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**Title Page**

# Identification of Amino Acid Determinants of Dopamine 2 Receptor Synthetic Agonist Function

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**Running Title Page**

Running Title: **Molecular Determinants of Dopamine D2 Receptor Function**

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**ABBREVIATIONS:** D2R, dopamine D2 receptor; hD2R, human dopamine D2 receptor; DD2R, *Drosophila* D2-like receptor; D1, dopamine D1 receptor; D3, dopamine D3 receptor; D4, dopamine D4 receptor; D5, dopamine D5 receptor; GPCR, G protein-coupled receptor; SCAM, substituted cysteine accessibility method; HEK, human embryonic kidney; SRE, serum response element; CRE, cyclic AMP response element; transmembrane domain, TM; cAMP, cyclic AMP.

**RECOMMENDED SECTION ASSIGNMENT:** Neuropharmacology

**Abstract**

The human dopamine 2 receptor (hD2R) modulates locomotor activity, hormone secretion, as well as neuropsychiatric function. Current knowledge of the hD2R structure is in large part derived from mutagenesis studies and molecular pharmacologic analysis together with homology modeling using bovine rhodopsin as a template. In this study, we utilized comparison of the *Drosophila* D2-like receptor (DD2R) with the hD2R as a novel approach for identifying candidate amino acids that are determinants of ligand potency and/or efficacy. We focused our studies on four dopaminergic ligands that are used in the treatment of Parkinson's disease: bromocriptine, pergolide, piribedil, and ropinirole. All four ligands are potent agonists at the wild type hD2R, whereas only bromocriptine shows comparable function at the DD2R. We performed site-directed mutagenesis to replace hD2R amino acids (modeled to project into the ligand binding pocket) with corresponding fly residues, and vice versa. Substitution of three amino acids in the hD2R with the homologous DD2R residues (V91A, C118S, and L170I) led to a pronounced loss of pergolide potency and efficacy. A converse triple amino acid substitution of human residues into the fly receptor (DD2R-A133V/S160C/I211L) markedly enhanced pergolide efficacy and potency at the mutant DD2R. The same substitutions also converted piribedil and ropinirole, which lacked appreciable activity on the DD2R, to partial agonists. These findings demonstrate an important role of these three residues in drug-receptor interactions. Our study illustrates that comparison of a

mammalian receptor with an invertebrate homolog complements previously described strategies for defining GPCR structure-function relationships.

## Introduction

The human dopamine 2 receptor (hD2R) modulates locomotor activity, cognition, emotion, reinforcement, and secretion of selected endocrine hormones. The dysregulation of dopamine-mediated neurotransmission has been linked to multiple pathologies, including Parkinson's disease, schizophrenia, Tourette's syndrome, and hyperprolactinemia. The hD2R is a class A G protein-coupled receptor (GPCR) with homology to rhodopsin and the β-adrenergic receptor. Dopamine receptors have been divided into two subgroups: D1- and D2-like based on amino acid homology as well as biochemical and pharmacological properties. Among these, the D1 and D5 subtypes (classified as D1-like receptors) signal primarily through Gs whereas hD2R as well as the D3 and D4 subtypes (classified as D2-like receptors) signal predominately through Gi/o (Missale et al., 1998; Emilien et al., 1999; Vallone et al., 2000).

Due to the inherent difficulties in crystallizing membrane proteins, including GPCRs, high-resolution structural information is only available for one class A family member, rhodopsin (Gether, 2000). As an alternative approach to understanding D2 receptor function, a variety of molecular and pharmacologic approaches have been utilized. Several laboratories have used the substituted cysteine accessibility method (SCAM) to map residues that project into the hD2R ligand pocket. By systematically substituting amino acids in transmembrane domains 2, 3, 4, 5, 6 and 7 with cysteines and reacting the altered receptors with

charged sulfhydryl-specific methanethiosulfonate derivatives, the accessibility of these mutated residues in the predicted binding crevice was determined (Javitch et al., 1994; Javitch et al., 1995a; Javitch et al., 1995b; Fu et al., 1996; Javitch et al., 1998; Javitch et al., 1999; Javitch et al., 2000; Shi et al., 2001). SCAM analysis along with a series of complementary approaches (e.g. studies of chimeric receptors and point mutants as well as computer based modeling using the known rhodopsin structure as a template) enabled the identification of amino acids that line the putative binding pocket of the hD2 receptor (Neve et al., 1991; Mansour et al., 1992; Javitch et al., 1994; Kozell et al., 1994; Javitch et al., 1995a; Javitch et al., 1995b; Naylor et al., 1995; Fu et al., 1996; Javitch et al., 1996; Javitch et al., 1998; Wilcox et al., 1998; Javitch et al., 1999; Simpson et al., 1999; Coley et al., 2000; Javitch et al., 2000; Wilcox et al., 2000; Ballesteros et al., 2001; Shi et al., 2001). These studies identified single residues as well as multiple amino acid combinations which play a role in determining ligand affinity (Javitch et al., 1996; Alberts et al., 1998; Simpson et al., 1999; Schetz and Sibley, 2000). In contrast, the contribution of hD2R amino acids as potential determinants of hD2R agonist function has been less extensively studied.

Our laboratory cloned and pharmacologically characterized the *Drosophila* D2-like receptor (DD2R), the first known invertebrate homolog of mammalian dopamine 2 receptors. Like its mammalian counterpart, the DD2R signals through the inhibitory G protein, Gi, shows highest potency for dopamine (versus other biogenic amines), and is fully activated by bromocriptine with nanomolar potency (Hearn et al., 2002). Bromocriptine is among a group of synthetic

agonists which are used clinically for the treatment of Parkinson's disease. In contrast to this ligand, other drugs in this group including piribedil, pergolide and ropinirole have little or no activity at the fly DD2R. The differential response to synthetic hD2R agonists, in combination with the relatively high conservation of the predicted fly binding pocket when aligned with its human counterpart (48/73 identical amino acids), suggests a limited number of candidate residues which confer synthetic ligand potency/efficacy. Consistent with this prediction, we demonstrate by exchanging homologous residues between the human and fly D2-like receptors that a combination of amino acids in the ligand pocket that are not conserved between the two receptors underlie the species-specific differences in pharmacology. Comparison between a mammalian versus a corresponding non-mammalian GPCR thus enabled the identification of a novel combination of residues that are molecular determinants of ligand activity at both the human and fly D2 receptors.

## Methods

**Materials.** The receptor ligands used in this study were purchased from Sigma-Aldrich (St. Louis, MO). These reagents include: (+)-bromocriptine mesylate ((+)-2-Bromo-12'-hydroxy-2'-(1-methylethyl)-5'-(2-methylpropyl)ergotaman-3',6'-18-trione methanesulfonate salt), piribedil maleate (2-[4-(1,3-Benzodioxol-5-ylmethyl)-1-piperazinyl]pyrimidine maleate salt), pergolide mesylate (8β-[(Methylthio)methyl]-6-propylergoline methanesulfonate salt), ropinirole hydrochloride (diethyl[2-(2-oxo-2,3-dihydro-1H-indol-4-yl)ethyl]ammonium chloride), dopamine hydrochloride (2-(3,4-Dihydroxyphenyl)ethylamine hydrochloride) and forskolin (7β-Acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one). All stock solutions for ligands were prepared in deionized water with the exception of bromocriptine, which was prepared in ethanol, and pergolide and forskolin which were prepared in DMSO. Aliquots of ligands were stored frozen at -80°C and were diluted in serum-free media immediately before use.

**Comparison of Amino Acid Sequences.** An amino acid alignment of the *Drosophila* D2-like receptor with the human D2 receptor was performed using ALIGN X with the blosum62mt2 scoring matrix (Vector NTI Suite, version 5.5, InforMax, North Bethesda, MD). An alignment comparing the human D2 receptor with mammalian and invertebrate species orthologs was performed using CLUSTALW version 3.2 (http://workbench.sdsc.edu).

**Generation of Mutant Receptors.** The fly and human D2 receptors (506 and 443 amino acids respectively) (Grandy et al., 1989; Hearn et al., 2002) were each subcloned into the expression vector pcDNA1.1 (Invitrogen, Carlsbad, CA). Mutants were generated by modifying the coding region of either the wild type human or fly D2 receptors. Amino acid substitutions were introduced via oligonucleotide-directed, site-specific mutagenesis, as described previously (Beinborn et al., 1993). Oligonucleotides of interest were synthesized at the Tufts University DNA Synthesis Core Facility (Boston, MA). Introduction of desired mutations was confirmed by restriction enzyme analyses followed by dideoxynucleotide sequencing of the entire protein-coding region using an automated ABI 37X DNA sequencer (Applied Biosystems, Foster City, CA).

