalen, E.J., Wisler, J.W., Shenoy, S.K., and Lefkowitz, R.J. (2008). β -Arrestin-biased Agonism at the β 2-Adrenergic Receptor. *J. Biol. Chem.* 283, 5669–5676.
- Erickson, C.E., Gul, R., Blessing, C.P., Nguyen, J., Liu, T., Pulakat, L., Bastepe, M., Jackson, E.K., and Andresen, B.T. (2013). The β -blocker Nebivolol Is a GRK/ β -arrestin Biased Agonist. *PLoS ONE* 8.
- Essali, A., Al-Haj Haasan, N., Li, C., and Rathbone, J. (2009). Clozapine versus typical neuroleptic medication for schizophrenia. *Cochrane Database Syst Rev* CD000059.
- Farooq, S., and Taylor, M. (2011). Clozapine: dangerous orphan or neglected friend? *Br. J. Psychiatry* 198, 247–249.
- Fishburn, C.S., Elazar, Z., and Fuchs, S. (1995a). Differential glycosylation and intracellular trafficking for the long and short isoforms of the D2 dopamine receptor. *J Biol Chem* 270, 29819–29824.
- Fishburn, C.S., Elazar, Z., and Fuchs, S. (1995b). Differential glycosylation and intracellular trafficking for the long and short isoforms of the D2 dopamine receptor. *J. Biol. Chem.* 270, 29819–29824.
- Foussias, G., and Remington, G. (2010). Antipsychotics and schizophrenia: from efficacy and effectiveness to clinical decision-making. *Can. J. Psychiatry Rev. Can. Psychiatr.* 55, 117–125.
- Frankel, J.S., and Schwartz, T.L. (2017). Brexpiprazole and cariprazine: distinguishing two new atypical antipsychotics from the original dopamine stabilizer aripiprazole. *Ther. Adv. Psychopharmacol.* 7, 29–41.
- Freedman, R. (2003). Schizophrenia. *N Engl J Med* 349, 1738–49.
- Friedman, J.H. (2013). Parkinson disease psychosis: Update. *Behav. Neurol.* 27, 469–477.
- Froemming, J.S., Lam, Y.W., Jann, M.W., and Davis, C.M. (1989). Pharmacokinetics of haloperidol. *Clin. Pharmacokinet.* 17, 396–423.
- Gurevich, E.V., Gainetdinov, R.R., and Gurevich, V.V. (2016). G protein-coupled receptor kinases as regulators of dopamine receptor functions. *Pharmacol. Res.* 111, 1–16.
- Han, Y., Moreira, I.S., Urizar, E., Weinstein, H., and Javitch, J.A. (2009). Allosteric communication between protomers of dopamine class A GPCR dimers modulates activation. *Nat. Chem. Biol.* 5, 688–695.
- Hanyaloglu, A.C., McCullagh, E., and von Zastrow, M. (2005). Essential role of Hrs in a recycling mechanism mediating functional resensitization of cell signaling. *EMBO J.* 24, 2265–2283.

- Hunter, M.R., Grimsey, N.L., and Glass, M. (2016). Sulfation of the FLAG epitope is affected by co-expression of G protein-coupled receptors in a mammalian cell model. *Sci. Rep.* 6, 27316.
- Iqbal, M.M., Rahman, A., Husain, Z., Mahmud, S.Z., Ryan, W.G., and Feldman, J.M. (2003). Clozapine: a clinical review of adverse effects and management. *Ann. Clin. Psychiatry Off. J. Am. Acad. Clin. Psychiatr.* 15, 33–48.
- Jibson, M.D., and Tandon, R. (1998a). New atypical antipsychotic medications. *J Psychiatr Res* 32, 215–28.
- Jibson, M.D., and Tandon, R. (1998b). New atypical antipsychotic medications. *J. Psychiatr. Res.* 32, 215–228.
- Kamal, M., Marquez, M., Vauthier, V., Leloire, A., Froguel, P., Jockers, R., and Couturier, C. (2009). Improved donor/acceptor BRET couples for monitoring β -arrestin recruitment to G protein-coupled receptors. *Biotechnol. J.* 4, 1337–1344.
- Kang, X., and Simpson, G.M. (2010). Clozapine: more side effects but still the best antipsychotic. *J Clin Psychiatry* 71, 982–983.
- Kapur, S., and Mamo, D. (2003a). Half a century of antipsychotics and still a central role for dopamine D2 receptors. *Prog Neuropsychopharmacol Biol Psychiatry* 27, 1081–90.
- Kapur, S., and Mamo, D. (2003b). Half a century of antipsychotics and still a central role for dopamine D2 receptors. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 27, 1081–1090.
- Kapur, S., and Remington, G. (2001). Dopamine D(2) receptors and their role in atypical antipsychotic action: still necessary and may even be sufficient. *Biol Psychiatry* 50, 873–883.
- Kerwin, R.W., and Bolonna, A.A. (2004). Is clozapine antisuicidal? *Expert Rev Neurother* 4, 187–190.
- Kim, O.-J., Ariano, M.A., Namkung, Y., Marinec, P., Kim, E., Han, J., and Sibley, D.R. (2008). D2 dopamine receptor expression and trafficking is regulated through direct interactions with ZIP. *J. Neurochem.* 106, 83–95.
- Kornhuber, J., Wiltfang, J., Riederer, P., and Bleich, S. (2006). Neuroleptic drugs in the human brain. *Eur. Arch. Psychiatry Clin. Neurosci.* 256, 274–280.
- Kovoor, A., Seyffarth, P., Ebert, J., Barghshoon, S., Chen, C.-K., Schwarz, S., Axelrod, J.D., Cheyette, B.N.R., Simon, M.I., Lester, H.A., et al. (2005a). D2 dopamine receptors colocalize regulator of G-protein signaling 9-2 (RGS9-2) via the RGS9 DEP domain, and RGS9 knock-out mice develop dyskinesias associated with dopamine pathways. *J. Neurosci. Off. J. Soc. Neurosci.* 25, 2157–2165.
- Kovoor, A., Seyffarth, P., Ebert, J., Barghshoon, S., Chen, C.-K., Schwarz, S., Axelrod, J.D., Cheyette, B.N.R., Simon, M.I., Lester, H.A., et al. (2005b). D2 Dopamine Receptors

Colocalize Regulator of G-Protein Signaling 9-2 (RGS9-2) via the RGS9 DEP Domain, and RGS9 Knock-Out Mice Develop Dyskinesias Associated with Dopamine Pathways. *J. Neurosci.* 25, 2157–2165.

Kubale, V., Blagotinšek, K., Nøhr, J., Eidne, K.A., and Vrecl, M. (2016). The Conserved Arginine Cluster in the Insert of the Third Cytoplasmic Loop of the Long Form of the D2 Dopamine Receptor (D2L-R) Acts as an Intracellular Retention Signal. *Int. J. Mol. Sci.* 17, 1152.

Kumagai, H., Ikeda, Y., Motozawa, Y., Fujishiro, M., Okamura, T., Fujio, K., Okazaki, H., Nomura, S., Takeda, N., Harada, M., et al. (2015). Quantitative Measurement of GPCR Endocytosis via Pulse-Chase Covalent Labeling. *PloS One* 10, e0129394.

Kuroki, T., Nagao, N., and Nakahara, T. (2008). Neuropharmacology of second-generation antipsychotic drugs: a validity of the serotonin-dopamine hypothesis. *Prog Brain Res* 172, 199–212.

Lan, T.-H., Liu, Q., Li, C., Wu, G., and Lambert, N.A. (2012). Sensitive and high resolution localization and tracking of membrane proteins in live cells with BRET. *Traffic Cph. Den.* 13, 1450–1456.

Lester, H.A., Miwa, J.M., and Srinivasan, R. (2012). Psychiatric drugs bind to classical targets within early exocytotic pathways: therapeutic effects. *Biol. Psychiatry* 72, 907–915.

Lewis, S., and Lieberman, J. (2008a). CATIE and CUtLASS: can we handle the truth? *Br J Psychiatry* 192, 161–163.

Lewis, S., and Lieberman, J. (2008b). CATIE and CUtLASS: can we handle the truth? *Br. J. Psychiatry* 192, 161–163.

Los, G.V., Encell, L.P., McDougall, M.G., Hartzell, D.D., Karassina, N., Zimprich, C., Wood, M.G., Learish, R., Ohana, R.F., Urh, M., et al. (2008). HaloTag: a novel protein labeling technology for cell imaging and protein analysis. *ACS Chem. Biol.* 3, 373–382.

Luttrell, L.M. (2014). Minireview: More than just a hammer: ligand “bias” and pharmaceutical discovery. *Mol. Endocrinol. Baltim. Md* 28, 281–294.

Makita, N., and Iiri, T. (2013). Biased agonism: a novel paradigm in G protein-coupled receptor signaling observed in acquired hypocalciuric hypercalcemia [Review]. *Endocr. J. advpub.*

Manschreck, T.C., and Boshes, R.A. (2007). The CATIE schizophrenia trial: results, impact, controversy. *Harv Rev Psychiatry* 15, 245–258.

Margolese, H.C., Chouinard, G., Kolivakis, T.T., Beauclair, L., and Miller, R. (2005). Tardive dyskinesia in the era of typical and atypical antipsychotics. Part 1: pathophysiology and mechanisms of induction. *Can J Psychiatry* 50, 541–7.

- Masuho, I., Martemyanov, K.A., and Lambert, N.A. (2015). Monitoring G protein activation in cells with BRET. *Methods Mol. Biol. Clifton NJ* 1335, 107–113.
- Meltzer, H.Y. (2012a). Clozapine: balancing safety with superior antipsychotic efficacy. *Clin. Schizophr. Relat. Psychoses* 6, 134–144.
- Meltzer, H.Y. (2012b). Clozapine. *Clin. Schizophr. Relat. Psychoses* 6, 134–144.
- Meltzer, H.Y. (2013a). Update on typical and atypical antipsychotic drugs. *Annu. Rev. Med.* 64, 393–406.
- Meltzer, H.Y. (2013b). Update on Typical and Atypical Antipsychotic Drugs. *Annu. Rev. Med.* 64, 393–406.
- Meltzer, H.Y., and Huang, M. (2008). In vivo actions of atypical antipsychotic drug on serotonergic and dopaminergic systems. *Prog Brain Res* 172, 177–197.
- Meltzer, H.Y., Li, Z., Kaneda, Y., and Ichikawa, J. (2003). Serotonin receptors: their key role in drugs to treat schizophrenia. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 27, 1159–1172.
- Millan, M.J., Morris, B.J., and Herz, A. (1988). Antagonist-induced opioid receptor up-regulation. I. Characterization of supersensitivity to selective mu and kappa agonists. *J. Pharmacol. Exp. Ther.* 247, 721–728.
- Min, C., Zheng, M., Zhang, X., Guo, S., Kwon, K.-J., Shin, C.Y., Kim, H.-S., Cheon, S.H., and Kim, K.-M. (2015). N-linked Glycosylation on the N-terminus of the dopamine D2 and D3 receptors determines receptor association with specific microdomains in the plasma membrane. *Biochim. Biophys. Acta* 1853, 41–51.
- Missale, C., Nash, S.R., Robinson, S.W., Jaber, M., and Caron, M.G. (1998). Dopamine receptors: from structure to function. *Physiol Rev* 78, 189–225.
- Naheed, M., and Green, B. (2001). Focus on clozapine. *Curr Med Res Opin* 17, 223–229.
- Namkung, Y., and Sibley, D.R. (2004). Protein kinase C mediates phosphorylation, desensitization, and trafficking of the D2 dopamine receptor. *J Biol Chem* 279, 49533–49541.
- Octeau, J.C. (2014). Compartmentalized D2-dopamine receptors: Roles in signaling & potential mechanism of antipsychotic drug action. *Diss. Masters Theses Campus Access* 1–111.
- Octeau, J.C., Schrader, J.M., Masuho, I., Sharma, M., Aiudi, C., Chen, C.-K., Kovoov, A., and Cerver, J. (2014). G Protein Beta 5 Is Targeted to D2-Dopamine Receptor-Containing Biochemical Compartments and Blocks Dopamine-Dependent Receptor Internalization. *PLoS ONE* 9.
- Opgen-Rhein, C., and Dettling, M. (2008). Clozapine-induced agranulocytosis and its genetic determinants. *Pharmacogenomics* 9, 1101–1111.

- Pirmohamed, M., and Park, K. (1997). Mechanism of clozapine-induced agranulocytosis : current status of research and implications for drug development. *CNS Drugs* 7, 139–158.
- Prou, D., Gu, W.J., Le Crom, S., Vincent, J.D., Salamero, J., and Vernier, P. (2001a). Intracellular retention of the two isoforms of the D(2) dopamine receptor promotes endoplasmic reticulum disruption. *J Cell Sci* 114, 3517–27.
- Prou, D., Gu, W.-J., Crom, S.L., Vincent, J.-D., Salamero, J., and Vernier, P. (2001b). Intracellular retention of the two isoforms of the D2 dopamine receptor promotes endoplasmic reticulum disruption. *J. Cell Sci.* 114, 3517–3527.
- Rajagopal, S., and Shenoy, S.K. (2017). GPCR desensitization: Acute and prolonged phases. *Cell. Signal.*
- Richtand, N.M., Welge, J.A., Logue, A.D., Keck, P.E., Strakowski, S.M., and McNamara, R.K. (2007). Dopamine and serotonin receptor binding and antipsychotic efficacy. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* 32, 1715–1726.
- Sahlholm, K., Marcellino, D., Nilsson, J., Ögren, S.O., Fuxe, K., and Århem, P. (2014). Typical and atypical antipsychotics do not differ markedly in their reversibility of antagonism of the dopamine D2 receptor. *Int. J. Neuropsychopharmacol.* 17, 149–155.
- Sahlholm, K., Zeberg, H., Nilsson, J., Ögren, S.O., Fuxe, K., and Århem, P. (2016). The fast-off hypothesis revisited: A functional kinetic study of antipsychotic antagonism of the dopamine D2 receptor. *Eur. Neuropsychopharmacol. J. Eur. Coll. Neuropsychopharmacol.* 26, 467–476.
- Schlager, B., Straessle, A., and Hafen, E. (2012). Use of anionic denaturing detergents to purify insoluble proteins after overexpression. *BMC Biotechnol.* 12, 95.
- Seeman, P. (2002). Atypical antipsychotics: mechanism of action. *Can J Psychiatry* 47, 27–38.
- Seeman, P. (2006). Targeting the dopamine D2 receptor in schizophrenia. *Expert Opin. Ther. Targets* 10, 515–531.
- Seeman, P. (2014). Clozapine, a fast-off-D2 antipsychotic. *ACS Chem. Neurosci.* 5, 24–29.
- Sharma, M., Cerver, J., Oceau, J.C., and Kovoov, A. (2013a). Plasma membrane compartmentalization of D2 dopamine receptors. *J. Biol. Chem.* 288, 12554–12568.
- Sharma, M., Cerver, J., Oceau, J.C., and Kovoov, A. (2013b). Plasma Membrane Compartmentalization of D2 Dopamine Receptors. *J. Biol. Chem.* 288, 12554–12568.
- Shaw, B.F., Lelie, H.L., Durazo, A., Nersissian, A.M., Xu, G., Chan, P.K., Gralla, E.B., Tiwari, A., Hayward, L.J., Borchelt, D.R., et al. (2008). Detergent-insoluble Aggregates Associated with Amyotrophic Lateral Sclerosis in Transgenic Mice Contain Primarily Full-length, Unmodified Superoxide Dismutase-1. *J. Biol. Chem.* 283, 8340–8350.