**Cell Culture.** Human embryonic kidney (HEK) 293 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Biotechnics Research Inc., Lake Forest, CA) and 100 units/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a humidified environment containing 5% $CO_2$.

**Transfection and Luciferase Assay.** HEK 293 cells were plated (5,000-7,000 per well) onto 96-well Primaria plates (BD Biosciences, Bedford, MA). Cells were transiently transfected using Lipofectamine reagent (Invitrogen, Carlsbad, CA) with cDNAs encoding (i) either wild type or mutant receptors (hD2R or DD2R), (ii)

a reporter gene construct consisting of five tandem repeats of the serum response element ($SRE_{5X}$) ligated upstream from a reporter gene encoding firefly luciferase (Feuerbach et al., 2000) and (iii) Gq5i, a chimeric G protein consisting of Gq with substitution of the five corresponding carboxy-terminal amino acids of Gi (Conklin et al., 1993; Elshourbagy et al., 2000). The 5 amino acids at the carboxy-terminus of Gq5i are sufficient to enable interaction with Gi coupled receptors (Conklin et al., 1993; Coward et al., 1999). The Gq domains of Gq5i enables receptor mediated signaling to the Gq pathway which can, in turn, be detected using the serum response element $(SRE)_{5x}$-luciferase reporter gene construct.

As a complementary approach to examine Gi mediated signaling, cells were stimulated with forskolin and dopamine receptor mediated inhibition of cyclic AMP (cAMP) induced transcription was examined. For these studies, cells were transiently transfected with cDNAs encoding (i) either wild type or mutant receptors (hD2R or DD2R) and (ii) a reporter gene construct consisting of six tandem repeats of the cyclic AMP response element ($CRE_{6X}$) (George et al., 1998; Kemp et al., 1999) ligated upstream from a reporter gene encoding firefly luciferase (Feuerbach et al., 2000).

Twenty-four hours after transfection, cells were exposed to ligand for 3 hours in serum-free medium either in the presence or absence of forskolin (as indicated in figure legends). The cells were then lysed, and luciferase activity quantified using LucLite reagents (PerkinElmer, Wellesley, MA). All measurements were repeated in at least three separate experiments, each

performed in triplicate. Ligand potencies were assessed by stimulating receptor expressing cells with increasing concentrations of agonists. $pEC_{50}$ values were calculated from concentration response curves using computerized nonlinear curve fitting (PRISM 3.0, GraphPad, San Diego, CA). Percent efficacies were calculated by dividing the maximum light unit value of an agonist by the maximum value for bromocriptine at the corresponding receptor.

**Statistical Analysis.** All statistical analyses were performed using one-way ANOVA and Dunnet's post-test (INSTAT, GraphPad, San Diego, CA).

## Results

**Functional assessment of dopamine D2 receptor agonists on the fly and human D2 receptors.** Four dopamine agonists used in the treatment of Parkinson's disease (Figure 1) were the focus of this structure-function analysis. Each of these agonists signals with relatively high potency at the hD2R; corresponding $EC_{50}$ values were < 10nM. However, among these four ligands, only bromocriptine is a strong agonist at the DD2R as well (Figure 2). In contrast, pergolide signals at the DD2 receptor with low potency and low efficacy, whereas piribedil and ropinirole do not trigger ligand induced signaling at the fly receptor (Figure 2).

**Comparison of the hD2 and the DD2 receptors reveals candidate molecular determinants of agonist efficacy/potency.** As a first step to identifying candidate residues which may underlie the pharmacological differences between the *Drosophila* and human D2 receptors, an amino acid alignment of the two GPCRs was made. Residues that are conserved or different between the two receptors are indicated in Figure 3. A total of 73 residues comprise the human D2 pocket (Javitch et al., 1994; Javitch et al., 1995a; Javitch et al., 1995b; Fu et al., 1996; Javitch et al., 1998; Javitch et al., 1999; Javitch et al., 2000; Ballesteros et al., 2001; Shi et al., 2001). Within this pocket, only 25 residues are not conserved between the fly and human homologs. Among these, 24 residues that are found in the outer two thirds of the transmembrane domain

pocket were examined in this study and are highlighted in Figure 3. We hypothesize that species divergence in these amino acids is the most likely explanation for the distinct pharmacologies of the fly versus the human D2 receptors.

**Selected hD2R mutants show a loss of agonist efficacy/potency.**

Since the pharmacologic profile of agonist function differs between the hD2 and DD2 receptors (Figure 2), amino acids in the ligand pocket that are not conserved between the two receptors (Figure 3) represent candidate residues underlying the species-specific differences. Site directed mutagenesis was utilized to exchange the divergent amino acids between the *Drosophila* and human D2-like receptor homologs, which had been identified as candidate potency/efficacy determinants. As a first step, human receptor residues were replaced with the corresponding fly amino acids. It was hypothesized that substitution of fly residues into the human D2 receptor would result in mutants with decreased potency for piribedil, pergolide and ropinirole while having little if any impact on the $EC_{50}$ of agonists which are not species-selective, i.e. bromocriptine (Figure 2) and/or dopamine (Hearn et al., 2002). To assess all of the candidate amino acids (24 residues), a total of 13 mutants were generated; each included one to five substitutions. The mutants were each expressed in HEK293 cells and stimulated with increasing concentrations of agonist. Full efficacy of bromocriptine or dopamine and conserved potency for at least one of these agonists (within 3-fold of either the wild type hD2R or DD2R values) was

utilized as an index of intact tertiary structure of the mutant receptors. The pharmacologic profile of each of the variants satisfied these criteria. Two mutants (hD2R-IFV109FYI and hD2R-C118S) exhibited decreased potency for bromocriptine (Table 1) but displayed $EC_{50}$ values for dopamine that were indistinguishable from the wild type human D2R value (mean $pEC_{50} \pm$ SEM for hD2R is $6.08 \pm 0.24$, for hD2R-IFV109FYI is $6.05 \pm 0.27$ and for hD2R-C118S is $5.84 \pm 0.26$, no significant differences by ANOVA). These findings suggest that the overall structure of the hD2R-IFV109FYI and hD2R-C118S mutants was conserved despite a slight decrease in bromocriptine potency.

When the human D2 receptor mutants were stimulated with pergolide, six of these constructs displayed a significant decrease in potency when compared to the wild type receptor (highlighted in Table 1). Corresponding substituted residues were thus considered potential contributors to the pharmacological species differences between the fly and human receptors.

**Selected mutant DD2 receptors show an increase in agonist potency/efficacy.** Further investigation focused on the six mutant human receptors that displayed a significant decrease in pergolide potency (Table 1). To explore whether converse substitutions confer increased agonist potency, human D2 receptor residues were introduced in place of the corresponding fly amino acids. Each of the generated receptors maintained conserved potency of at least bromocriptine and/or dopamine suggesting that the DD2R mutants retained intact tertiary structure (Table 2). Two mutants (DD2R-FA132WV and DD2R-FL136LE)

displayed decreased bromocriptine potency (Table 2) but retained dopamine potency within 3-fold of the wild type DD2R value (mean $pEC_{50} \pm$ SEM for DD2R is $6.07 \pm 0.26$, for DD2R-FA132WV is $6.05 \pm 0.24$ for DD2R-FL136LE is $6.68 \pm 0.30$, no significant differences by ANOVA). Four mutants, DD2R-S160C, DD2R-FA132WV, DD2R-IVl211LLF and DD2R-LS239IV showed an increase in pergolide potency however the change did not quite reach statistical significance (Table 2). Among these mutants with a tendency toward gain of function, the DD2R-S160C variant was chosen for further analysis since the complementary hD2R construct (hD2R-C118S) showed the most pronounced loss of function phenotype with regard to pergolide potency (Table 1). Taken together, the findings with the C118S and S160C substitution mutants provided preliminary evidence that the respective residues in DD2R and hD2R are important determinants of agonist potency/efficacy. To further explore this possibility, a series of additional *Drosophila* mutants was generated. Substitutions in each of these constructs included the S160C alteration in combination with one of the other putative gain of function mutations shown in Table 2. Of the five different constructs generated, two (DD2R-S160C/FA132WV and DD2R-S160C/IVL211LLF) showed a significant increase in both pergolide potency and efficacy versus the wild type DD2R (Table 3). Findings with the two combination mutants suggested that a subset of the six substituted residues act in concert to define pergolide activity at the human D2 receptor.