- Siskind, D., McCartney, L., Goldschlager, R., and Kisely, S. (2016). Clozapine v. first- and second-generation antipsychotics in treatment-refractory schizophrenia: systematic review and meta-analysis. *Br. J. Psychiatry J. Ment. Sci.* 209, 385–392.
- Tandon, R. (2011). Antipsychotics in the treatment of schizophrenia: an overview. *J Clin Psychiatry* 72 *Suppl 1*, 4–8.
- Tandon, R., Belmaker, R.H., Gattaz, W.F., Lopez-Ibor, J.J., Okasha, A., Singh, B., Stein, D.J., Olie, J.P., Fleischhacker, W.W., and Moeller, H.J. (2008). World Psychiatric Association Pharmacopsychiatry Section statement on comparative effectiveness of antipsychotics in the treatment of schizophrenia. *Schizophr Res.*
- Tandon R, Carpenter WT, and Davis JM (2007). First- and second-generation antipsychotics: Learning from cutlass and catie. *Arch. Gen. Psychiatry* 64, 977–978.
- Thibault, D., Albert, P.R., Pineyro, G., and Trudeau, L.-É. (2011). Neurotensin Triggers Dopamine D2 Receptor Desensitization through a Protein Kinase C and β -Arrestin1-dependent Mechanism. *J. Biol. Chem.* 286, 9174–9184.
- Tirotta, E., Fontaine, V., Picetti, R., Lombardi, M., Samad, T.A., Oulad-Abdelghani, M., Edwards, R., and Borrelli, E. (2008). Signaling by dopamine regulates D2 receptors trafficking at the membrane. *Cell Cycle* 7, 2241–2248.
- Unterwald, E.M., Rubinfeld, J.M., Imai, Y., Wang, J.-B., Uhl, G.R., and Kreek, M.J. (1995). Chronic opioid antagonist administration upregulates mu opioid receptor binding without altering mu opioid receptor mRNA levels. *Mol. Brain Res.* 33, 351–355.
- Urs, N.M., Peterson, S.M., and Caron, M.G. (2017). New Concepts in Dopamine D2 Receptor Biased Signaling and Implications for Schizophrenia Therapy. *Biol. Psychiatry* 81, 78–85.
- Vallone, D., Picetti, R., and Borrelli, E. (2000). Structure and function of dopamine receptors. *Neurosci Biobehav Rev* 24, 125–132.
- Violin, J.D., and Lefkowitz, R.J. (2007). β -Arrestin-biased ligands at seven-transmembrane receptors. *Trends Pharmacol. Sci.* 28, 416–422.
- Walker, J.K., Premont, R.T., Barak, L.S., Caron, M.G., and Shetzline, M.A. (1999). Properties of secretin receptor internalization differ from those of the beta(2)-adrenergic receptor. *J. Biol. Chem.* 274, 31515–31523.
- Wenthur, C.J., and Lindsley, C.W. (2013). Classics in chemical neuroscience: clozapine. *ACS Chem. Neurosci.* 4, 1018–1025.
- Wüller, S., Wiesner, B., Löffler, A., Furkert, J., Krause, G., Hermosilla, R., Schaefer, M., Schüle, R., Rosenthal, W., and Oksche, A. (2004). Pharmacochaperones post-translationally enhance cell surface expression by increasing conformational stability of wild-type and mutant vasopressin V2 receptors. *J. Biol. Chem.* 279, 47254–47263.

Xu, W., Ozdener, F., Li, J.G., Chen, C., de Riel, J.K., Weinstein, H., and Liu-Chen, L.Y. (1999). Functional role of the spatial proximity of Asp114(2.50) in TMH 2 and Asn332(7.49) in TMH 7 of the mu opioid receptor. *FEBS Lett.* 447, 318–324.

Zurek, N., Sparks, L., and Voeltz, G. (2011). Reticulon short hairpin transmembrane domains are used to shape ER tubules. *Traffic Cph. Den.* 12, 28–41.

APPENDIX 1: Introduction and Review of the Problem

Schizophrenia is a seriously debilitating mental disorder affecting ~1% of the world's population. Although antipsychotic drugs have been on the market for the last 70 years, they lack efficacy in treating schizophrenia's most incapacitating symptoms, and produce serious and sometime irreversible side-effects. One drug, clozapine, has shown clinical efficacy superior to all other available antipsychotics, resulting in what clinicians describe as patient awakening. However utility of clozapine is drastically limited as it produces a fatal adverse effect in 1% of treated patients. There is currently no molecular/mechanistic explanation for clozapine's uniquely superior efficacy. I have observed a unique molecular action of clozapine, which could potentially explain its unique efficacy. This study will further investigate the nature of that molecular action, and seek to provide evidence of a unique interaction between clozapine and the shared target of all antipsychotic drugs.

D2-dopamine receptors (D2R): D2R are seven-pass transmembrane domain G-protein coupled receptors (GPCRs)(Beaulieu and Gainetdinov, 2011). They belong to the D2-like, or pertussis toxin (PTX) sensitive $G_{i/o}$ -coupled dopamine receptors, inhibiting the activation of the cAMP pathway (Beaulieu and Gainetdinov, 2011; De Mei et al., 2009). Most highly concentrated in the striatum, D2R are the most widely expressed D2-like receptors, found also in the substantia nigra, cortex, and many other brain areas (Beaulieu and Gainetdinov, 2011b; De Mei et al., 2009). D2R is a clinically relevant receptor, as all clinically available antipsychotic drugs (APDs) bind D2R at therapeutic concentrations (Ginovart and Kapur, 2012; Nord and Farde, 2011). Alternative splicing leads to two isoforms of D2R, D2R Long (D2L) and D2R Short (D2S), differing by 29 amino acids in the 3rd intracellular loop (Beaulieu and Gainetdinov, 2011b). D2L is primarily expressed

post-synaptically while D2S functions mainly as an autoreceptor pre-synaptically (Beaulieu and Gainetdinov, 2011).

Schizophrenia and APDs: Schizophrenia is an extremely debilitating mental disease, affecting about 1% of the world's population (Freedman, 2003b). The disease is characterized by three categories of symptoms: 1) "Positive", psychotic symptoms, like hearing voices, 2) negative symptoms, like attention deficit or anhedonia, and 3) Cognitive dysfunction, often leading to social and vocational impairment (Freedman, 2003b). The severity of these symptoms is such that 10% of patients diagnosed with schizophrenia ultimately commit suicide (Siris, 2001). Introduction of APDs 70 years ago proved revolutionary in the treatment of schizophrenia, reducing many of the positive symptoms, and allowing for discharge of many institutionalized patients (Freedman, 2003b; Muench and Hamer, 2010; Nord and Farde, 2011). Clinical potency of the APDs is closely correlated with their affinity for D2R (Freedman, 2003b; Howes and Kapur, 2009; Muench and Hamer, 2010; Nord and Farde, 2011; Seeman, 2010). But, APD efficacy does not share this correlation, and the underlying cellular mechanisms relating D2R blockade and APD efficacy are not understood (Howes and Kapur, 2009; Muench and Hamer, 2010). The only common link between APDs is they target D2R at therapeutic concentrations (Freedman, 2003b; Muench and Hamer, 2010; Seeman, 2010). Therefore, focus was predominantly laid on D2R blockade, and drugs designed in this manner are collectively called the first generation antipsychotics (FGAs) (Howes and Kapur, 2009; Muench and Hamer, 2010).

FGAs and Extrapyramidal Symptoms (EPS): Rudimentary hypotheses of APD action state that clinical APD efficacy results from D2R blockade in the cortex (Howes and

Kapur, 2009; Tandon, 2011b). However, signaling of D2R expressed in the striatum is heavily involved in controlling voluntary locomotor activity, and blockade of D2R by APDs in the striatum can produce serious locomotive side-effects known as EPS (Beaulieu and Gainetdinov, 2011b; Muench and Hamer, 2010; Nord and Farde, 2011; Seeman, 2010; Tandon, 2011b). Acutely, FGAs have the potential to produce Parkinsonism, but, with chronic treatment, also have the potential to cause an irreversibly debilitating dyskinetic disorder, tardive dyskinesia (TD). Due to their lack of efficacy in treating the negative and cognitive symptoms, and the common incidence of serious EPS, management of schizophrenia with FGAs is critically inadequate (Tandon, 2011b).

Clozapine and Second Generation Antipsychotics (SGAs): In the 1970s clozapine was introduced with superior clinical efficacy compared to FGAs (Nord and Farde, 2011; Wahlbeck et al., 2000; Wenthur and Lindsley, 2013b). Clozapine displayed efficacy against the negative and cognitive symptoms, as well as the positive symptoms, along with the ability to treat patients refractory to the FGAs (Tandon, 2011b; Wahlbeck et al., 2000; Wenthur and Lindsley, 2013b). Furthermore, clozapine displayed a markedly reduced propensity of inducing EPS (Muench and Hamer, 2010; Seeman, 2010). However, it was discovered that treatment with clozapine causes life-threatening agranulocytosis in nearly 1% of treated patients (Alvir et al., 1993; Farooq and Taylor, 2011; Pirmohamed and Park, 1997). As a result of these findings, use of clozapine has been limited to patients unresponsive to other neuroleptics, and only with stringent haematological monitoring (Farooq and Taylor, 2011; Pirmohamed and Park, 1997).

Clozapine's superior clinical efficacy provided motivation to develop a second generation of antipsychotic drugs, with the goal of reproducing clozapine's efficacy and reduced induction of EPS, while also avoiding agranulocytosis (Jibson and Tandon, 1998;

Kuroki et al., 2008). Identifying and mimicking unique pharmacological properties of clozapine as compared to the FGAs became the focus of antipsychotic development. Along with a reduced affinity for D2R, it was found that clozapine displayed higher binding affinities for other brain receptors, most notably serotonin receptors. Mimicking this “atypicality” of clozapine gave rise to the second generation antipsychotics (SGAs), or therapeutic agents designed specifically with a reduced affinity for D2R and higher affinity for serotonin receptors (Jibson and Tandon, 1998b; Kuroki et al., 2008b; Nord and Farde, 2011).

In the midst of promised superior clinical efficacy, and despite much higher cost, SGA use has become the standard for schizophrenia pharmacotherapy (Freedman, 2003b; Meltzer, 2013b). However, despite their decreased tendency to cause EPS, SGAs cause many of their own serious side effects including: weight gain, type II diabetes mellitus, and dyslipidemia (Meltzer, 2013b). Furthermore, multiple non-commercial, independently funded clinical studies such as CUtLASS, CATIE, and EUFEST revealed no difference in efficacy between FGAs and SGAs, and notwithstanding their reduction in EPS, SGAs were found to be no more tolerable than FGAs (Leucht et al., 2009; Lewis and Lieberman, 2008b; Naber and Lambert, 2009; Tandon R et al., 2007)

Clozapine’s clinical efficacy is superior to both FGAs and SGAs: Clozapine, however, evidenced by the above mentioned clinical studies and other private meta-analysis, continues to outperform FGAs and SGAs in terms of clinical efficacy, tolerability and reduction of schizophrenia related mortality (Agid et al., 2010b; Kerwin and Bolonna, 2004b; Leucht et al., 2009; Lewis and Lieberman, 2008b; Meltzer, 2013b). Time and again, clozapine succeeds in the wake of disappointing outcomes with other APDs; consistently effective in treatment-resistant patients (Farooq and Taylor, 2011; Meltzer, 2013b). While some consider clozapine the “Gold Standard” SGA, others view its

unduplicated success as reason to discuss it separately from other “atypical” APDs (Meltzer, 2013b). Outcomes of clozapine treatment have even been described as “awakenings”, as patients experience “restoration of [their] lost souls to near normal existence” (Stahl, 1997). Evidence even suggests that clozapine may exhibit antiparkinsonian effects, further distinguishing its superior motor system side effect profile (Casey, 1989b). Unfortunately, agranulocytosis has critically limited the utility of this fabulous APD (Farooq and Taylor, 2011; Pirmohamed and Park, 1997).

Pharmacological basis for Clozapine’s activity: Though many explanations have been suggested, the molecular mechanisms behind clozapine’s pharmacological superiority remain unknown (Wenthur and Lindsley, 2013b). Initially, it was believed to be clozapine’s enhanced ability to target serotonin receptors centrally (Kuroki et al., 2008b). As previously discussed, SGAs were designed in this line of thinking, and results from the aforementioned clinical studies has greatly diminished support for this theory (Leucht et al., 2009; Lewis and Lieberman, 2008b; Naber and Lambert, 2009; Tandon R et al., 2007). Investigation of clozapine’s binding of D4DR has also proven unfruitful, as drugs that share this property do not replicate clozapine’s efficacy (Kramer et al., 1997).

As other hypotheses continue to fail, many researchers accept that binding of D2R by APDs is central for their clinical efficacy (Kapur and Mamo, 2003b; Vauquelin et al., 2012). It has been suggested that clozapine’s “atypicality”, while not a function of serotonin receptor activity, is due to lower D2R occupancy/quicker dissociation, or “fast-off” antagonism (Vauquelin et al., 2012). Fast-off binding to D2R by clozapine or other SGAs reduces interference of D2R signaling, and in the striatum may explain the reduced incidence of EPS seen with many of the atypical APDs (Seeman et al., 1997; Vauquelin et al., 2012). The reduced D2R occupancy is likely the result of the lowered affinity for D2R

that clozapine and the SGAs share, and this concept is the basis for the “fast-off theory” of their “atypicality” (Seeman et al., 1997; Vauquelin et al., 2012).

Yet neither the serotonin theory nor the fast-off theory explain the unique clinical efficacy of clozapine. As previously stated, drugs that share these properties have been developed, and pale in comparison with clozapine in multiple independent clinical trials (Leucht et al., 2009; Lewis and Lieberman, 2008b; Meltzer, 2013b; Naber and Lambert, 2009; Tandon R et al., 2007). Keeping in mind that the common link between all available antipsychotics is binding of D2R at therapeutic concentrations, one can deduce that the superior clinical efficacy of clozapine results from a unique and currently undescribed molecular action of clozapine at D2R not shared by any other available antipsychotic drug. This unique property of clozapine is unlikely to be the cause of cardio-metabolic side effects, as these are shared by the SGAs (Meltzer, 2013b). However, it is possible that this property could be the cause of the agranulocytosis, as this adverse effect seems to be specific to treatment with clozapine (Farooq and Taylor, 2011; Pirmohamed and Park, 1997).

In preliminary experimentation, I have observed the first evidence of such unique actions of clozapine at D2R. 1% of the world’s population suffers from schizophrenia, and 1 in 10 patients will take their own life (Freedman, 2003b; Siris, 2001). In other words, 1 in every 1000 people will take their own life as a direct result of schizophrenia. Further investigation of our discovery could result in better drugs to treat this severely debilitating and unfortunately prevalent disease.

Recent discoveries have begun to change receptor-ligand theory. It is well established that GPCRs can exist in many functional conformations that can each illicit specific signaling (Hall et al., 1999). It has also been shown that ligands can stabilize unique conformations

of receptors; allowing for one ligand to antagonize one receptor mediated signal, while activating/preserving another function of that same receptor (Drake et al., 2008; Luttrell, 2014; Makita and Iiri, 2013; Violin and Lefkowitz, 2007). Thus, many unique confirmations of a receptor are possible in response to different ligands. As previously mentioned, it is now being accepted again that binding of D2R plays a central role in antipsychotic activity (Kapur and Mamo, 2003b). Here, I am suggesting that clozapine's unique therapeutic effect is neither a function of binding other brain targets nor fast-off kinetics, but an ability to stabilize D2R in a conformation distinct from that produced by the other APDs. Here, I have described molecular and cellular actions of APDs produced via binding to at D2-dopamine receptors that suggest that clozapine-binding induces a D2R conformation distinct from that produced by every APD that was tested. Binding of other APDs produce D2R conformational alterations that enhance detergent-solubility and enhance insertion into the cell-surface.

Initial Observation of Unique Clozapine Activity

As my project design and specific aims were an outcome of my initial observation, I will first describe this observation.

Membrane trafficking of GPCRs is a highly regulated process, involving a variety of chaperone proteins, and depending heavily on the biochemical properties of the receptor itself (Dong et al., 2007). Our lab studies many properties of D2R, including the nature of its expression in the cellular membrane (Sharma et al., 2013b). Along these lines, we investigated the ability of APDs to effect D2R membrane expression using a cell-surface ELISA in a 96-well plate format. Briefly, Human Embryonic Kidney 293T cells (HEK293T) were transiently transfected with plasmid DNA coding for Human D2R. 24 hr post transfection, the cells were treated with receptor saturating concentrations of various

antipsychotic drugs. Following 24 hr treatment the non-permeable intact cells were probed with a horseradish peroxidase (HRP) conjugate antibody targeting a specific epitope fused to the N-terminus of D2R. Addition of substrate for HRP, and subsequent reading with a Glomax plate reader, revealed relative levels of cell membrane surface D2R expression. I observed that this long-term (24 hr) APD treatment, in the absence of dopamine treatment, dramatically increases levels of cell-surface D2R with respect to vehicle, while clozapine produced the smallest increase (Fig. 1). This method of measuring cell-surface receptor has been previously described and validated (Cerver et al., 2012b; Octeau et al., 2014), and saturation concentrations were used to achieve maximum effect to remove any confounders. The dose-response curves depicted in figure 1 prove that concentrations used were sufficient to reach maximal response and binding saturation. There was no observed correlation between affinity for D2R and propensity to cause increased surface expression. For example, haloperidol has a much higher affinity for D2R than clozapine, while quetiapine has a much lower affinity. In our assay, both haloperidol and quetiapine caused dramatically greater increases in surface D2R than that of clozapine. This suggests that the displayed phenomenon is not resulting from the extent of D2R binding achieved by each drug, but rather unique binding interactions with the receptor. This observation led to our suspicion that clozapine's unique therapeutic effect results from an ability to stabilize D2R in a conformation distinct from that produced by the other APDs, evidenced by its reduced tendency to increase D2R cell-surface expression. One other APD, aripiprazole, also showed significantly less cell-surface D2R expression than all other APDs, except for clozapine. While all other tested APDs were antagonists of D2R, aripiprazole, a relatively new APD, is a partial agonist of D2R (Burris et al., 2002). Aripiprazole's partial agonism of D2R may cause agonist induced internalization of D2R or down-regulation through

other signaling pathways (Marchese et al., 2008). Further investigation of aripiprazole was required to distinguish its action from that of clozapine, and is discussed below.

Any display of unique actions of clozapine at D2R is exciting in its own right. However, for this observation to result in development of better pharmacotherapies for the treatment of schizophrenia, we first had to further investigate the nature of this phenomenon. Below I have discussed 3 specific research aims I addressed to better describe clozapine's individuality among the other APDs in mediation of cell-surface expression of D2R. The experimental strategy to address each aim is listed and described in Appendix 2.

APPENDIX 2: Study Design and Specific Methodology

Specific Aim 1: Describe the origins of and further characterize the observed increase in cell-surface D2R

As previously mentioned, membrane trafficking of GPCRs is a heavily regulated process, mediated by many chaperone proteins, and dependent upon many biochemical properties of the receptor itself (Dong et al., 2007). At any given point in time, cell-surface expression levels are a function of both newly synthesized membrane-inserted receptor and removal of “older” cell-surface receptor to endocytic vesicles (Dong et al., 2007; Marchese et al., 2008). Therefore, APDs could be affecting either side of this balance between new receptor insertion and old receptor removal. However, a third explanation is also possible. It is logical to predict that simply increasing translation of D2R would increase receptor levels in any cellular pool, including both intracellular and cell-surface populations. Therefore, the question must be answered, is the phenomenon a function of an increase in total D2R expression, increased D2R membrane insertion, or decreased cell-surface D2R removal? Secondly, it is important to know if this observation is conserved between both D2L and D2S isoforms. Thirdly, it is essential to demonstrate that the increased cell-surface expression is specific to D2R, and similar results are not observed in other classes of GPCRs.

There is much overlap of export chaperone proteins across many classes of GPCRs, and it is possible that the ADPs may be targeting one of these proteins (Dong et al., 2007). Thus, this possibility must be ruled out. Finally, further distinction must be explored between aripiprazole mediated D2 surface expression and that of clozapine. It is possible that aripiprazole causes unique down regulation of D2R, its partial-agonist activity leads to agonist induced internalization of the receptor, or it shares clozapine’s unique

functionality. Still, head to head comparison with clozapine must be performed in order to investigate this potential commonality.

APD treatments achieve effect saturation. It was important to display that the drugs used in our experiments were reaching saturation binding, or a maximum effect on surface expression to remove confounders of varying drug affinities. Therefore, we generated dose-response curves using increasing doses (10-10,000 nM) haloperidol, clozapine, and olanzapine, to display that they are reaching their maximum effect at the concentrations used in previous experiments. These results are discussed in the manuscript and Appendix 3, and the data is summarized in Fig. 1A.

Is increase in D2R simply a function of increase in total receptor? To answer this question, I performed a variation of the previously described ELISA method that allowed for access to surface and intracellular D2R pools. HEK293T cells were cultured in a 96 well plate, and transiently transfected with cDNA coding for human D2R. 24 hr post-transfection, cells were treated with receptor saturating concentrations of the indicated APDs and appropriate vehicle. 48 hr post-transfection, cells were fixed and permeabilized by incubating with ice cold methanol at -20 °C for 15 min. This method of permeabilization for antibody probing of intracellular antigens/proteins has been well validated (Jamur and Oliver, 2010; Krutzik and Nolan, 2003). Wells were blocked for 1 hr at 4 °C in 5% w/v nonfat milk dissolved in PBS. Total FLAG-tagged D2R signal was assessed by probing with HRP-conjugated anti-FLAG M2 antibody (1:5,000 dilution in 5% w/v nonfat milk dissolved in PBS)(Sigma). Supersignal West Femto chemiluminescent substrate (Pierce-ThermoFisher Scientific) was added to each well, and the luminescent signal was measured using a multi-plate luminometer (Glomax). Thus any APD mediated increase in total D2R was determined by comparing chemiluminescent

signals from APD treated cells with vehicle treated cells. Results of this experiment are discussed above in the manuscript, and data is depicted in Figure 1C.

Is increase due to reduced removal from the surface or increased membrane insertion? I investigated this question in two ways. First, to exclusively estimate removal of D2R from the cell-surface, I used a variation of a pulse-chase assay utilizing specific covalent labeling of surface D2R fused with HaloTag®(Promega). Pulse-chase assays used for similar purposes have been previously described (Yamaguchi et al., 2009). HaloTag is a dehalogenase enzyme designed to form specific irreversible covalent bonds with linkers in specifically designed HaloTag ligands *in-vitro* (Los et al., 2008b; Urh and Rosenberg, 2012). This experiment will utilize the HaloTag-PEG-Biotin ligand (Promega, cat#G8591) that is membrane impermeable, and therefore only labels surface receptor. A fusion HaloTag-D2R cDNA construct was created as described in the manuscript, with the HaloTag enzyme fused to the N-terminus of D2R, which is extracellular. This construct was used in a similar cell-surface ELISA, however, 24 hr after transfection, and prior to APD treatment, surface HaloTag-D2R was specifically labeled by incubation with membrane-impermeable HaloTag-PEG-biotin ligand (100 nM in complete DMEM, 1 hr, 37 °). Unreacted reagent was removed by washing 2X with 1X PBS. In this manner, specifically the population of surface D2R was labeled immediately prior to APD treatment. Cells were then treated with receptor- saturating concentration of APDs for 24hr. Remaining PEG-biotin labeled surface receptor was then probed for by incubating with HRP-conjugated streptavidin (1:10,000 3% w/v BSA in 1X PBS), which covalently binds biotin. Therefore, addition of chemiluminescent substrate will reveal signal only for receptor left of the population labeled 24 hr post-transfection. Because newly-synthesized receptor will not have this label, the surface receptor signal will be merely a function of *what has not been removed*, and therefore allows for comparison of rates of removal of

surface-D2R between different APD treatments as well as the vehicle. If, for example, in this experiment the APD treated cells display increased surface-D2R levels compared to vehicle, then this indicates a reduced rate of removal of surface D2R with APD treatment. If surface D2R signal appears no different from that of vehicle treated cells, this indicates that the rates of surface D2R removal are similar, and the explanation for the previously observed APD mediated increase of surface D2R is likely an increase in membrane insertion of D2R. Results of this experiment are explained in the manuscript, and the data is depicted in figure 2A. Control ELISAs were conducted to display similar behavior between the HaloTag-D2R and WT-D2R constructs, such as agonist-induced receptor internalization, and APD enhance surface receptor expression. Results of these experiments are also discussed in the manuscript, and the data summarized in figure 2B-C. Secondly, I conducted a cell-surface ELISA with a D2R mutant, IYIV212-215A. This mutation blocks D2R- β -arrestin binding required for agonist-induced internalization of D2R in mammalian cells (Lan et al., 2009). Thus cell-surface ELISAs using this mutant under conditions of APD treatment will display an APD's ability to influence membrane insertion. The data from these experiments were not included in the manuscript, but is summarized in Figure 11 in Appendix 4.

Is the surface-D2R increase conserved with both D2R and D2Rsh? To address this question, the original cell-surface ELISA was repeated with cDNA coding for FLAG-D2Rsh, in a head to head comparison with D2R, displaying any differences/similarities. Total D2Rsh expression following 24 hr APD treatment was also assessed in the same manner as D2R

Is the effect specific to D2R? I answered this question by simply repeating the cell-surface ELISA and APD treatment with a non-dopamine receptor GPCR. To this end, APD effect

on FLAG-tagged β 2-adrenergic receptor (β 2-AR) and FLAG-tagged Mu Opioid Receptor (MOR) expression was assessed in both surface and total cellular receptor ELISAs.

Is Clozapine's activity unique to that of Aripiprazole? Further distinction between clozapine and aripiprazole was realized in the previously described HaloTag pulse-chase experiment, and is discussed in Appendix 3. Additionally, aripiprazole could cause increased D2R removal from the surface due to its partial-agonist activity, through agonist induced internalization. I explored aripiprazole's ability to cause endocytosis of D2R in response to short-term (45 min) treatment. HEK293T were seeded in a 96-well plate at 5×10^4 cells/well, and transiently transfected with D2R. 48 hr post-transfection, cells were treated with 10 μ M aripiprazole, dopamine, or vehicle for 45 min. Cells were then fixed with ice cold methanol-free paraformaldehyde (4% v/v in 1XPBS). Wells were blocked in 5% w/v nonfat milk in 1XPBS for 1 hr at 4 °C, and remaining surface receptor was probed for with HRP-conjugated anti-FLAG M2 antibody (1:5,000 in 5% w/v nonfat milk dissolved in 1X PBS). Supersignal West Femto chemiluminescent substrate was added to each well, and the HRP-catalyzed luminescent signal was detected and quantified by a multiplate-luminometer (Glomax). Reduced signal in either of the treated groups compared to vehicle indicates agonist-induced internalization of the receptor. Dopamine treatment was used as a positive control for receptor internalization. If aripiprazole causes receptor internalization, then it is likely that the lower surface signal observed in cells treated with aripiprazole for 24 hr is a result of increased removal of receptor from the membrane. The results of this experiment were not included in the manuscript, but are discussed in Appendix 3, and the data is summarized in Figure 12.

Visually Characterizing Cell-Surface D2R Increase: Cellular localization of D2R, along with β 2-AR and β 2-AR_{W158A} (discussed below) was visually characterized using Immunocytochemistry (ICC) and confocal microscopy. I conducted the initial experiments concerning β 2-AR localization, and D2R localization in response to clozapine and haloperidol (images not shown). The confocal images and co-localization data presented in the manuscript were the result of experiments conducted by my lab-mate, Craig Irving. We assessed the co-localization characteristics of D2R, β 2-AR, and β 2-AR_{W158A} with mCh-Sec61-beta a known ER marker fused with the red fluorescent protein mCherry (Shu et al., 2006; Zurek et al., 2011b). HEK293T cells were cultured on cover slips, coated with poly-D-lysine, in a 12-well plate at a density of 1×10^5 cells/well, and transfected with indicated cDNA constructs. 24 hr post transfection, cells were treated with indicated antipsychotics or vehicle. 48 hr post-transfection, cells were fixed and permeabilized by incubation with ice cold methanol for 10 min on ice or at 4 °C. Cells were washed 3x in 1X PBS, and blocked in 5% (w/v in 1X PBS) nonfat milk for 1 hr at 20 °C. FLAG-tagged receptor was probed for with Anti-FLAG M2 Antibody (1:1000 dilution in 5% nonfat milk) overnight at 4 °C. Cells were then washed 2x with PBS for 15 min at 20 °C, and then probed with fluorophore-conjugated anti-mouse secondary antibody (1:1,000 dilution in 5% w/v Nonfat milk) for imaging under fluorescent microscopy. Images were taken with a Zeiss Confocal Microscope, and Pearson co-localization coefficients (PCCs) with indicated cellular markers determined using the Zeiss imaging software. Pearson coefficients provides a percentage of locational overlap between the two fluorescent emissions, but does not normalize for signal intensity.