**A triple mutant markedly enhances the potency of pergolide at the DD2 receptor.** To further define the residues that are important determinants of pergolide activity, different combinations of amino acids were selected from S160C, FA132WV, and IVL211LLF. A series of six additional mutants (DD2R-A133V/S160C/I211L, DD2R-F132W/S160C/I211L, DD2R-A133V/S160C/V212L, DD2R-F132W/S160C/V212L, DD2R-A133V/S160C, and DD2R-F132W/S160C) was generated and assessed sequentially with bromocriptine, pergolide, piribedil and ropinirole) (data not shown). We focused on two of these constructs that conferred the most pronounced gain of function to compounds with either minor or no detectable activity at the wild type fly DD2R. A triple amino acid substitution in the DD2R (DD2R-A133V/S160C/I211L) markedly increased pergolide efficacy and enhanced the potency of this compound to within 10-fold of the wild type human D2 receptor (Figure 4). In addition, a double mutant (DD2R-A133V/S160C) increased the efficacy and potency of pergolide albeit to a lesser degree than the triple substitution. Whereas, piribedil and ropinirole fail to stimulate the wild type fly DD2 receptor, introduction of either the double or triple combination of human amino acids into the wild type fly DD2R enabled these agonists to induce a limited degree of signaling (Figure 4).

To further validate the importance of the corresponding amino acids for ligand function, respective converse substitutions were introduced into the wild type human D2 receptor. The double and triple amino acid human mutants were assessed with each of the four agonists. Bromocriptine $EC_{50}$ was conserved

(Figure 5); however, a decrease in potency was observed with pergolide, piribedil and ropinirole.

A complementary reporter gene assay, which relies on receptor coupling to the endogenous Gi, was used to confirm the pharmacologic significance of the human D2R residues V91, C118 and L170. Cells were treated with forskolin to stimulate cAMP induced expression of a $CRE_{6x}$-luciferase reporter gene. Gi mediated inhibition of this activity in response to dopamine receptor agonists was assessed. As anticipated, bromocriptine triggered a concentration dependent decrease in forskolin-induced cyclic AMP mediated reporter gene ($CRE_{6x}$-luciferase) transcription (Figure 6A). Consistent with our findings using the Gq5i/$SRE_{5x}$-luciferase reporter assay (Figures 4 and 5), the function of bromocriptine when assessed via the endogenous Gi protein and the $CRE_{6x}$-luciferase construct was similar at the wild type and mutant hD2Rs and DD2Rs (Figure 6A). Also supporting our prior observations, pergolide showed markedly less activity at the wild type *Drosophila* versus the human D2 receptor (Figure 6B). This difference was largely reversed when examined at the triple mutant receptors (hD2R-V91A/C118S/L170I versus DD2R-A133V/S160C/I211L) where three critical species-selective residues were exchanged between the two receptor isoforms (Figure 6B). As predicted, while piribedil and ropinirole showed significant endogenous Gi mediated activity at the wild type hD2R, essentially no activity of these ligands was appreciable when the wild type DD2R was examined (Figure 6C and 6D). The introduction of human residues into the fly receptor (DD2R-A133V/S160C/I211L) conferred enhanced piribedil and

ropinirole function, whereas the converse substitution of fly residues in the hD2R (hD2R-V91A/C118S/L170I) led to a loss of pergolide and ropinirole activity (Figure 6C and 6D).

Taken together, two complementary readouts of signal transduction (which rely on either recombinant Gq5i or endogenous Gi mediated signaling) provide consistent results. The findings suggest that V91, C118, and L170 (i.e. the residues which are represented by the double and triple amino acid substitutions discussed above) are important determinants of activity for selected synthetic agonists at the human dopamine 2 receptor.

## Discussion

In the present study, the *Drosophila* DD2R provided a new tool to identify molecular determinants of ligand activity at the human D2 receptor. This report focuses on four synthetic agonists used as treatment options for Parkinson's disease (i.e. bromocriptine, piribedil, pergolide and ropinirole). For many GPCRs (e.g. the cholecystokinin type 2, neurokinin type 1, and serotonin type 1B receptors), species-specific (dog or rat versus human) differences in ligand activity have been identified (Fong et al., 1992; Oksenberg et al., 1992; Beinborn et al., 1993; Cascieri et al., 1994) and provided an experimental basis for exploring the molecular determinants of ligand function. In contrast, known dopamine 2 receptor agonists appear to act comparably on mammalian D2 receptor orthologs (Grandy et al., 1989). The similarity in the pharmacological profiles of mammalian D2 receptors therefore does not enable these isoforms to be used as molecular probes to identify functionally important residues. Consistent with these observations, a comparison of mammalian D2 receptors (human versus mouse versus rat) reveals a fully conserved (100% identical) putative ligand pocket.

The cloning and the characterization of the *Drosophila* D2-like receptor (Hearn et al., 2002) presented a novel opportunity to identify synthetic agonist efficacy/potency determinants. Both the fly and human D2 receptors signal through Gi and both are activated by dopamine and bromocriptine with relatively high potency. At the same time, these two receptors showed marked differences

in sensitivity to piribedil, pergolide and ropinirole (Figure 2). The receptor ligand pockets show 66% identity and 77% similarity at the amino acid level leaving a finite number of residues that may underlie the distinct pharmacological profiles.

Systematic exchange of residues between the fly and human D2 receptors ultimately led to the identification of a three amino acid combination (A133V/ S160C/I211L) that when transferred from the human D2 receptor into the fly receptor resulted in a marked increase in pergolide efficacy (converting this compound into a full DD2R agonist) and potency (to within 10-fold of the wild type human value) (Figure 4). These residue substitutions in the DD2R also increased the function of the two other species-selective synthetic agonists that were tested (i.e. piribedil and ropinirole) suggesting that these amino acids are functionally important to multiple structurally diverse ligands. The observed sequential increase in drug function with single vs. double vs. triple amino acid substitutions in the DD2R is consistent with current models of synthetic agonist-receptor interaction where combinations of selected amino acids within a receptor's binding pocket are postulated to interact with ligands to define potency and/or efficacy (Javitch et al., 1996).

The most pronounced mutation induced potency and efficacy increases in this study were observed with the agonist, pergolide (Figure 4). The observation that limited alterations in the DD2R binding pocket were sufficient to enhance pergolide function close to that of bromocriptine (which acts as a potent agonist at the fly receptor) suggests that the mode of action of these two ligands may be similar. Consistent with this hypothesis, an inspection of the structure of the

ligands used in this study reveals that pergolide and bromocriptine are structurally related compounds and on this basis are classified as ergoline derivatives. In contrast, the two ligands that were less affected by the receptor mutations that were studied, piribedil and ropinirole, are both nonergots. Future studies will be needed to identify the additional pharmacological determinants which further contribute to potency/activity differences of piribedil and ropinirole at the fly versus human D2 receptors.

Our studies suggest that V91, C118, and L170 are critical to synthetic agonist function at the human D2 receptor. A model of the hD2R was proposed by Kalani et al. using computational techniques to predict the binding sites and relative binding energies of various dopaminergic ligands (Kalani et al., 2004). Based on in silico analysis, a model was thus proposed in which C118 is among the residues that form a predominately hydrophobic pocket where dopamine, as well as, class I (exemplified by clozapine) and class II (exemplified by haloperidol) antagonists bind to the receptor. Using the same approach, V91 is modeled to lie in a largely hydrophobic pocket for class II antagonists (Kalani et al., 2004). It is of note that the predicted relevance of the C118 and V91 for receptor-ligand interactions is in line with our experimental findings. However, according to the in silico model, L170 (which we found to be pharmacologically relevant) is not predicted to interact with any of the ligands that were studied. It is well established that only some of the residues forming the surface of a GPCR binding crevice are in direct contact with agonists or antagonists, whereas others

primarily play a structural role in stabilizing the overall pocket configuration (Javitch et al., 1996). L170 may fall into the latter class of amino acids.

In addition to modeling approaches, our conclusion that residues C118 and V91 are important determinants of synthetic agonist potency/efficacy at the human D2R is also supported by complementary biochemical/pharmacological investigations. Javitch et al. have shown by SCAM analysis that covalently linking C118 to sulfhydryl-reactive reagents results in an inhibition of ligand affinity (Javitch et al., 1994). This transmembrane domain 3 amino acid was therefore postulated to reside within the binding site crevice of the human D2 receptor. In addition, serial substitutions in place of C118 (i.e. K, S, M, and A), resulted in mutant receptors exhibiting variable reductions in the binding affinities of dopamine and a synthetic D2 receptor ligand, sulpiride. These observations led the authors to conclude that the residue occupying position 118 in the hD2R plays an important role in ligand binding affinity, an effect that depends on both the size and the charge of the corresponding side chain (Javitch et al., 1996). Our study extends these findings by demonstrating that C118 is an important determinant not only of affinity but also of potency and efficacy for multiple structurally diverse drugs that are commonly used for the treatment of Parkinson's disease (i.e. pergolide, piribedil and ropinirole).

Previous studies have also explored the role of valine 91 as a molecular determinant of ligand affinity (Simpson et al., 1999; Kortagere et al., 2004; Floresca et al., 2005). Valine 91 in the hD2R was found to be in the water-accessible surface of the receptor by SCAM analysis (Javitch et al., 1999). The

potential relevance of the residue occupying position 91 was inferred from studies on the D4 receptor. When the corresponding residue in the rat D4 receptor (F88V) was substituted with its hD2R homolog (V91), there was a 100-fold decrease in affinity for the D4R selective ligand L-750,667 (Schetz et al., 2000). Conversely, when V91 in the hD2R was substituted with phenylalanine (the corresponding residue in the hD4R), there was ~100-fold increase in affinity for the highly selective D4R compound CPPMA compared to the wild type hD2 receptor (Simpson et al., 1999). The latter observation, although focused on the binding properties of a D4R selective compound, support our conclusion that V91 in the hD2R is an important determinant of receptor-ligand interaction.

An amino acid alignment comparing the human D2 receptor with mammalian and invertebrate species orthologs (Figure 7) highlights the residues that were found to be determinants of synthetic agonist activity (C118/V91/L170). Of note, residue C118 in the human D2 receptor is conserved among mammalian D2 receptors whereas its corresponding amino acid is a serine in both the fly DD2R and *C. elegans* D2 receptors. Residue V91 is also highly conserved among mammalian receptors while the corresponding residue in both the fly and *C. elegans* D2 receptors is an alanine. Leucine 170 is also conserved among the mammlain D2 receptors however in the fly and *C. elegans* D2-like receptors, it is an isoleucine and methionine, respectively. Variability in the amino acid sequence of a given mammalian GPCR subtype (e.g. the cholecystokinin type 2 and neurokinin type 1 receptors) has been previously used to identify residues underlying ligand affinity and/or efficacy differences between species

(e.g. dog versus human, and mouse versus human) (Fong et al., 1992; Beinborn et al., 1993). The conservation of critical amino acids (i.e. C118, V91 and L170) among mammalian D2 receptors precluded the use of these receptors in mapping the efficacy/potency determinants of synthetic ligands. In contrast, comparison of hD2R with the *Drosophila* D2 receptor homolog provided a greater degree of divergence between the corresponding binding pockets. This strategy thus enabled the identification of functionally important amino acids that could not have been appreciated by comparison of only mammalian receptor isoforms.

In summary our study demonstrates that pharmacologic differences that occur between vertebrate and invertebrate receptor homologs may be utilized to map residues that are relevant to synthetic agonist binding and function. This approach revealed three amino acids in the human D2 receptor which together serve as important potency/efficacy determinants for selected anti-Parkinsonian drugs. We suggest that parallel strategies utilizing other invertebrate orthologous GPCRs may have utility in exploring the interplay between receptor-selective ligands and their cognate GPCRs.

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## Footnotes

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**Legends for Figures**

**Figure 1. Structure of selected hD2R agonists used in the treatment of Parkinson's disease.**

**Figure 2. Activity of D2 receptor ligands at the human D2 receptor versus the *Drosophila* DD2R.** HEK293 cells were co-transfected with receptor (either hD2R or DD2R) cDNA in addition to a $SRE_{5x}$-luciferase reporter gene construct and a plasmid encoding the chimeric G protein, Gq5i. After a 3 hour stimulation with increasing concentrations of ligand, luciferase activity was quantified. Data represent the mean $\pm$ SEM of multiple independent experiments (n=11 for hD2R and n=14 for DD2R). Luciferase activity is expressed as a percentage of the bromocriptine induced maximum for each receptor. Calculated $EC_{50}$ values (in nM) for the hD2R were 0.11 (bromocriptine), 0.15 (pergolide), 9.3 (piribedil), and 6.7 (ropinirole). Corresponding $EC_{50}$ values (in nM) for the DD2R were 0.44 (bromocriptine) and 82 (pergolide). Piribedil and ropinirole did not stimulate the DD2R. The maximum levels of bromocriptine induced signaling for the hD2R and DD2R were comparable: 21,568 $\pm$ 1916 versus 27,707 $\pm$ 4090 counts/second (mean $\pm$ SEM) respectively.

**Figure 3. Schematic representation of the human dopamine 2 receptor.** Gray circles illustrate residues that are conserved between the human dopamine 2 receptor and fly DD2R based on amino acid sequence alignment. Black circles

illustrate 24 residues that differ between the two receptors and are predicted to comprise part of the putative ligand binding pocket. White circles represent amino acids that are not conserved.

**Figure 4. Double and triple amino acid substitutions in the DD2R result in enhanced agonist potency/efficacy.** HEK293 cells were co-transfected with receptor cDNA in addition to a $SRE_{5x}$-luciferase reporter gene construct and a plasmid encoding the chimeric G protein, Gq5i. After a 3 hour stimulation with increasing concentrations of ligand, luciferase activity was quantified. Data represent the mean $\pm$ SEM of multiple independent experiments (n=11 for hD2R, n=14 for DD2R, n=4 for DD2R-A133V/S160C/I211L and n=4 for DD2R-A133V/S160C). Luciferase activity is expressed as a percentage of the bromocriptine induced maximum for each receptor. Calculated $EC_{50}$ values (in nM) for DD2R-A133V/S160C/I211L were 0.22 (bromocriptine) and 1.7 (pergolide). Corresponding $EC_{50}$ values (in nM) for DD2R-A133V/S160C were 0.40 (bromocriptine) and 4.0 (pergolide). In contrast to findings with the wild type DD2R, piribedil and ropinirole had detectable efficacies at the mutant receptors although the potencies of these compounds remained relatively low ($EC_{50}$ values $> 10^6$M). The maximum levels of bromocriptine induced signaling were as follows (in counts/second; mean $\pm$ SEM): hD2R (11,908 $\pm$ 1813), DD2R (13,531 $\pm$ 600), DD2R-A133V/S160C/I211L (13,004 $\pm$ 1175) and DD2R-A133V/S160C (13,846 $\pm$ 2094).

**Figure 5. Double and triple amino acid substitutions in the hD2R result in decreased agonist potency/efficacy.** HEK293 cells were co-transfected with receptor cDNA in addition to a $SRE_{5x}$-luciferase reporter gene construct and a plasmid encoding the chimeric G protein, Gq5i. After a 3 hour stimulation with increasing concentrations of ligand, luciferase activity was quantified. Data represent the mean $\pm$ SEM of multiple independent experiments (n=11 for hD2R, n=14 for DD2R, n=4 for hD2R-V91A/C118S/L170I and n=4 for hD2R-V91A/C118S). Luciferase activity is expressed as a percentage of the bromocriptine induced maximum for each receptor. Calculated $EC_{50}$ values (in nM) for hD2R-V91A/C118S/L170I were 1.3 (bromocriptine), 74 (pergolide), and 1243 (ropinirole). Corresponding $EC_{50}$ values (in nM) for hD2R-V91A/C118S were 0.58 (bromocriptine), 38 (pergolide), and 406 (ropinirole). Low efficacy of piribedil at these mutant receptors precluded calculation of $EC_{50}$ values. The maximum levels of bromocriptine induced signaling were as follows (in counts/second; mean $\pm$ SEM): hD2R (3946 $\pm$ 464), DD2R (5706 $\pm$ 389), hD2R-V91A/C118S/L170I (3673 $\pm$ 374) and hD2R-V91A/C118S (3557 $\pm$ 208).

**Figure 6. Cyclic AMP dependent changes in gene transcription provide a complementary measure of ligand function of wild type and mutant dopamine receptors.** HEK293 cells were co-transfected with receptor cDNA in addition to a $CRE_{6x}$-luciferase reporter gene construct. After a 3 hour stimulation with 0.5μM of forskolin in the absence or presence of increasing concentrations

of ligand, luciferase activity was quantified. Data represent the mean ± SEM of three independent experiments. Luciferase activity is expressed as a percentage of the forskolin induced maximum for each receptor.

**Figure 7. Amino acid alignment between mammalian and invertebrate D2 receptor orthologs.** Portions of transmembrane domains (TM) 2, 3 and 4 encompassing human D2 receptor residues (V91, C118, and L170) are shown. The position of hD2R amino acids are indicated above the alignment. Residues that correspond to positions 91, 118 and 170 of the human D2 receptor are highlighted. The sequence labeled ‘mammalian D2R’ reflects conserved sequences in the human, rat, mouse and dog D2 receptors.