AIM 2: Display the above APD effect is a result of direct and unique binding interactions with D2R Aim 2 will be addressed in 3 ways, using head to head

comparisons between the APD displaying the most dramatic increase in surface D2R (haloperidol), and clozapine.

Is the above listed APD effect conserved in a ligand-binding deficient D2R mutant? It has been reported that mutation of a conserved aspartic acid residue in GPCRs abolishes ligand binding (Han et al., 2009b). Creation of an N-terminal FLAG tagged mutant D2R, D114A, allowed for investigation APD-mediated enhanced D2R surface expression in the absence of ligand binding. I repeated the above described ELISA with D2R_{D114A} to explore whether or not the APD enhancement D2R membrane insertion remains. If so, this would indicate that the APDs are binding a target other than D2R, perhaps a chaperone protein. However, if the effect is lost, then APD enhanced surface expression of D2R is a result of direct and specific binding interactions between D2R and APDs. Results of this experiment are discussed in the manuscript and the data is summarized in figure 3C. Control experiments assessing total cellular D2R_{D114A} in response to APD treatment were also completed, and the data is summarized in figure 3D.

Can Haloperidol's marked increase of surface D2R expression be inhibited by Clozapine co-treatment? As stated earlier, Haloperidol causes the largest increase in surface D2R, while clozapine causes the least increase. If our hypothesis that clozapine binding stabilizes D2R in a unique confirmation is true, then competitive binding between haloperidol and clozapine should, to some degree, reduce haloperidol's effect. I generated two dose response curves for haloperidol; one in the presence and one in the absence of 10 μ M clozapine. If clozapine is able to block or shift haloperidol's dose response curve to the right, it would indicate competitive binding between the two drugs. This would be further evidence that binding to D2R is required, as that is the common property of these

drugs, and that clozapine's interaction with D2R is unique compared to that of haloperidol. Results of this experiment are discussed in the manuscript and in Appendix 3, and the data is summarized in figure 1A.

Can APDs rescue misfolded D2R for proper membrane insertion?

It has been shown that ligands can rescue misfolded protein from aggregation in the ER, binding and stabilizing them in a conformation suitable for export (Lan et al., 2012; Lester et al., 2012). Furthermore, mutant misfolding GPCRs have been created by mutating a conserved tryptophan residue to alanine, and used to display pharmacochaperone abilities of ligands (Lan et al., 2012). Utilizing the corresponding D2R mutant, D2R_{W160A}, I assessed APD ability to rescue the misfolded receptor for export to the cell membrane. D2R_{W160A} surface receptor expression in response to APD treatment was assessed in the same manner as above, and total cellular receptor ELISA controls were executed to ensure that observed differences were not due to changes in total receptor expression.

Specific Aim 3: Investigate the actions of different APDs on the cell biology and biochemistry/biophysics of the intracellular accumulations of D2R.

Is the observed increase in cell-surface D2R a result of drug binding at the cell membrane or intracellularly? If direct binding of D2R by the APDs is required for D2R increase at the cell surface, then this binding could occur either at the membrane or intracellularly. Knowing where the interaction must occur is essential in determining the mechanism for increased membrane expression of D2R. If the crucial interaction occurs intracellularly, then anything facilitating APD movement across the cell membrane will improve the ability of APD-mediated enhancement cell-surface D2R expression. Some APDs, such as

amisulpride or sulpride, are poorly membrane permeable, but are substrates for the membrane transporter OCT1 (Dos Santos Pereira et al., 2014). Therefore, transient of OCT1 will increase intracellular concentrations of amisulpride or sulpride. An enhanced potency of amisulpride to increase surface D2R in cells also expressing OCT1, would indicate drug movement into the cell is required for the effect, and the key interaction is intracellular. Therefore, utilizing the cell-surface ELISA, I generated two dose response curves for amisulpride-induced enhancement of surface D2R expression, in the presence and absence of OCT1. To further confirm this result, the experiment will be repeated with a drug, tiapride, which is not a substrate for OCT1. No observed change in tiapride's potency with OCT1 present would further validate the conclusion that OCT1's increase in amisulpride's potency was a direct result of its ability to facilitate amisulpride's movement across the cell membrane. The results of the experiments are summarized in the manuscript and discussed in Appendix 3, and the data is depicted in figure 6A-B.

Characterizing D2R localized intracellularly. Following translation, properly folded transmembrane proteins, such as GPCRs, are threaded through the membrane of the endoplasmic reticulum (ER), as they are trafficked to the cell surface (Alberts et al., 2008; Dong et al., 2007). However, many studies have reported D2R displays atypical localization in intracellular perinuclear compartments when transiently expressed, in multiple cell lines, or endogenously expressed in neurons (Fishburn et al., 1995b; Kim et al., 2008b; Kovoov et al., 2005b; Prou et al., 2001b; Sharma et al., 2013b; Tirotta et al., 2008b). Because most APDs are seemingly altering this cellular localization of D2R, while clozapine appears to conserve this distribution, I wanted to further characterize the intracellular D2R. To this end I compared functional co-localization between D2R and ER targeted proteins with other GPCRs primarily targeted to the membrane or the ER. I

utilized a bioluminescence energy transfer (BRET) assay in which a receptor-Rluciferase fusion constructs, excited by a substrate, emits light that, when within 100 Å, will excite an acceptor protein, PTP1B-Venus, which will then emit light. This quantifiable light is therefore indicative of the extent two proteins, in this case D2R, β 2-AR, or β 2-AR_{W158A}, and PTP1B co-localize. PTP1B is a known ER marker, and therefore can be used to evaluate D2R localization in the ER, and the extent to which APDs facilitate D2R export from the ER (Monteleone et al., 2012). β 2-AR, and β 2-AR_{W158A} have been previously with PTP1B-Venus for this purpose (Lan et al., 2012). β 2-AR_{W158A} is a misfolding β 2-AR mutant, with a mutation in the same conserved tryptophan residue as D2R_{W160A}, and displays strong ER localization. On the other hand, in previous ICC experiments, I had shown β 2-AR primarily located at the cell surface. Thus, these two receptors constructs were chosen to compare and contrast with the cellular localization of D2R. BRET requires a dual emission multidetection microplate reader, such as a POLARstar OPTIMA. The experiment was carried out as described in the “Materials and Methods” section of the manuscript. Results are summarized in the manuscript and discussed in Appendix 3, and the data is depicted in figure 9B.

Is glycosylation of D2R important for APD mediated increase in surface expression? It is well established that protein maturation often requires post-translational modifications (PTM), and many GPCRs are glycosylated as part of this maturation process on the excretory pathway (Dong et al., 2007). It is also known that N-linked glycosylation at the N-terminus of D2R is important in regulating proper membrane expression of D2R, (Min et al., 2015). Initially, I had proposed to use ICC and image D2R localization in APD treated cells in the presence and absence of N-linked glycosylation. The antibiotic tunicamycin can be used *in-vitro* to inhibit N-linked glycosylation, and has been

previously described for this use (Girard-Bock et al., 2016). However, tunicamycin treatment for such extended periods of time interfered with cell fixation, in this proved difficult to image. Alternatively, I created a glycosylation deficient mutant D2R construct (N5,17,23Q) removing all known putative glycosylation sites from D2R (Min et al., 2015). I then repeated the initial cell-surface ELISA with the D2R_{N5,17,23Q} construct, to alternatively show the significance of glycosylation to APD mediated cell-surface increase. If, for example, APDs were changing the rate at which D2R was being glycosylated, and promoting membrane insertion, then inhibiting all glycosylation of D2R should remove APD-mediated enhancement of D2R surface expression. To control for APD-mediated changes in total D2R_{N5,17,23Q}, total cellular D2R_{N5,17,23Q} signal following 24 hr APD treatment was assessed as described above. Results of these experiment are summarized and discussed in the manuscript as well as Appendix 3, and the data is depicted in figure 7C-D.

What is the role of G-protein Signaling in the above listed APD effect at D2R? It is established that a commonality between APDs is there inhibition of D2R mediated activation of G-proteins (Masri et al., 2008). I investigated the potential role of G-protein signaling on the above listed APD effect at D2R. D2R is coupled to the pertussis-toxin (PTX) sensitive G α_i (Beaulieu and Gainetdinov, 2011b). Thus, co-expressing D2R in HEK293 cells over-expressing PTX will inhibit any D2R mediated basal level G-protein signaling. Therefore I repeated the cell-surface ELISA with HEK293 cells transiently transfected with both D2R and PTX, to investigate whether the removal of G-protein signaling alters the APD mediated surface D2R increase. The results are discussed in the manuscript and data presented in Figure 10A.

How is G-protein signaling affected by D2R increase at the surface? To assess the effect of APD-mediated enhancement of surface D2R expression on receptor function, I monitored dopamine-induced D2R-mediated G-protein activation via bioluminescence resonance energy transfer (BRET) measuring recruitment of G-protein coupled receptor kinase. The assay exploits G-protein coupled receptor kinase 3 (GRK3) fused with luciferase as the BRET donor, and a G γ fused with Venus as the acceptor. Agonist induced G-protein activation results in GRK recruitment for receptor phosphorylation (Beaulieu and Gainetdinov, 2011b), bringing the BRET donor and acceptor into adequate proximity for energy transfer. Utilization of BRET in this manner has been well validated (Kamal et al., 2009; Masuho et al., 2015). For this experiment we again utilized the POLARstar OPTIMA multidetection microplate reader. The POLARstar is equipped with automatic injectors that allow for automatic addition of agonist to each well. Resulting emissions for both wave lengths are then measured over time for each individual well, thus removing confounders of timing differences. HEK293T cells were seeded in 6cm diameter culture dishes at a density of 1×10^6 cells/dish, and transfected with D2R, G α_i , Venus 155-239 G β_1 , Venus 155 G γ_2 , and the masGRK3ct NanoLuc cDNA. 24 hr post transfection, media was replaced with fresh completed DMEM. 48 hr post-transfection, cells were washed 2x in 1X PBS, harvested by trituration, and seeded in opaque white-bottomed 96-well plates. Cells were then treated with specified concentrations of dopamine or vehicle, and luminescence measurements were made using the POLARstar OPTIMA multidetection microplate reader. Raw BRET ratios were calculated as the emission intensity at 520-545 nm (acceptor) divided by the emission intensity at 475-495 (donor). Net BRET was found by subtracting the average raw BRET ratio before the addition of drug from the average raw BRET ratio after the addition of drug. Dopamine response was calculated by subtracted the net BRET in dopamine treated cells from that

observed in vehicle treated cells. The results of this experiment are summarized and discussed in the manuscript, and the data is depicted in figure 10B.

Effect of clozapine treatment on D2R-mediated changes in biochemical profile of Gβ5—

WE have previously reported that co-expression with D2R significantly increases the detergent-insolubility of and stabilizes Gβ5 (Octeau et al., 2014). In order to investigate potential functional outcomes of clozapine's unique activity at D2R, we explored the effect of clozapine treatment on the biochemical interactions between D2R and Gβ5.

HEK293T cells co-expressing D2R and Gβ5 were treated with 10 μM clozapine or vehicle for 24 hr. Triton X-100-soluble and insoluble protein fractions were isolated as described in the “Materials and Methods” section of the manuscript. Relative expression of Gβ5 in TX100-soluble and –insoluble fractions was measured as described in the “Western Blotting” section of the “Materials and Methods”. Gβ5 protein was probed for as previously described, by incubating blots for 1 hr at 4 °C with rabbit polyclonal antibody CT215 (anti- Gβ5, 1:5,000 in 3% w/v BSA dissolved in 1X PBS) (Octeau et al., 2014; Watson et al., 1994).

APPENDIX 3: Theoretical/Speculative Discussion and Implications

APD's act as pharmacochaperones of D2R. Several studies have reported on the ability of ligands to act as pharmacochaperones for cell surface receptors (Lan et al., 2012; Lester et al., 2012; Wüller et al., 2004). Furthermore, these pharmacochaperones can serve to both rescue misfolded receptors, and overcome coded ER retention signals to produce enhanced export trafficking of GPCRs (Lan et al., 2012; Lester et al., 2012; Wüller et al., 2004). The data presented here clearly show that APD treatment results in increased cell surface expression of D2R, with clozapine and aripiprazole displaying the lowest ability to do so. These observed differences in surface receptor expression cannot be attributed to enhanced protein expression, as total receptor signal among all APD treated cells, except for aripiprazole, was similar, suggesting that APDs cause a redistribution of D2R, increasing the percentage of total D2R residing in the cell membrane. Cells treated with aripiprazole displayed significantly lower total receptor expression compared to the other APDs, which could explain the observed lower surface D2R expression. If aripiprazole treated cells display similar surface D2R expression to those treated with clozapine, but dramatically less total receptor expression, then the percentage of total cellular D2R residing in the membrane must be much higher in aripiprazole treated cells. Thus, the percentage of total cellular D2R residing at the cell surface was higher for all other APDs than in clozapine or vehicle treated cells. Furthermore, the Halotag D2R results show that the increased surface D2R signal is not a result of reduced receptor loss from the cell surface, but indicates that APDs cause an increase in membrane insertion of D2R. Again, aripiprazole was unique in this experiment, as receptor loss from the surface was enhanced in cells treated with aripiprazole. Because aripiprazole treated cells display similar surface D2R expression to cells treated with clozapine, but less total receptor expression and increased surface receptor loss compared to both vehicle and clozapine

treated cells, we can deduce that the rate of membrane insertion of D2R in response to aripiprazole treatment compared to clozapine or vehicle treatment is enhanced. We therefore conclude that APD treatment results in a re-distribution of D2R to the cell membrane, via increased membrane insertion of D2R, with clozapine uniquely producing the smallest effect. This conclusion is confirmed by ICC. Confocal images and PCCs obtained in these experiments clearly display D2R constrained to intracellular pools and strong co-localization with ER marker Sec61 in vehicle and clozapine treated cells. Conversely, cells treated with haloperidol display greatly enhanced membrane D2R staining, and significantly reduced PCCs with Sec61. Thus, in cells treated with vehicle or clozapine, D2R is primarily residing in the ER, while in cells treated with haloperidol, D2R is primarily targeted to the cellular membrane.