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## Tables

**Table 1. Agonist potencies and efficacies at wild type versus mutant human dopamine 2 receptors.**

| Mutation | Bromocriptine | Pergolide | | n |
|---|---|---|---|---|
| | $pEC_{50}$ mean ± SEM | $pEC_{50}$[a] mean ± SEM | %Efficacy[b] mean ± SEM | |
| hD2R | 9.95 ± 0.04 | 9.81 ± 0.10 | 77.3 ± 7.1 | 11 |
| hD2R-WV90FA | 9.76 ± 0.07 | 9.08 ± 0.09[d] | 71.1 ± 5.2 | 4 |
| hD2R-LE94FL | 9.65 ± 0.08 | 9.03 ± 0.16[d] | 54.9 ± 3.5 | 4 |
| hD2R-IFV109FYI | 8.86 ± 0.06[c] | 9.39 ± 0.07 | 65.1 ± 4.8 | 4 |
| hD2R-C118S | 8.96 ± 0.07[c] | 7.78 ± 0.20[d] | 88.0 ± 8.1 | 4 |
| hD2R-F164A | 9.83 ± 0.08 | 9.87 ± 0.10 | 63.4 ± 3.7 | 4 |
| hD2R-S167G | 9.61 ± 0.20 | 10.46 ± 0.14[d] | 96.8 ± 22.4 | 4 |
| hD2R-LLF170IVL | 9.32 ± 0.10 | 8.91 ± 0.07[d] | 75.0 ± 13.1 | 3 |
| hD2R-VV190IL | 9.69 ± 0.10 | 10.49 ± 0.21[d] | 77.2 ± 7.3 | 4 |
| hD2R-IV195LS | 9.16 ± 0.11 | 9.04 ± 0.10[d] | 77.2 ± 11.1 | 4 |
| hD2R-V200I | 9.78 ± 0.13 | 10.07 ± 0.08 | 80.2 ± 5.5 | 4 |
| hD2R-TH392CN | 9.59 ± 0.11 | 10.03 ± 0.15 | 67.0 ± 5.1 | 4 |
| hD2R-I397A | 9.73 ± 0.10 | 9.81 ± 0.11 | 64.0 ± 5.2 | 4 |

| | | | | |
|---|---|---|---|---|
| hD2R-LYSAF407AYMMT | 9.60 ± 0.03 | 9.03 ± 0.08[d] | 69.1 ± 5.4 | 4 |

[a] Mutants with a pergolide $pEC_{50}$ value that is significantly lower than the wild type human D2 receptor are highlighted.

[b] For all receptors, pergolide efficacy (expressed as a percentage of the corresponding value for bromocriptine) is comparable (no significant differences by ANOVA).

[c] Value is more than 3-fold lower than either the wild type hD2R or DD2R values (see Table 2 for DD2R value) but the dopamine $pEC_{50}$ values are conserved (see text).

[d] Value significantly different ($P<0.01$) than wild type hD2R value.

**Table 2. Agonist potencies and efficacies of wild type versus mutant *Drosophila* D2 receptors.**

| Mutation | Bromocriptine | Pergolide | | n |
|---|---|---|---|---|
| | $pEC_{50}$ mean ± SEM | $pEC_{50}$ mean ± SEM | %Efficacy[a] mean ± SEM | |
| DD2R | 9.36 ± 0.02 | 7.08 ± 0.01 | 34.6 ± 2.6 | 14 |
| DD2R-FA132WV | 7.99 ± 0.08[d] | 7.43 ± 0.10 | 54.5 ± 10.1 | 4 |
| DD2R-FL136LE | 7.55 ± 0.15[d] | N.D.[e] | 0.5 ± 0.3[c] | 3 |
| DD2R-S160C | 10.09 ± 0.12 | 7.29 ± 0.22 | 50.9 ± 10.1 | 5 |
| DD2R-IVL211LLF | 9.03 ± 0.08 | 7.27 ± 0.10 | 61.5 ± 6.5[b] | 3 |
| DD2R-LS239IV | 9.25 ± 0.12 | 7.34 ± 0.10 | 40.8 ± 2.4 | 3 |
| DD2R-AYMMT468LYSAF | 8.79 ± 0.13 | 6.82 ± 0.28 | 10.1 ± 2.8[b] | 3 |

[a] Pergolide efficacy is expressed as a percentage of the corresponding value for bromocriptine.

[b] Value significantly different ($P<0.05$) versus wild type DD2R value.

[c] Value significantly different ($P<0.01$) versus wild type DD2R value.

[d] Value more than 3-fold lower than either the wild type hD2R or DD2R values but the dopamine $pEC_{50}$ values are conserved (see text).

[e] N.D. = not determined due to lack of efficacy.

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**Table 3. Agonist potencies and efficacies of pergolide at the DD2R are enhanced through the introduction of combination mutants.**

| Mutation | Bromocriptine | Pergolide[a] | | n |
|---|---|---|---|---|
| | $pEC_{50}$ mean ± SEM | $pEC_{50}$[a] mean ± SEM | %Efficacy[a,b] mean ± SEM | |
| DD2R | 9.36 ± 0.02 | 7.08 ± 0.01 | 34.6 ± 2.6 | 14 |
| DD2R-S160C | 10.09 ± 0.12 | 7.29 ± 0.22 | 50.9 ± 10.1 | 5 |
| DD2R-S160C/IVL211LLF | 9.82 ± 0.03 | 7.80 ± 0.03[d] | 58.4 ± 10.9[c] | 5 |
| DD2R-S160C/FA132WV | 9.53 ± 0.03 | 8.65 ± 0.03[d] | 79.3 ± 6.8[d] | 7 |
| DD2R-S160C/FL136LE | 9.37 ± 0.08 | 7.15 ± 0.04 | 23.6 ± 4.7 | 4 |
| DD2R-S160C/AYMMT468LYSAF | 9.86 ± 0.03 | 7.01 ± 0.03 | 30.6 ± 1.5 | 3 |
| DD2R-S160C/LS239IV | 9.76 ± 0.08 | 7.73 ± 0.06[d] | 51.0 ± 1.9 | 3 |

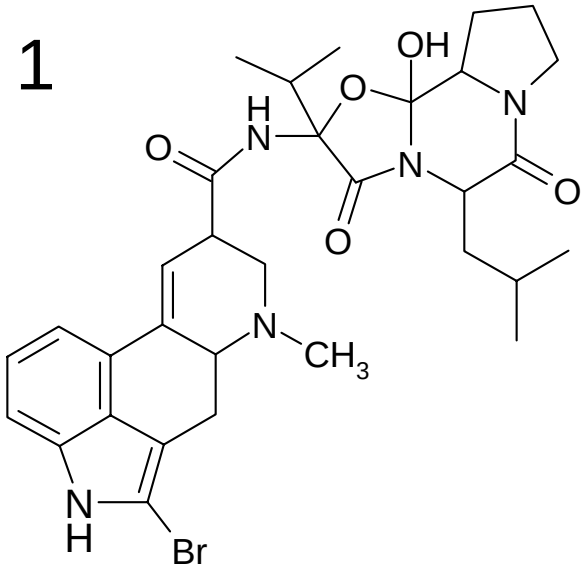
[a] Mutants with pergolide potency and/or efficacy values that are significantly higher than the wild type DD2 receptor are highlighted.

[b] Pergolide efficacy is expressed as a percentage of the corresponding value for bromocriptine.

[c] Value significantly different (P<0.05) than wild type DD2R value.

[d] Value significantly different (P<0.01) than wild type DD2R value.

# Figure 1

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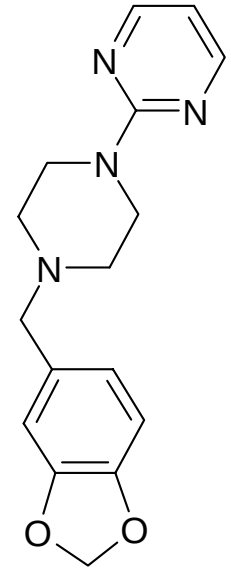


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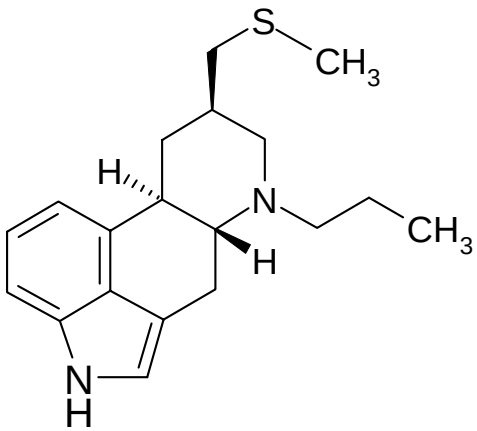


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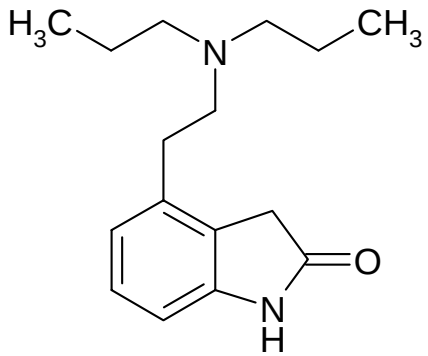
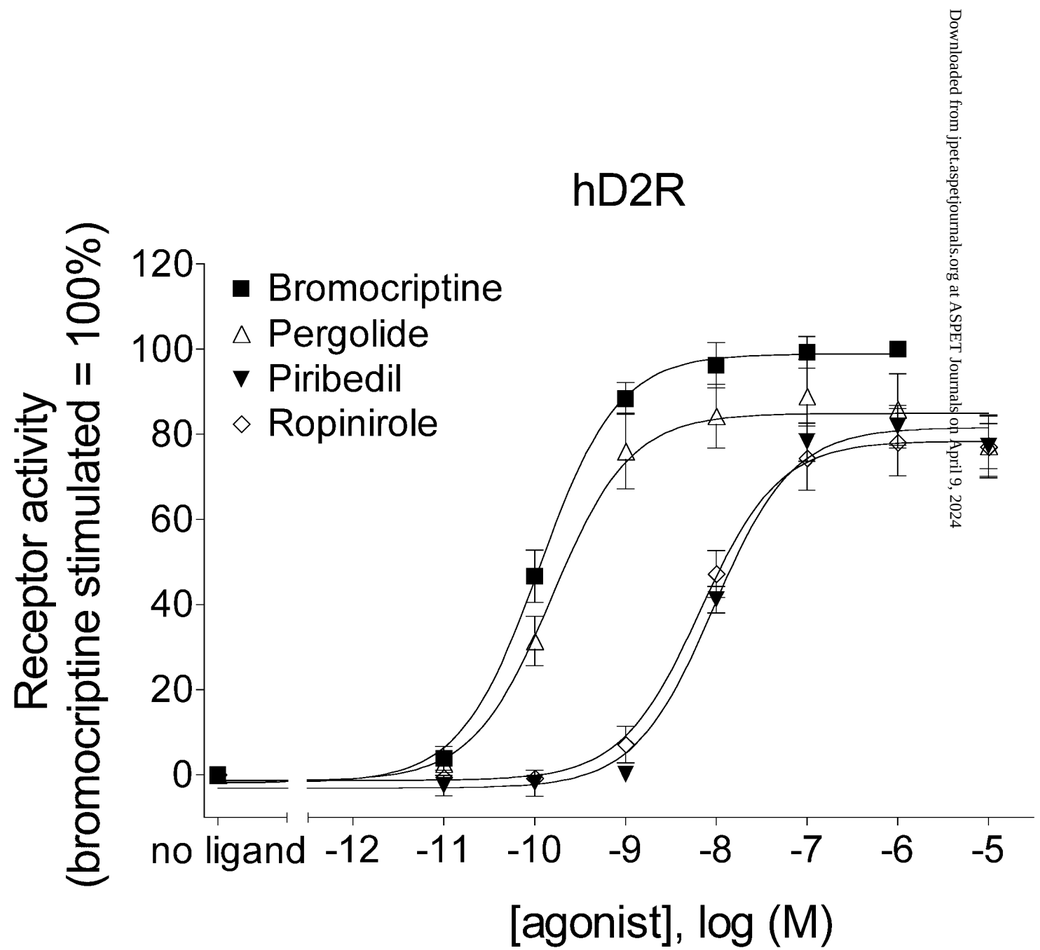
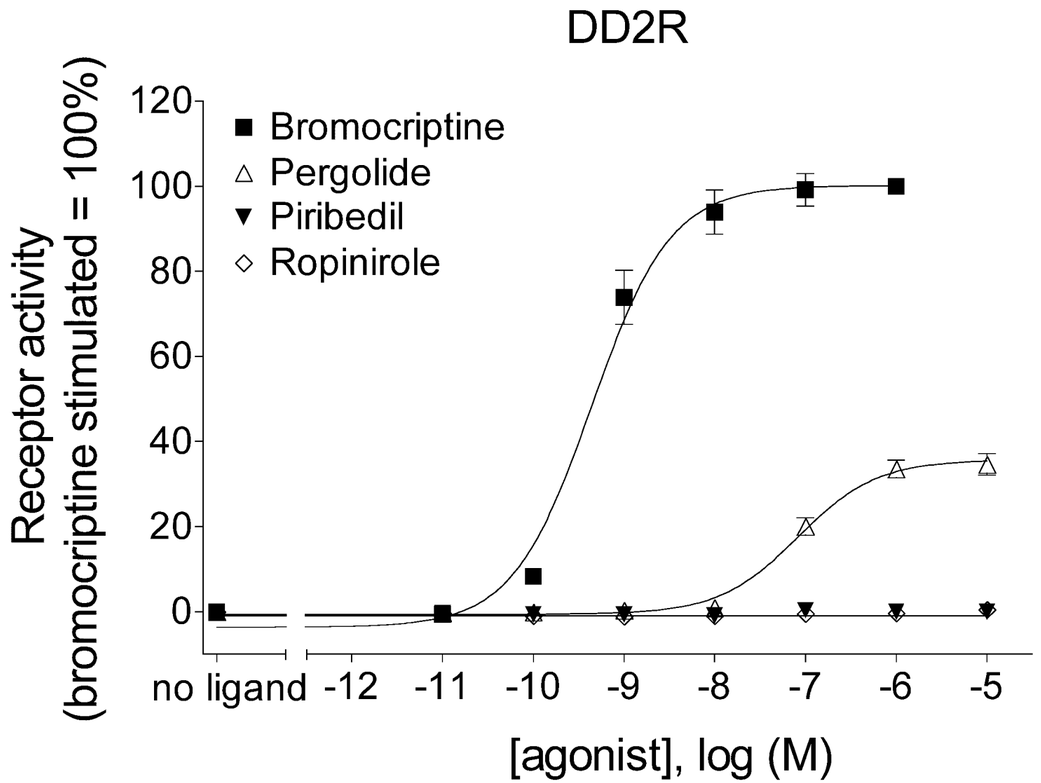



Ropinirole

# Figure 2





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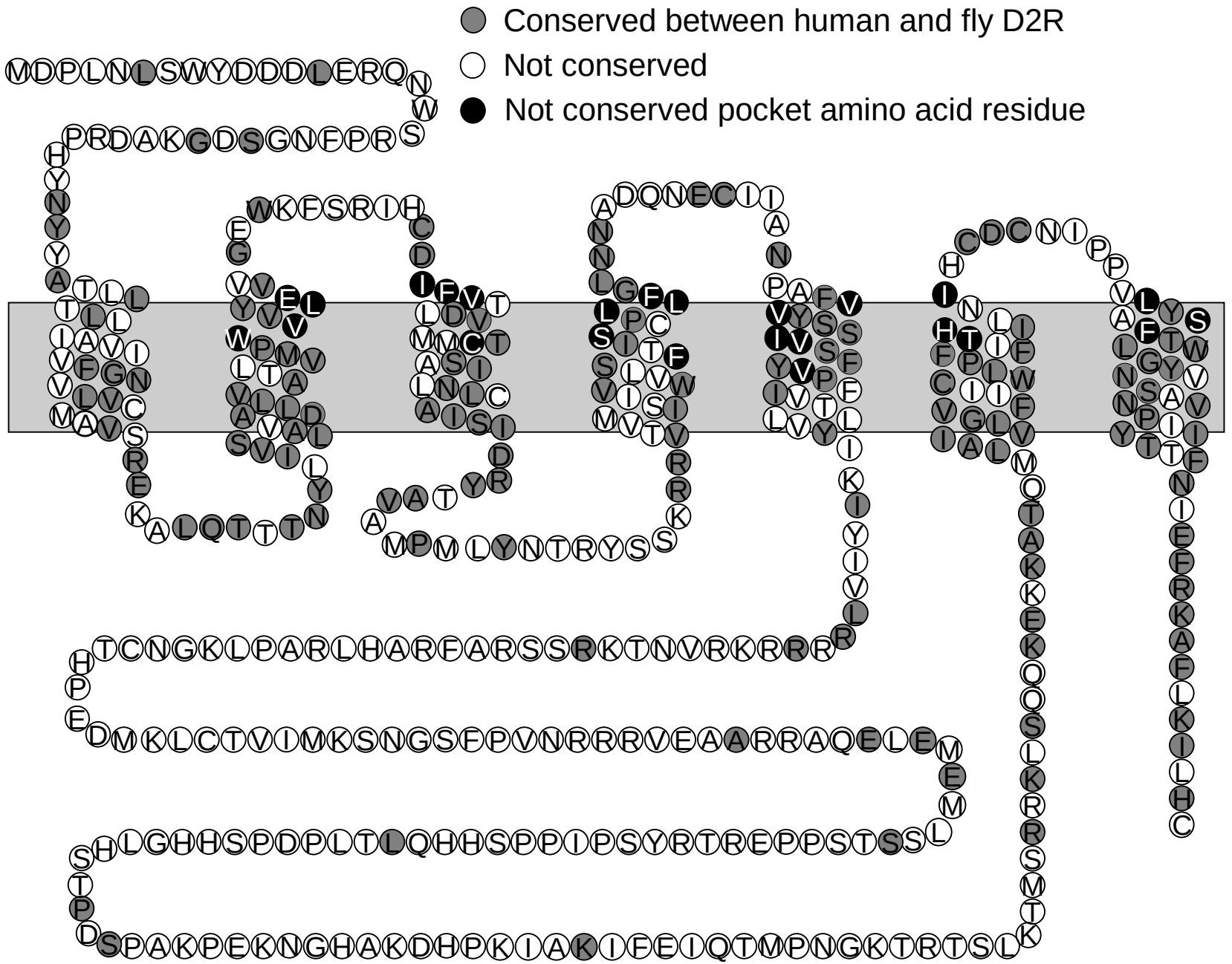