Aripiprazole did not cause internalization of D2R. It is well known that D2R undergoes agonist-induced endocytosis, possibly followed by trafficking to lysosomes and receptor degradation (Beaulieu and Gainetdinov, 2011b). Because of D2R surface expression in cells treated with haloperidol was similar to that of clozapine, we investigated the possibility that aripiprazole was causing agonist-induced D2R internalization. HEK293T cells were transiently expressing D2R were treated with 10 μ M concentrations of aripiprazole, dopamine, or vehicle for 30 min, and surface receptor was measured as described in Appendix 2. While dopamine produced nearly 50% loss of surface receptor relative to vehicle, aripiprazole treatment resulted in no significant change in D2R surface receptor (Fig. 12). Therefore, aripiprazole did not acutely cause agonist-induced D2R endocytosis.

Non-internalizing D2R Mutant, D2R_{T225,S228,S229→A}, similarly effected by APDs.

Mechanisms of D2R trafficking, and receptor endocytosis are not fully understood.

Traditionally, it has been assumed that like other GPCRs, phosphorylation by G-protein receptor kinases (GRK) initiates the process of receptor internalization (Beaulieu and Gainetdinov, 2011b). However, recent studies have shown D2R desensitization and receptor internalization in a GRK-independent manner (Clever et al., 2013; Namkung and Sibley, 2004). Moreover, it has been demonstrated that D2R is basally phosphorylated by protein kinase C, and that treatment with PKC activators can induce D2R internalization independently of agonist treatment (Namkung and Sibley, 2004b; Thibault et al., 2011). Thus, it is plausible, that constitutive internalization of D2R occurs through a PKC-dependent mechanism. Furthermore, it has been reported that mutation of PKC phosphorylation sites in D2R (T225, S228, S229) inhibit agonist-induced internalization (Clever et al., 2013). Therefore, it is likely, though not one hundred percent certain, that the D2R_{T225,S228,S229→A} PKC phosphorylation deficient mutant blocks both constitutive and agonist-induced internalization. Utilizing the D2R_{T225,S228,S229→A} allows for investigation of APD-mediated effects of D2R membrane trafficking under conditions of significantly less, if not completely absent, receptor endocytosis. In this case, D2R surface expression is primarily a function of newly inserted receptor, rather than a balance of receptor membrane insertion and receptor endocytosis. Both haloperidol and clozapine treatment produced significant enhanced membrane expression of D2R_{T225,S228,S229→A} relative to vehicle, however haloperidol's effect was significantly higher at 250% relative to vehicle (Fig 11). Thus, under conditions where surface receptor is likely primarily a function of newly inserted receptor, APDs produced an effect similar to previously observed with D2R. These results therefore support the conclusion, that differences in

enhanced surface D2R expression among APD treatments results from differences in rates of D2R membrane insertion.

Enhanced cell surface D2R expression is an intrinsic effect of APDs requiring specific and direct binding to D2R. A possible explanation for this phenomenon is that in addition to D2R, APDs bind a separate distinct cellular target important for export trafficking of transmembrane proteins. However, an important finding in this study is that increased membrane insertion of D2R is a result of direct, specific binding of APDs to D2R. We display with two other GPCRs, β 2-AR and MuOR, that APD treatment is unable to reproduce this effect with other transmembrane receptors. Additionally, we utilized a ligand-binding deficient D2R mutant, D2R_{D114A}, to clearly show that the effect on surface D2R is lost when APD binding to the receptor is abolished. These data indicate that enhanced membrane insertion of D2R is a result of direct APD binding to D2R, and APDs can be considered to have efficacy for this response. This is a significant observation, as this is the first time APDs have been shown to have a direct action at D2R other than antagonism. Thus, we reasonably suggest that enhanced membrane insertion of D2R is a cellular response induced by APD binding, and APDs be considered agonists for this response. According to this reasoning, review of the dose response curves displayed in figure 1 would define haloperidol as a full-agonist of enhanced membrane insertion of D2R, while clozapine's efficacy is significantly reduced compared to both olanzapine and haloperidol, should be considered a partial agonist. Partial agonists, as seen with aripiprazole, are often used clinically to effectively inhibit the actions of full agonists (Burris et al., 2002; Frankel and Schwartz, 2017). Hence, dose-response curves generated for haloperidol in the presence of clozapine lend further support for this conclusion, as the efficacy of proposed full-agonist haloperidol is significantly reduced in the presence of

proposed partial agonist clozapine. This suggests a conserved target for clozapine and haloperidol, further adding support to the conclusion that enhanced membrane insertion of D2R is an intrinsic effect of APDs, for which haloperidol is a full agonist and clozapine is a partial agonist.

Intracellular binding of D2R by APDs critical for pharmacochaperone effects. Another important observation is the increase in potency of amisulpride as a pharmacochaperone of D2R with the expression of membrane transporter OCT1. It has been reported that the D2R binding pocket exists within extracellular domains of the transmembrane protein (Shi and Javitch, 2004). With the exception of a few newer partial agonists (Burris et al., 2002; Frankel and Schwartz, 2017), APDs act as D2R antagonists (Howes and Kapur, 2009; Muench and Hamer, 2010; Seeman, 2010), and are thus assumed to bind only the extracellular domains of D2R at the cell surface. However, the increase in potency of the poorly membrane permeable amisulpride in the presence of OCT1 indicates that transport into the cell is critical for pharmacochaperone efficacy, and enhanced membrane insertion is a result of APD binding to D2R intracellularly. This is the first evidence of APD binding of D2R other than at the cell membrane eliciting a response other than antagonism.

Interestingly, despite their marked ability to increase membrane insertion of D2R, the concentration response curves for amisulpride and tiapride show the response has not yet saturated at 10 μ M concentrations. As previously mentioned, both amisulpride and tiapride, along with sulpiride, display poor membrane permeability (Dos Santos Pereira et al., 2014). While these drugs do display reduced incidence of EPS, it could be simply because these drugs do not efficiently cross the blood brain barrier. Here we have shown accumulation of APDs inside the cell enhances the APD-mediated increase in membrane insertion of D2R. While 24 hr treatment will allow for an equilibrium of diffusion across

the cell membrane even for poorly permeable drugs, it is reasonable to assume that less amisulpiride, sulpiride, and tiapride accumulates inside of the cell. Yet, at similar doses, these APDs are achieving similar maximum effect to many of the other APDs. It is possible that these drugs may be most potent in producing this enhancement of D2R membrane insertion, as lower amounts of the drug at the site of action produce similar effects to the other APDs. Their concentration response curves suggest that higher doses will yet achieve higher responses. If there is a link between this pharmacochaperone phenomenon of APDs and incidence of EPS, then these results are expected. Just as drugs that move poorly across the cell membrane are limited in their ability to enhance surface expression of D2R, so would those same drugs that also move poorly across the BBB be limited in their propensity to cause EPS.

APDs can rescue misfolded receptor. Properties of pharmacochaperones include overcoming coded ER retention signals and protein misfolding to promote trafficking of transmembrane receptors to the cell surface (Lan et al., 2012; Lester et al., 2012; Wüller et al., 2004). An important feature of the graph depicted in Figure 9B is the observed lack of BRET occurring between D2R and ER marker PTP1B despite the fact that D2R shows strong co-localization with ER marker Sec61 (Figure 4). This suggests that D2R found in the ER may be misfolded, and incapable of participating in BRET. Previous studies have utilized mutant, misfolding GPCRs, created by mutating a conserved amino acid residue (W160A) to report on pharmacochaperone abilities of ligands (Lan et al., 2012). Utilizing a D2R_{W160A} mutant, we have shown here that haloperidol is able to rescue misfolded D2R protein, and promote proper receptor expression in the cell membrane, with expression levels approximately 20-fold greater than that of clozapine or vehicle treated cells. Clozapine treatment was barely able to raise surface receptor expression above background, while vehicle treated cells displayed no significant surface D2R expression.

This observation is a strong indication that APDs are behaving as pharmacochaperones for D2R, possibly rescuing misfolded receptor, with clozapine displaying a dramatically lower ability to do so.

D2R ER retention and drug effective doses highlight physiological relevance. A common criticism of studies such as this is that drug doses used exceed physiologically relevant concentrations. APD concentrations have been measured in the 1-5 μM range in both human and rat brains (Baldessarini et al., 1993; Froemming et al., 1989; Kornhuber et al., 2006). The dose response curves displayed in figure 1B show significant drug responses at drug concentrations in the 100 nM range, well below the in-brain measured concentrations. The physiological relevance of a model where D2R, a transmembrane receptor, is primarily targeted to intracellular compartments might also be questioned. A critical explanation of our observations is that transiently expressing D2R in HEK293T cells has artificially caused the membrane receptor to misfold or aggregate in intracellular pools due to over-expression, and these results are simply irrelevant artefacts. However, in numerous previously reported studies, when transiently expressed across multiple cell lines, or endogenously expressed in neurons, D2R is atypically localized in intracellular perinuclear compartments (Fishburn et al., 1995b; Kim et al., 2008b; Kovoov et al., 2005b; Prou et al., 2001b; Sharma et al., 2013b; Tirotta et al., 2008b). Recently it was reported that a di-arginine motifs can function as ER retention signals, and conserved arginine clusters in the 3rd cytoplasmic loop of D2R function as such a signal (Kubale et al., 2016b). Kubale et al. reported, that while WT D2R localized primarily to the endoplasmic reticulum, and disruption of amino acids 267, 268, and 269, all arginines in the 3rd cytoplasmic loop of D2R resulted in dramatically enhanced membrane targeting of D2R (Kubale et al., 2016b). Hence, this intracellular accumulation of D2R is not surprising, and should be expected, as it appears naturally coded to do so. Interestingly, it

has been reported that the two receptor isoforms, D2R and D2Rsh, differ in their cellular localization, with D2Rsh displaying much greater membrane targeting (Prou et al., 2001b). As previously stated, D2Rsh is a splicing variant of D2R, lacking 29 amino acids within the 3rd cytoplasmic loop, including the three arginine residues forming the ER retention motif in D2R long isoform. Since the intracellular localization of D2R has been shown to be heavily influenced by the A267-269 ER retention motif, it is not surprising that D2sh, which lacks this motif, displays much greater surface expression. It has been reported that actions of pharmacochaperones include overcoming ER retention signals to promote export of transmembrane receptors to the cell surface (Wüller et al., 2004). If, as we propose, that APDs are acting as pharmacochaperones, and, as has been reported, D2RL contains a strong ER retention motif that D2Rsh lacks, then APD treatment should have a much greater effect on the surface expression of D2R long isoform than D2Rsh. This is exactly what we observed. Haloperidol induced increase of surface D2Rsh was only half that of D2RL, and while clozapine increased surface expression of D2RL by 60% over vehicle treated cells, surface D2Rsh expression in clozapine and vehicle treated cells were not different. Therefore, we conclude that our observed intracellular, perinuclear expression of D2R is not an artifact, and APDs act as pharmacochaperones of D2R evidenced by their ability to rescue misfolded receptor and overcome coded ER retention motifs to increase D2R export from the ER to the cell surface. Moreover this highlights the physiological relevance of our finding, as D2R is endogenously coded for ER targeting, and APD treatment drastically alters that distribution.

Repeatability with multiple detection methods confirm results are not artifacts. Some may criticize these observations as merely a product of poor Anti-FLAG M2 antibody binding. Recently, it has been reported that sulfation of N-terminus linked FLAG epitope-tagged

D2R greatly reduces binding of Anti-FLAG M2 antibody (Hunter et al., 2016), and that surface D2R expression was underestimated because of FLAG epitope sulfation. Thus, some might argue that our observations are simply a function of APDs effecting FLAG-epitope post-translational modification, specifically its sulfation, rather than pharmacochaperone effects on D2R itself, and the differences we observe are related to changes in M2 antibody binding. We believe this is not the case for two reasons. First, the experiments revealing the differences in FLAG D2R recognition because of sulfation were done in HEK293 cells stably expressing FLAG D2R (Hunter et al., 2016). Therefore, they were dealing with D2R expression levels dramatically lower than in our model, and any differences in receptor recognition observed based on sulfation would be much more dramatic than in our system. Secondly, we have repeated the initial observation done with Anti-FLAG M2 antibody with multiple detection methods. The Halotag experiments (Figure 2) utilize covalent linkage of PEG-biotin reagent, probed for with HRP-conjugated streptavidin, which cannot be explained away by FLAG-epitope sulfation. ICC experiments, completed in our lab, using D2R fused to yellow fluorescent protein display the same punctate, intracellular, and perinuclear distribution of FLAG D2R labeled with fluorophore-conjugated Anti-FLAG M2 antibody (data not shown). Thus, we have repeated observations acquired with Anti-FLAG M2 antibody with multiple detection methods, suggesting that the pattern of D2R localization we observe is not a function of differences in post-translation modification of the FLAG epitope.

First evidence of unique action of clozapine at D2R. Perhaps the most important aspect of our data is the unique activity of clozapine at D2R. Since its introduction in the 1970s, clozapine has displayed superior clinical efficacy in treatment of the positive, negative, and cognitive symptoms of schizophrenia compared with all other available APDs (Agid

et al., 2010b; Kerwin and Bolonna, 2004b; Leucht et al., 2009; Lewis and Lieberman, 2008b; Meltzer, 2013b). Despite the belief that this superior efficacy came through clozapine action at serotonin receptors (Kuroki et al., 2008b), results from multiple clinical trial comparing clozapine to drugs designed in this manner have largely disproven this theory (Leucht et al., 2009; Lewis and Lieberman, 2008b). As a result many researchers have returned to the belief that D2R remains central to APD efficacy (Kapur and Mamo, 2003b; Vauquelin et al., 2012). Until now, no one has provided any evidence for unique actions of clozapine at D2R that could explain its superior efficacy. The dose response curves depicted in figure 1A clearly show that treatment with haloperidol and close structural relative of clozapine, olanzapine both cause dramatically higher increases in surface D2R expression than clozapine; an effect we have further characterized as an intrinsic pharmacochaperone effect of APDs. The graph depicted in Figure 1B shows that clozapine treatment causes lower surface D2R increases than any of the other tested APDs, except for aripiprazole. As previously discussed, because aripiprazole causes increased removal of D2R from the cell surface, and cells treated with aripiprazole display lower total D2R expression, it can be deduced that aripiprazole also causes increased membrane insertion of D2R with respect to clozapine. We have displayed in using multiple detection methods and D2R mutants that APDs can rescue misfolded D2R, and overcome endogenous coded ER retention motifs in D2R to redistribute D2R from intracellular perinuclear pools to enhanced cell membrane expression. However, the surface receptor ELISAs, along with PCC values and confocal images obtained via ICC show that clozapine treatment does not change the cellular distribution of D2R compared with vehicle treated cells. Furthermore, while saturating doses of clozapine can only produce 30% of haloperidol's pharmacochaperone effect, in G-protein activation assays 24 hr treatment with clozapine can produce equal D2R blockade (data not shown). This

implies a unique binding interaction of clozapine with D2R; able to produce equivalent blockade, but far inferior pharmacochaperone abilities at equal levels of binding. Consider again the dose response curves depicted in Figure 1A. Inhibition of haloperidol by clozapine co-treatment does not follow the pattern of competitive inhibition (dose response curve shift to the right), but of non-competitive. Again this suggests unique binding of clozapine to D2R compared with that of haloperidol. However, in experiments done with D2R_{D114A}, the ligand-binding deficient D2R mutant, effects on surface and total receptor of both haloperidol *and* clozapine are abolished, implying a like binding pocket for both drugs. In order to reconcile these results, one can conclude, that while clozapine and haloperidol share a like binding site, clozapine stabilizes D2R in a unique confirmation, that does not promote increased export to the plasma membrane, and, as seen in the competition curve in Figure 1A, is able to abolish haloperidol binding. This conclusion is further supported by the solubility data presented in Figure 8B, where clozapine treatment is shown to induce unique biochemical properties of D2R relative to all other tested APD and vehicle treatments. Whereas all other drugs increase the detergent solubility of D2R, clozapine uniquely reduces its detergent solubility.