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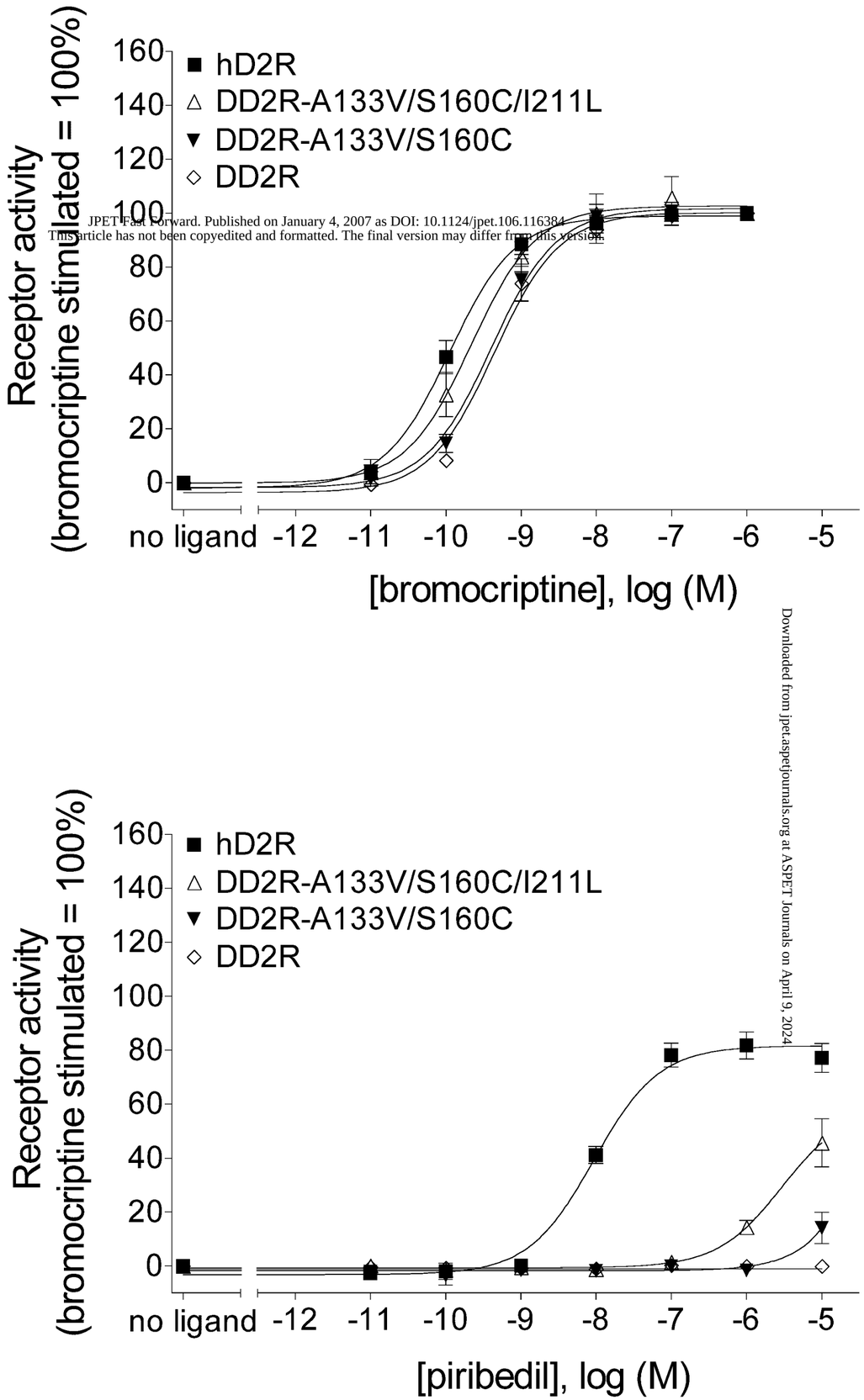