Speculative discussion of functional impact of clozapine treatment. Previously our lab reported on biochemical interaction of D2R and G β 5 (Octeau et al., 2014). Co-expression of D2R with G β 5 produces dramatic increase in G β 5 detergent insolubility, and a marked increase in G β 5 stability. G β 5 expression also displayed the ability to inhibit agonist-induced internalization of D2R but not MuOR (Octeau et al., 2014). It has been reported that accumulation of insoluble protein can be evidence of protein aggregation (Basso et al., 2009; Schlager et al., 2012; Shaw et al., 2008). Recall that ER targeted D2R was unable to participate in BRET with another ER targeted protein, suggesting that it

might be misfolded or aggregated. D2R also displays significant detergent insolubility. Therefore, it is possible that D2R has a natural propensity to aggregate, and as seen with G β 5, may cause other proteins to aggregate as well. Protein aggregation has been implicated in neurodegenerative diseases such as ALS disease and Huntington's disease (Basso et al., 2009; Furlong et al., 2000; Hatters, 2008). Huntington's chorea is almost indistinguishable from the tardive dyskinesia seen in psychiatric patients (David et al., 1987). Hypothetically speaking, D2R could be causing aggregation of other proteins that can lead to neurodegeneration similar to that seen in Huntington's disease, and APD's induce tardive dyskinesia by somehow accelerating this process.

The tri-arginine ER retention motif suggests that D2R is naturally coded to accumulate in the ER. Moreover, clozapine, which displays the smallest incidence of inducing tardive dyskinesia, *increases* the detergent insolubility of D2R. Again, hypothetically speaking, perhaps D2R is targeted to accumulate in the ER to prevent it from causing other proteins to aggregate, and clozapine, unlike the other APDs, preserves this protective cellular distribution of D2R. Along this line of thinking, I investigated the effects of clozapine treatment on D2R-mediated changes in the biochemical profile of G β 5. HEK293T cells co-expressing G β 5 and D2R were treated for 24 hr with 10 μ M clozapine or vehicle, and the detergent solubility of G β 5 was assessed as described in Appendix 2. Interestingly, treatment with clozapine nearly doubled the detergent solubility of G β 5 relative to vehicle treatment (Fig. 13). Thus, clozapine treatment displays inhibition of D2R-mediated changes in the biochemical profile of G β 5. This observation is striking and intriguing, and certainly begs further investigation into the nature of D2R's interaction with G β 5. If D2R induced protein aggregation does lead to neurodegeneration, and D2R accumulation is a purposeful protective mechanism against this outcome, then the ability of clozapine to conserve these biochemical properties of D2R could yield neuroprotective effects.

Furthermore, since treatment with all other tested APDs mediates D2R export from the ER and increases its detergent solubility, then perhaps they are overcoming this “protective aggregation” of D2R, and lead to neurodegeneration and tardive dyskinesia. Certainly these arguments are purely speculative at this point. However, the preliminary result of clozapine’s “protective” actions over the biochemical characteristics of Gβ5 are intriguing. Future experimentation should be done to 1) investigate the nature of D2R interaction with Gβ5, and other implicated proteins and 2) how APDs alter these interactions.

Implications for drug discovery and pharmacotherapy of schizophrenia— Perhaps this evidenced unique binding interaction of clozapine with D2R is the key for its superior clinical efficacy. The concept of ligand bias, where ligands bind the same receptor, but produce distinct conformational changes resulting in selective receptor signal activation, has been reported on for quite some time (Drake et al., 2008; Erickson et al., 2013; Luttrell, 2014; Makita and Iiri, 2013; Violin and Lefkowitz, 2007). Here we have reported that APDs are pharmacochaperone agonists of D2R membrane insertion in addition to antagonism of G-protein signaling. Perhaps, clozapine’s seemingly bias-ligand binding profile, antagonizing D2R-mediated G-protein signaling while avoiding the pharmacochaperone effects of the other APDs, is the key to its superior clinical efficacy. If so, these findings harbor great potential for the future of schizophrenia pharmacotherapy.

As previously mentioned, because of the fatal agranulocytosis produced by clozapine, its utility and impact in the treatment of schizophrenia is drastically limited (Farooq and Taylor, 2011; Pirmohamed and Park, 1997). However, these results could provide a model for APD development, in which investigational compounds can be compared to

clozapine to for similar receptor-ligand interactions, while avoiding agranulocytosis. The D2R surface ELISA could theoretically be used as a high-throughput screen for investigation APDs; identifying D2R antagonist that do not cause increased surface D2R expression. Yet, as seen in the case of aripiprazole, it is also necessary to distinguish between APDs displaying lower D2R surface expression because of increased surface receptor loss and lower total receptor expression. Additionally, clozapine binding of D2R produces unique biochemical effects on the receptor protein. Thus, I propose a novel APD development strategy identifying test compounds that display the following characteristics: 1) Antagonism of D2R, 2) Insignificant increase of surface D2R expression with prolonged treatment, 3) No effect on the rate of surface D2R removal, and 4) Do not increase D2R detergent-solubility. Unlike FGAs, clozapine does display metabolic and cardiovascular side effects. Yet, these side effects are common to of the SGAs (Meltzer, 2013b), and are therefore unlikely to be a result of the aforementioned unique characteristics of clozapine treatment. However, the side effect of agranulocytosis is unique to clozapine, so it is theoretically possible that agranulocytosis could correlate with the unique effect profile of clozapine. Currently, the mechanism of clozapine-induced agranulocytosis is unknown (Alvir et al., 1993; Pirmohamed and Park, 1997; Rajagopal, 2005), and developing an *in-vitro* assay to assess the risk of agranulocytosis is not at this point plausible. Nonetheless, these findings are tremendously exciting, as it is the first description of a unique effect profile of clozapine at D2R. Perhaps these results are the basis for the development of safe and tolerable antipsychotics that, like clozapine, effectively treat all aspects of schizophrenia.

APPENDIX 4: Supplemental Figures

Fig.11 (Left). Effect of APD treatment on surface expression of the of the PKC phosphorylation mutant, D2R_{T225,S228,S229→A}.

Fig 12 (Right). Effect of aripiprazole treatment on the internalization of D2R

Fig. 13. Effect of clozapine treatment on D2R modulation of Gβ5 solubility

FIGURE LEGENDS:

Fig.11. Effect of APD treatment on surface expression of the of the PKC phosphorylation mutant, D2R_{T225,S228,S229→A}.

HEK293 cells transiently expressing an extracellular N-terminal FLAG-tagged

D2R_{T225,S228,S229→A} construct were treated with the indicated APDs (10 μ M, 24 hr). The relative levels of cell-surface receptor were then quantified by probing intact cells with an anti-FLAG antibody and are reported as a percent of the signal from vehicle-treated cells.

[†]The levels of cell-surface receptor after either haloperidol or clozapine treatment was significantly higher than with vehicle and significantly different from each other (mean $\pm$ SEM, n = 24-56, Tukey's HSD, p < 0.05).

Fig 12 (Right). Effect of aripiprazole treatment on the internalization of D2R.

HEK293 cells transiently expressing the D2R construct were treated with either vehicle, dopamine (10 μ M), or aripiprazole (10 μ M) for 45 min. D2R contains an extracellular FLAG epitope and the levels of the receptor construct remaining at the cell surface after dopamine treatment were quantified by probing the intact, non-permeabilized cells with anti-FLAG antibody and are reported as a percent of the signal from the vehicle-treated cells. [†]Surface receptor levels were significantly less after dopamine treatment, but not aripiprazole treatment (mean $\pm$ SEM, n =16, Student's t-test, p < 0.05).

Fig. 13. Effect of clozapine treatment on D2R modulation of G β 5 solubility

A. Effect of clozapine treatment on D2R modulation of TX100 solubility of G β 5.

Representative Western blot depicting the effect of treatment with the clozapine (10 μ M, 24 hr) on D2R modulation of the segregation of G β 5, transiently expressed in HEK293 cells, into TX100-soluble (S) and insoluble (I) biochemical fractions.

B. Quantification of the TX100 solubility of D2R after APD treatment. Quantification of the percent of total cellular G β 5, that segregates into the TX100-soluble fraction, as shown in "A", after treatment with clozapine (10 μ M, 24 hr). [†]Treatment with clozapine, increased the TX100-solubility of G β 5 compared to vehicle (mean $\pm$ SEM; n = 4-6, Tukey's HSD, p < 0.05).

REFERENCES

- Agid, O., Kapur, S., and Remington, G. (2008). Emerging drugs for schizophrenia. *Expert Opin Emerg Drugs* 13, 479–495.
- Agid, O., Foussias, G., Singh, S., and Remington, G. (2010a). Where to position clozapine: re-examining the evidence. *Can J Psychiatry* 55, 677–684.
- Agid, O., Foussias, G., Singh, S., and Remington, G. (2010b). Where to position clozapine: re-examining the evidence. *Can. J. Psychiatry Rev. Can. Psychiatr.* 55, 677–684.
- Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2008). *Molecular Biology of the Cell* (Garland Science).
- Allen, J.A., Yost, J.M., Setola, V., Chen, X., Sassano, M.F., Chen, M., Peterson, S., Yadav, P.N., Huang, X.P., Feng, B., et al. (2011). Discovery of beta-arrestin-biased dopamine D2 ligands for probing signal transduction pathways essential for antipsychotic efficacy. *Proc Natl Acad Sci U A* 108, 18488–18493.
- Alvir, J.M.J., Lieberman, J.A., Safferman, A.Z., Schwimmer, J.L., and Schaaf, J.A. (1993). Clozapine-Induced Agranulocytosis – Incidence and Risk Factors in the United States. *N. Engl. J. Med.* 329, 162–167.
- Baldessarini, R.J., Centorrino, F., Flood, J.G., Volpicelli, S.A., Huston-Lyons, D., and Cohen, B.M. (1993). Tissue concentrations of clozapine and its metabolites in the rat. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* 9, 117–124.
- Basso, M., Samengo, G., Nardo, G., Massignan, T., D’Alessandro, G., Tartari, S., Cantoni, L., Marino, M., Cheroni, C., Biasi, S.D., et al. (2009). Characterization of Detergent-Insoluble Proteins in ALS Indicates a Causal Link between Nitritative Stress and Aggregation in Pathogenesis. *PLOS ONE* 4, e8130.
- Beaulieu, J.-M., and Gainetdinov, R.R. (2011a). The physiology, signaling, and pharmacology of dopamine receptors. *Pharmacol. Rev.* 63, 182–217.
- Beaulieu, J.-M., and Gainetdinov, R.R. (2011b). The Physiology, Signaling, and Pharmacology of Dopamine Receptors. *Pharmacol. Rev.* 63, 182–217.
- Beaulieu, J.-M., Espinoza, S., and Gainetdinov, R.R. (2015). Dopamine receptors - IUPHAR Review 13. *Br. J. Pharmacol.* 172, 1–23.
- Boxberger, K.H., Hagenbuch, B., and Lampe, J.N. (2014). Common drugs inhibit human organic cation transporter 1 (OCT1)-mediated neurotransmitter uptake. *Drug Metab. Dispos. Biol. Fate Chem.* 42, 990–995.
- Boyd, K.N., and Mailman, R.B. (2012). Dopamine receptor signaling and current and future antipsychotic drugs. *Handb. Exp. Pharmacol.* 53–86.

Burris, K.D., Molski, T.F., Xu, C., Ryan, E., Tottori, K., Kikuchi, T., Yocca, F.D., and Molinoff, P.B. (2002). Aripiprazole, a Novel Antipsychotic, Is a High-Affinity Partial Agonist at Human Dopamine D2 Receptors. *J. Pharmacol. Exp. Ther.* **302**, 381–389.

Casey, D.E. (1989a). Clozapine: neuroleptic-induced EPS and tardive dyskinesia. *Psychopharmacology (Berl.)* **99 Suppl**, S47-53.

Casey, D.E. (1989b). Clozapine: neuroleptic-induced EPS and tardive dyskinesia. *Psychopharmacology (Berl.)* **99 Suppl**, S47-53.

Celver, J., Sharma, M., and Kovoov, A. (2010). RGS9-2 mediates specific inhibition of agonist-induced internalization of D2-dopamine receptors. *J. Neurochem.* **114**, 739–749.

Celver, J., Sharma, M., and Kovoov, A. (2012a). D(2)-Dopamine receptors target regulator of G protein signaling 9-2 to detergent-resistant membrane fractions. *J. Neurochem.* **120**, 56–69.

Celver, J., Sharma, M., and Kovoov, A. (2012b). D2-Dopamine Receptors Target Regulator of G Protein Signaling 9-2 (RGS9-2) to Detergent-Resistant Membrane Fractions. *J. Neurochem.* **120**, 56–69.

Celver, J., Sharma, M., Thanawala, V., Oceau, J.C., and Kovoov, A. (2013). Arrestin–dependent but G–protein coupled receptor kinase–independent uncoupling of D2–dopamine receptors. *J. Neurochem.* **127**, 57–65.

Clayton, C.C., Donthamsetti, P., Lambert, N.A., Javitch, J.A., and Neve, K.A. (2014). Mutation of three residues in the third intracellular loop of the dopamine D2 receptor creates an internalization-defective receptor. *J. Biol. Chem.* **289**, 33663–33675.

Daigle, T.L., Kearn, C.S., and Mackie, K. (2008). Rapid CB1 cannabinoid receptor desensitization defines the time course of ERK1/2 MAP kinase signaling. *Neuropharmacology* **54**, 36–44.

David, A.S., Jeste, D.V., Folstein, M.F., and Folstein, S.E. (1987). Voluntary movement dysfunction in Huntington's disease and tardive dyskinesia. *Acta Neurol. Scand.* **75**, 130–139.

Dayalu, P., and Chou, K.L. (2008). Antipsychotic-induced extrapyramidal symptoms and their management. *Expert Opin. Pharmacother.* **9**, 1451–1462.

De Mei, C., Ramos, M., Iitaka, C., and Borrelli, E. (2009). Getting specialized: presynaptic and postsynaptic dopamine D2 receptors. *Curr. Opin. Pharmacol.* **9**, 53–58.

Dong, C., Filipeanu, C.M., Duvernay, M.T., and Wu, G. (2007). Regulation of G protein-coupled receptor export trafficking. *Biochim. Biophys. Acta* **1768**, 853–870.

Donthamsetti, P., Quejada, J.R., Javitch, J.A., Gurevich, V.V., and Lambert, N.A. (2015). Using bioluminescent resonance energy transfer (BRET) to characterize agonist-induced arrestin recruitment to modified and unmodified G protein-coupled receptors (GPCRs). *Curr. Protoc. Pharmacol.* Editor. Board SJ Enna Ed.--Chief AI **70**, 2.14.1-2.14.14.

- Dos Santos Pereira, J.N., Tadjerpisheh, S., Abu Abed, M., Saadatmand, A.R., Weksler, B., Romero, I.A., Couraud, P.-O., Brockmöller, J., and Tzvetkov, M.V. (2014). The poorly membrane permeable antipsychotic drugs amisulpride and sulpiride are substrates of the organic cation transporters from the SLC22 family. *AAPS J.* 16, 1247–1258.
- Drake, M.T., Violin, J.D., Whalen, E.J., Wisler, J.W., Shenoy, S.K., and Lefkowitz, R.J. (2008). β -Arrestin-biased Agonism at the β 2-Adrenergic Receptor. *J. Biol. Chem.* 283, 5669–5676.
- Erickson, C.E., Gul, R., Blessing, C.P., Nguyen, J., Liu, T., Pulakat, L., Bastepe, M., Jackson, E.K., and Andresen, B.T. (2013). The β -blocker Nebivolol Is a GRK/ β -arrestin Biased Agonist. *PLoS ONE* 8.
- Essali, A., Al-Haj Haasan, N., Li, C., and Rathbone, J. (2009). Clozapine versus typical neuroleptic medication for schizophrenia. *Cochrane Database Syst Rev* CD000059.
- Farooq, S., and Taylor, M. (2011). Clozapine: dangerous orphan or neglected friend? *Br. J. Psychiatry* 198, 247–249.
- Fishburn, C.S., Elazar, Z., and Fuchs, S. (1995a). Differential glycosylation and intracellular trafficking for the long and short isoforms of the D2 dopamine receptor. *J Biol Chem* 270, 29819–29824.
- Fishburn, C.S., Elazar, Z., and Fuchs, S. (1995b). Differential glycosylation and intracellular trafficking for the long and short isoforms of the D2 dopamine receptor. *J. Biol. Chem.* 270, 29819–29824.
- Foussias, G., and Remington, G. (2010). Antipsychotics and schizophrenia: from efficacy and effectiveness to clinical decision-making. *Can. J. Psychiatry Rev. Can. Psychiatr.* 55, 117–125.
- Frankel, J.S., and Schwartz, T.L. (2017). Brexpiprazole and cariprazine: distinguishing two new atypical antipsychotics from the original dopamine stabilizer aripiprazole. *Ther. Adv. Psychopharmacol.* 7, 29–41.
- Freedman, R. (2003a). Schizophrenia. *N Engl J Med* 349, 1738–49.
- Freedman, R. (2003b). Schizophrenia. *N. Engl. J. Med.* 349, 1738–1749.
- Friedman, J.H. (2013). Parkinson disease psychosis: Update. *Behav. Neurol.* 27, 469–477.
- Froemming, J.S., Lam, Y.W., Jann, M.W., and Davis, C.M. (1989). Pharmacokinetics of haloperidol. *Clin. Pharmacokinet.* 17, 396–423.
- Furlong, R.A., Narain, Y., Rankin, J., Wyttenbach, A., and Rubinsztein, D.C. (2000). alpha-synuclein overexpression promotes aggregation of mutant huntingtin. *Biochem. J.* 346, 577–581.
- Ginovart, N., and Kapur, S. (2012). Role of dopamine D(2) receptors for antipsychotic activity. *Handb. Exp. Pharmacol.* 27–52.

- Girard-Bock, C., Benoit-Biancamano, M.-O., Villeneuve, L., Desjardins, S., and Guillemette, C. (2016). A rare UGT2B7 variant creates a novel N-glycosylation site at codon 121 with impaired enzyme activity. *Drug Metab. Dispos. Biol. Fate Chem.*
- Gurevich, E.V., Gainetdinov, R.R., and Gurevich, V.V. (2016). G protein-coupled receptor kinases as regulators of dopamine receptor functions. *Pharmacol. Res.* *111*, 1–16.
- Hall, R.A., Premont, R.T., and Lefkowitz, R.J. (1999). Heptahelical Receptor Signaling: Beyond the G Protein Paradigm. *J. Cell Biol.* *145*, 927–932.
- Han, Y., Moreira, I.S., Urizar, E., Weinstein, H., and Javitch, J.A. (2009a). Allosteric communication between protomers of dopamine class A GPCR dimers modulates activation. *Nat. Chem. Biol.* *5*, 688–695.
- Han, Y., Moreira, I.S., Urizar, E., Weinstein, H., and Javitch, J.A. (2009b). Allosteric communication between protomers of dopamine class A GPCR dimers modulates activation. *Nat. Chem. Biol.* *5*, 688–695.
- Hanyaloglu, A.C., McCullagh, E., and von Zastrow, M. (2005). Essential role of Hrs in a recycling mechanism mediating functional resensitization of cell signaling. *EMBO J.* *24*, 2265–2283.
- Hatters, D.M. (2008). Protein misfolding inside cells: The case of huntingtin and Huntington's disease. *IUBMB Life* *60*, 724–728.
- Howes, O.D., and Kapur, S. (2009). The Dopamine Hypothesis of Schizophrenia: Version III--The Final Common Pathway. *Schizophr. Bull.* *35*, 549–562.
- Hunter, M.R., Grimsey, N.L., and Glass, M. (2016). Sulfation of the FLAG epitope is affected by co-expression of G protein-coupled receptors in a mammalian cell model. *Sci. Rep.* *6*, 27316.
- Iqbal, M.M., Rahman, A., Husain, Z., Mahmud, S.Z., Ryan, W.G., and Feldman, J.M. (2003). Clozapine: a clinical review of adverse effects and management. *Ann. Clin. Psychiatry Off. J. Am. Acad. Clin. Psychiatr.* *15*, 33–48.
- Jamur, M.C., and Oliver, C. (2010). Permeabilization of cell membranes. *Methods Mol. Biol. Clifton NJ* *588*, 63–66.
- Jibson, M.D., and Tandon, R. (1998a). New atypical antipsychotic medications. *J Psychiatr Res* *32*, 215–28.
- Jibson, M.D., and Tandon, R. (1998b). New atypical antipsychotic medications. *J. Psychiatr. Res.* *32*, 215–228.
- Kamal, M., Marquez, M., Vauthier, V., Leloire, A., Froguel, P., Jockers, R., and Couturier, C. (2009). Improved donor/acceptor BRET couples for monitoring β -arrestin recruitment to G protein-coupled receptors. *Biotechnol. J.* *4*, 1337–1344.

- Kang, X., and Simpson, G.M. (2010). Clozapine: more side effects but still the best antipsychotic. *J Clin Psychiatry* 71, 982–983.
- Kapur, S., and Mamo, D. (2003a). Half a century of antipsychotics and still a central role for dopamine D2 receptors. *Prog Neuropsychopharmacol Biol Psychiatry* 27, 1081–90.
- Kapur, S., and Mamo, D. (2003b). Half a century of antipsychotics and still a central role for dopamine D2 receptors. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 27, 1081–1090.
- Kapur, S., and Remington, G. (2001). Dopamine D(2) receptors and their role in atypical antipsychotic action: still necessary and may even be sufficient. *Biol Psychiatry* 50, 873–883.
- Kerwin, R.W., and Bolonna, A.A. (2004a). Is clozapine antisuicidal? *Expert Rev Neurother* 4, 187–190.
- Kerwin, R.W., and Bolonna, A.A. (2004b). Is clozapine antisuicidal? *Expert Rev. Neurother.* 4, 187–190.
- Kim, O.-J., Ariano, M.A., Namkung, Y., Marinec, P., Kim, E., Han, J., and Sibley, D.R. (2008a). D2 dopamine receptor expression and trafficking is regulated through direct interactions with ZIP. *J. Neurochem.* 106, 83–95.
- Kim, O.-J., Ariano, M.A., Namkung, Y., Marinec, P., Kim, E., Han, J., and Sibley, D.R. (2008b). D2 dopamine receptor expression and trafficking is regulated through direct interactions with ZIP. *J. Neurochem.* 106, 83–95.
- Kornhuber, J., Wiltfang, J., Riederer, P., and Bleich, S. (2006). Neuroleptic drugs in the human brain. *Eur. Arch. Psychiatry Clin. Neurosci.* 256, 274–280.
- Kovoor, A., Seyffarth, P., Ebert, J., Barghshoon, S., Chen, C.-K., Schwarz, S., Axelrod, J.D., Cheyette, B.N.R., Simon, M.I., Lester, H.A., et al. (2005a). D2 dopamine receptors colocalize regulator of G-protein signaling 9-2 (RGS9-2) via the RGS9 DEP domain, and RGS9 knock-out mice develop dyskinesias associated with dopamine pathways. *J. Neurosci. Off. J. Soc. Neurosci.* 25, 2157–2165.
- Kovoor, A., Seyffarth, P., Ebert, J., Barghshoon, S., Chen, C.-K., Schwarz, S., Axelrod, J.D., Cheyette, B.N.R., Simon, M.I., Lester, H.A., et al. (2005b). D2 Dopamine Receptors Colocalize Regulator of G-Protein Signaling 9-2 (RGS9-2) via the RGS9 DEP Domain, and RGS9 Knock-Out Mice Develop Dyskinesias Associated with Dopamine Pathways. *J. Neurosci.* 25, 2157–2165.
- Kramer, M.S., Last, B., Getson, A., and Reines, S.A. (1997). The effects of a selective D4 dopamine receptor antagonist (L-745,870) in acutely psychotic inpatients with schizophrenia. D4 Dopamine Antagonist Group. *Arch. Gen. Psychiatry* 54, 567–572.
- Krutzik, P.O., and Nolan, G.P. (2003). Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. *Cytom. Part J. Int. Soc. Anal. Cytol.* 55, 61–70.

- Kubale, V., Blagotinšek, K., Nøhr, J., Eidne, K.A., and Vrecl, M. (2016a). The Conserved Arginine Cluster in the Insert of the Third Cytoplasmic Loop of the Long Form of the D2 Dopamine Receptor (D2L-R) Acts as an Intracellular Retention Signal. *Int. J. Mol. Sci.* **17**, 1152.
- Kubale, V., Blagotinšek, K., Nøhr, J., Eidne, K.A., and Vrecl, M. (2016b). The Conserved Arginine Cluster in the Insert of the Third Cytoplasmic Loop of the Long Form of the D2 Dopamine Receptor (D2L-R) Acts as an Intracellular Retention Signal. *Int. J. Mol. Sci.* **17**, 1152.
- Kumagai, H., Ikeda, Y., Motozawa, Y., Fujishiro, M., Okamura, T., Fujio, K., Okazaki, H., Nomura, S., Takeda, N., Harada, M., et al. (2015). Quantitative Measurement of GPCR Endocytosis via Pulse-Chase Covalent Labeling. *PloS One* **10**, e0129394.
- Kuroki, T., Nagao, N., and Nakahara, T. (2008a). Neuropharmacology of second-generation antipsychotic drugs: a validity of the serotonin-dopamine hypothesis. *Prog Brain Res* **172**, 199–212.
- Kuroki, T., Nagao, N., and Nakahara, T. (2008b). Neuropharmacology of second-generation antipsychotic drugs: a validity of the serotonin-dopamine hypothesis. *Prog. Brain Res.* **172**, 199–212.
- Lan, H., Liu, Y., Bell, M.I., Gurevich, V.V., and Neve, K.A. (2009). A dopamine D2 receptor mutant capable of G protein-mediated signaling but deficient in arrestin binding. *Mol. Pharmacol.* **75**, 113–123.
- Lan, T.-H., Liu, Q., Li, C., Wu, G., and Lambert, N.A. (2012). Sensitive and high resolution localization and tracking of membrane proteins in live cells with BRET. *Traffic Cph. Den.* **13**, 1450–1456.
- Lester, H.A., Miwa, J.M., and Srinivasan, R. (2012). Psychiatric drugs bind to classical targets within early exocytotic pathways: therapeutic effects. *Biol. Psychiatry* **72**, 907–915.
- Leucht, S., Kissling, W., and Davis, J.M. (2009). Second-generation antipsychotics for schizophrenia: can we resolve the conflict? *Psychol. Med.* **39**, 1591–1602.
- Lewis, S., and Lieberman, J. (2008a). CATIE and CUTLASS: can we handle the truth? *Br J Psychiatry* **192**, 161–163.
- Lewis, S., and Lieberman, J. (2008b). CATIE and CUTLASS: can we handle the truth? *Br. J. Psychiatry* **192**, 161–163.
- Los, G.V., Encell, L.P., McDougall, M.G., Hartzell, D.D., Karassina, N., Zimprich, C., Wood, M.G., Learish, R., Ohana, R.F., Urh, M., et al. (2008a). HaloTag: a novel protein labeling technology for cell imaging and protein analysis. *ACS Chem. Biol.* **3**, 373–382.
- Los, G.V., Encell, L.P., McDougall, M.G., Hartzell, D.D., Karassina, N., Zimprich, C., Wood, M.G., Learish, R., Ohana, R.F., Urh, M., et al. (2008b). HaloTag: A Novel Protein Labeling Technology for Cell Imaging and Protein Analysis. *ACS Chem. Biol.* **3**, 373–382.

Luttrell, L.M. (2014). Minireview: More than just a hammer: ligand “bias” and pharmaceutical discovery. *Mol. Endocrinol. Baltim. Md* 28, 281–294.

Makita, N., and Iiri, T. (2013). Biased agonism: a novel paradigm in G protein-coupled receptor signaling observed in acquired hypocalciuric hypercalcemia [Review]. *Endocr. J. advpub.*

Manschreck, T.C., and Boshes, R.A. (2007). The CATIE schizophrenia trial: results, impact, controversy. *Harv Rev Psychiatry* 15, 245–258.

Marchese, A., Paing, M.M., Temple, B.R.S., and Trejo, J. (2008). G Protein-Coupled Receptor Sorting to Endosomes and Lysosomes. *Annu. Rev. Pharmacol. Toxicol.* 48, 601–629.

Margolese, H.C., Chouinard, G., Kolivakis, T.T., Beaulieu, L., and Miller, R. (2005). Tardive dyskinesia in the era of typical and atypical antipsychotics. Part 1: pathophysiology and mechanisms of induction. *Can J Psychiatry* 50, 541–7.

Masri, B., Salahpour, A., Didriksen, M., Ghisi, V., Beaulieu, J.-M., Gainetdinov, R.R., and Caron, M.G. (2008). Antagonism of dopamine D2 receptor/beta-arrestin 2 interaction is a common property of clinically effective antipsychotics. *Proc. Natl. Acad. Sci. U. S. A.* 105, 13656–13661.

Masuho, I., Martemyanov, K.A., and Lambert, N.A. (2015). Monitoring G protein activation in cells with BRET. *Methods Mol. Biol. Clifton NJ* 1335, 107–113.

Meltzer, H.Y. (2012a). Clozapine: balancing safety with superior antipsychotic efficacy. *Clin. Schizophr. Relat. Psychoses* 6, 134–144.

Meltzer, H.Y. (2012b). Clozapine. *Clin. Schizophr. Relat. Psychoses* 6, 134–144.

Meltzer, H.Y. (2013a). Update on typical and atypical antipsychotic drugs. *Annu. Rev. Med.* 64, 393–406.

Meltzer, H.Y. (2013b). Update on Typical and Atypical Antipsychotic Drugs. *Annu. Rev. Med.* 64, 393–406.

Meltzer, H.Y., and Huang, M. (2008). In vivo actions of atypical antipsychotic drug on serotonergic and dopaminergic systems. *Prog Brain Res* 172, 177–197.

Meltzer, H.Y., Li, Z., Kaneda, Y., and Ichikawa, J. (2003). Serotonin receptors: their key role in drugs to treat schizophrenia. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 27, 1159–1172.

Millan, M.J., Morris, B.J., and Herz, A. (1988). Antagonist-induced opioid receptor up-regulation. I. Characterization of supersensitivity to selective mu and kappa agonists. *J. Pharmacol. Exp. Ther.* 247, 721–728.

Min, C., Zheng, M., Zhang, X., Guo, S., Kwon, K.-J., Shin, C.Y., Kim, H.-S., Cheon, S.H., and Kim, K.-M. (2015). N-linked Glycosylation on the N-terminus of the dopamine D2 and D3 receptors

determines receptor association with specific microdomains in the plasma membrane. *Biochim. Biophys. Acta* 1853, 41–51.

Missale, C., Nash, S.R., Robinson, S.W., Jaber, M., and Caron, M.G. (1998). Dopamine receptors: from structure to function. *Physiol Rev* 78, 189–225.

Monteleone, M.C., González Wusener, A.E., Burdisso, J.E., Conde, C., Cáceres, A., and Arregui, C.O. (2012). ER-bound protein tyrosine phosphatase PTP1B interacts with Src at the plasma membrane/substrate interface. *PloS One* 7, e38948.

Muench, J., and Hamer, A.M. (2010). Adverse effects of antipsychotic medications. *Am. Fam. Physician* 81, 617–622.

Naber, D., and Lambert, M. (2009). The CATIE and CUTLASS studies in schizophrenia: results and implications for clinicians. *CNS Drugs* 23, 649–659.

Naheed, M., and Green, B. (2001). Focus on clozapine. *Curr Med Res Opin* 17, 223–229.

Namkung, Y., and Sibley, D.R. (2004a). Protein kinase C mediates phosphorylation, desensitization, and trafficking of the D2 dopamine receptor. *J Biol Chem* 279, 49533–49541.

Namkung, Y., and Sibley, D.R. (2004b). Protein Kinase C Mediates Phosphorylation, Desensitization, and Trafficking of the D2 Dopamine Receptor. *J. Biol. Chem.* 279, 49533–49541.

Nord, M., and Farde, L. (2011). Antipsychotic Occupancy of Dopamine Receptors in Schizophrenia. *CNS Neurosci. Ther.* 17, 97–103.

Octeau, J.C. (2014). Compartmentalized D2-dopamine receptors: Roles in signaling & potential mechanism of antipsychotic drug action. *Diss. Masters Theses Campus Access* 1–111.

Octeau, J.C., Schrader, J.M., Masuho, I., Sharma, M., Aiudi, C., Chen, C.-K., Kovoov, A., and Cerver, J. (2014). G Protein Beta 5 Is Targeted to D2-Dopamine Receptor-Containing Biochemical Compartments and Blocks Dopamine-Dependent Receptor Internalization. *PLoS ONE* 9.

Opgen-Rhein, C., and Dettling, M. (2008). Clozapine-induced agranulocytosis and its genetic determinants. *Pharmacogenomics* 9, 1101–1111.

Pirmohamed, M., and Park, K. (1997). Mechanism of clozapine-induced agranulocytosis : current status of research and implications for drug development. *CNS Drugs* 7, 139–158.

Prou, D., Gu, W.J., Le Crom, S., Vincent, J.D., Salamero, J., and Vernier, P. (2001a). Intracellular retention of the two isoforms of the D(2) dopamine receptor promotes endoplasmic reticulum disruption. *J Cell Sci* 114, 3517–27.

- Prou, D., Gu, W.-J., Crom, S.L., Vincent, J.-D., Salamero, J., and Vernier, P. (2001b). Intracellular retention of the two isoforms of the D2 dopamine receptor promotes endoplasmic reticulum disruption. *J. Cell Sci.* *114*, 3517–3527.
- Rajagopal, S. (2005). Clozapine, agranulocytosis, and benign ethnic neutropenia. *Postgrad. Med. J.* *81*, 545–546.
- Rajagopal, S., and Shenoy, S.K. (2017). GPCR desensitization: Acute and prolonged phases. *Cell. Signal.*
- Richtand, N.M., Welge, J.A., Logue, A.D., Keck, P.E., Strakowski, S.M., and McNamara, R.K. (2007). Dopamine and serotonin receptor binding and antipsychotic efficacy. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* *32*, 1715–1726.
- Sahlholm, K., Marcellino, D., Nilsson, J., Ögren, S.O., Fuxe, K., and Århem, P. (2014). Typical and atypical antipsychotics do not differ markedly in their reversibility of antagonism of the dopamine D2 receptor. *Int. J. Neuropsychopharmacol.* *17*, 149–155.
- Sahlholm, K., Zeberg, H., Nilsson, J., Ögren, S.O., Fuxe, K., and Århem, P. (2016). The fast-off hypothesis revisited: A functional kinetic study of antipsychotic antagonism of the dopamine D2 receptor. *Eur. Neuropsychopharmacol. J. Eur. Coll. Neuropsychopharmacol.* *26*, 467–476.
- Schlager, B., Straessle, A., and Hafen, E. (2012). Use of anionic denaturing detergents to purify insoluble proteins after overexpression. *BMC Biotechnol.* *12*, 95.
- Seeman, P. (2002). Atypical antipsychotics: mechanism of action. *Can J Psychiatry* *47*, 27–38.
- Seeman, P. (2006). Targeting the dopamine D2 receptor in schizophrenia. *Expert Opin. Ther. Targets* *10*, 515–531.
- Seeman, P. (2010). Dopamine D2 receptors as treatment targets in schizophrenia. *Clin. Schizophr. Relat. Psychoses* *4*, 56–73.
- Seeman, P. (2014). Clozapine, a fast-off-D2 antipsychotic. *ACS Chem. Neurosci.* *5*, 24–29.
- Seeman, P., Corbett, R., and Van Tol, H.H. (1997). Atypical neuroleptics have low affinity for dopamine D2 receptors or are selective for D4 receptors. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* *16*, 93-110; discussion 111-135.
- Sharma, M., Cerver, J., Oceau, J.C., and Kovoov, A. (2013a). Plasma membrane compartmentalization of D2 dopamine receptors. *J. Biol. Chem.* *288*, 12554–12568.
- Sharma, M., Cerver, J., Oceau, J.C., and Kovoov, A. (2013b). Plasma Membrane Compartmentalization of D2 Dopamine Receptors. *J. Biol. Chem.* *288*, 12554–12568.
- Shaw, B.F., Lelie, H.L., Durazo, A., Nersissian, A.M., Xu, G., Chan, P.K., Gralla, E.B., Tiwari, A., Hayward, L.J., Borchelt, D.R., et al. (2008). Detergent-insoluble Aggregates Associated with Amyotrophic Lateral Sclerosis in Transgenic Mice Contain Primarily Full-length, Unmodified Superoxide Dismutase-1. *J. Biol. Chem.* *283*, 8340–8350.

Shi, L., and Javitch, J.A. (2004). The second extracellular loop of the dopamine D2 receptor lines the binding-site crevice. *Proc. Natl. Acad. Sci. U. S. A.* *101*, 440–445.

Shu, X., Shaner, N.C., Yarbrough, C.A., Tsien, R.Y., and Remington, S.J. (2006). Novel chromophores and buried charges control color in mFruits. *Biochemistry (Mosc.)* *45*, 9639–9647.

Siris, S.G. (2001). Suicide and schizophrenia. *J. Psychopharmacol. (Oxf.)* *15*, 127–135.

Siskind, D., McCartney, L., Goldschlager, R., and Kisely, S. (2016). Clozapine v. first- and second-generation antipsychotics in treatment-refractory schizophrenia: systematic review and meta-analysis. *Br. J. Psychiatry J. Ment. Sci.* *209*, 385–392.

Stahl, S.M. (1997). “Awakening” from schizophrenia: intramolecular polypharmacy and the atypical antipsychotics. *J. Clin. Psychiatry* *58*, 381–382.

Tandon, R. (2011a). Antipsychotics in the treatment of schizophrenia: an overview. *J Clin Psychiatry* *72 Suppl 1*, 4–8.

Tandon, R. (2011b). Antipsychotics in the treatment of schizophrenia: an overview. *J. Clin. Psychiatry* *72 Suppl 1*, 4–8.

Tandon, R., Belmaker, R.H., Gattaz, W.F., Lopez-Ibor, J.J., Okasha, A., Singh, B., Stein, D.J., Olie, J.P., Fleischhacker, W.W., and Moeller, H.J. (2008). World Psychiatric Association Pharmacopsychiatry Section statement on comparative effectiveness of antipsychotics in the treatment of schizophrenia. *Schizophr Res.*

Tandon R, Carpenter WT, and Davis JM (2007). First- and second-generation antipsychotics: Learning from cutlass and catie. *Arch. Gen. Psychiatry* *64*, 977–978.

Thibault, D., Albert, P.R., Pineyro, G., and Trudeau, L.-É. (2011). Neurotensin Triggers Dopamine D2 Receptor Desensitization through a Protein Kinase C and β -Arrestin1-dependent Mechanism. *J. Biol. Chem.* *286*, 9174–9184.

Tirotta, E., Fontaine, V., Picetti, R., Lombardi, M., Samad, T.A., Oulad-Abdelghani, M., Edwards, R., and Borrelli, E. (2008a). Signaling by dopamine regulates D2 receptors trafficking at the membrane. *Cell Cycle* *7*, 2241–2248.

Tirotta, E., Fontaine, V., Picetti, R., Lombardi, M., Samad, T.A., Oulad-Abdelghani, M., Edwards, R., and Borrelli, E. (2008b). Signaling by dopamine regulates D2 receptors trafficking at the membrane. *Cell Cycle Georget. Tex* *7*, 2241–2248.

Unterwald, E.M., Rubenfeld, J.M., Imai, Y., Wang, J.-B., Uhl, G.R., and Kreek, M.J. (1995). Chronic opioid antagonist administration upregulates mu opioid receptor binding without altering mu opioid receptor mRNA levels. *Mol. Brain Res.* *33*, 351–355.

Urh, M., and Rosenberg, M. (2012). HaloTag, a Platform Technology for Protein Analysis. *Curr. Chem. Genomics* *6*, 72–78.

Urs, N.M., Peterson, S.M., and Caron, M.G. (2017). New Concepts in Dopamine D2 Receptor Biased Signaling and Implications for Schizophrenia Therapy. *Biol. Psychiatry* 81, 78–85.

Vallone, D., Picetti, R., and Borrelli, E. (2000). Structure and function of dopamine receptors. *Neurosci Biobehav Rev* 24, 125–132.

Vauquelin, G., Bostoen, S., Vanderheyden, P., and Seeman, P. (2012). Clozapine, atypical antipsychotics, and the benefits of fast-off D2 dopamine receptor antagonism. *Naunyn. Schmiedeberg Arch. Pharmacol.* 385, 337–372.

Violin, J.D., and Lefkowitz, R.J. (2007). β -Arrestin-biased ligands at seven-transmembrane receptors. *Trends Pharmacol. Sci.* 28, 416–422.

Wahlbeck, K., Cheine, M., and Essali, M.A. (2000). Clozapine versus typical neuroleptic medication for schizophrenia. *Cochrane Database Syst. Rev.* CD000059.

Walker, J.K., Premont, R.T., Barak, L.S., Caron, M.G., and Shetzline, M.A. (1999). Properties of secretin receptor internalization differ from those of the beta(2)-adrenergic receptor. *J. Biol. Chem.* 274, 31515–31523.

Watson, A.J., Katz, A., and Simon, M.I. (1994). A fifth member of the mammalian G-protein beta-subunit family. Expression in brain and activation of the beta 2 isotype of phospholipase C. *J. Biol. Chem.* 269, 22150–22156.

Wenthur, C.J., and Lindsley, C.W. (2013a). Classics in chemical neuroscience: clozapine. *ACS Chem. Neurosci.* 4, 1018–1025.

Wenthur, C.J., and Lindsley, C.W. (2013b). Classics in Chemical Neuroscience: Clozapine. *ACS Chem. Neurosci.* 4, 1018–1025.

Wüller, S., Wiesner, B., Löffler, A., Furkert, J., Krause, G., Hermosilla, R., Schaefer, M., Schüle, R., Rosenthal, W., and Oksche, A. (2004). Pharmacochaperones post-translationally enhance cell surface expression by increasing conformational stability of wild-type and mutant vasopressin V2 receptors. *J. Biol. Chem.* 279, 47254–47263.

Xu, W., Ozdener, F., Li, J.G., Chen, C., de Riel, J.K., Weinstein, H., and Liu-Chen, L.Y. (1999). Functional role of the spatial proximity of Asp114(2.50) in TMH 2 and Asn332(7.49) in TMH 7 of the mu opioid receptor. *FEBS Lett.* 447, 318–324.

Yamaguchi, K., Inoue, S., Ohara, O., and Nagase, T. (2009). Pulse-chase experiment for the analysis of protein stability in cultured mammalian cells by covalent fluorescent labeling of fusion proteins. *Methods Mol. Biol. Clifton NJ* 577, 121–131.

Zurek, N., Sparks, L., and Voeltz, G. (2011a). Reticulon short hairpin transmembrane domains are used to shape ER tubules. *Traffic Cph. Den.* 12, 28–41.

Zurek, N., Sparks, L., and Voeltz, G. (2011b). Reticulon short hairpin transmembrane domains are used to shape ER tubules. *Traffic Cph. Den.* 12, 28–41.