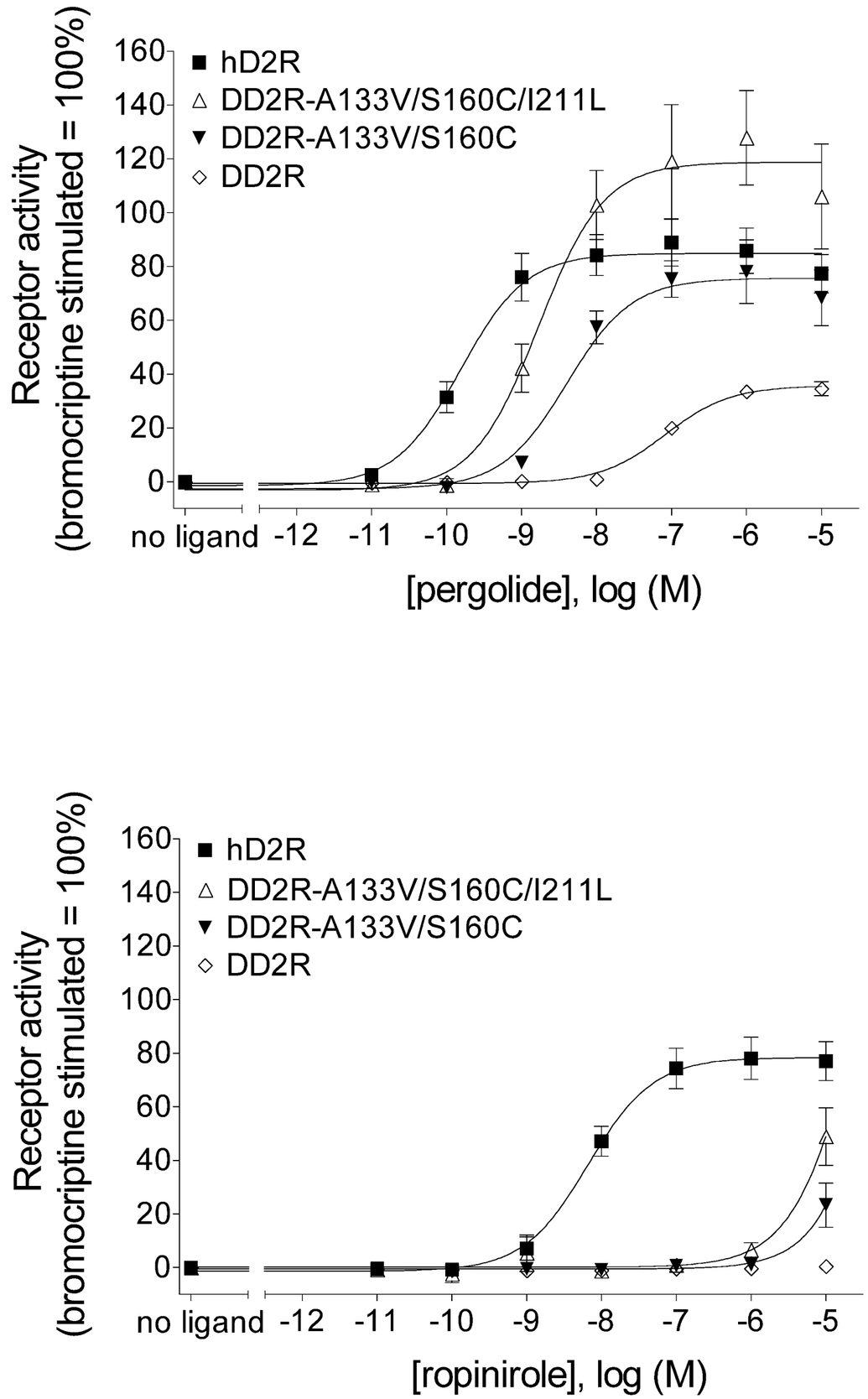

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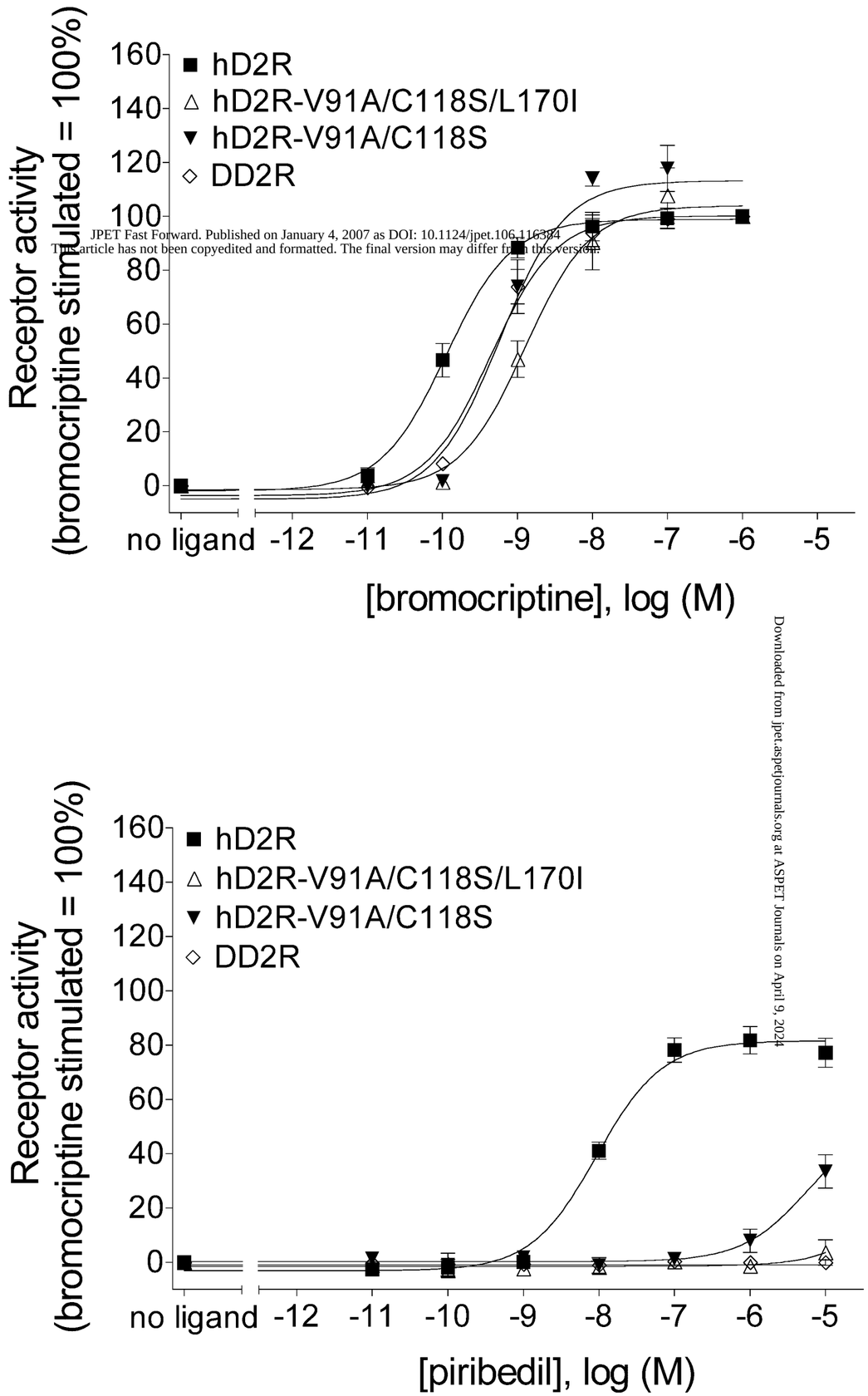


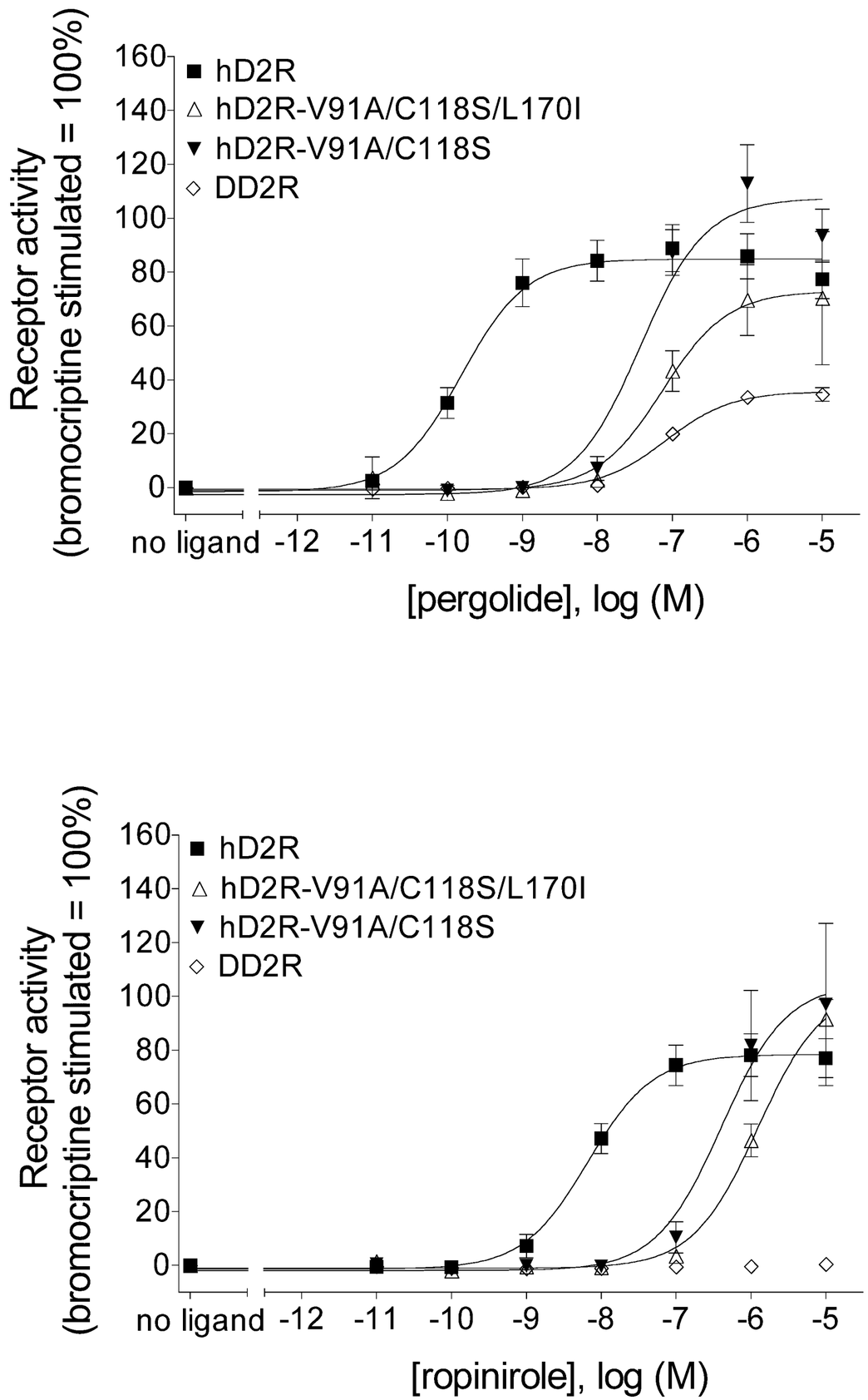
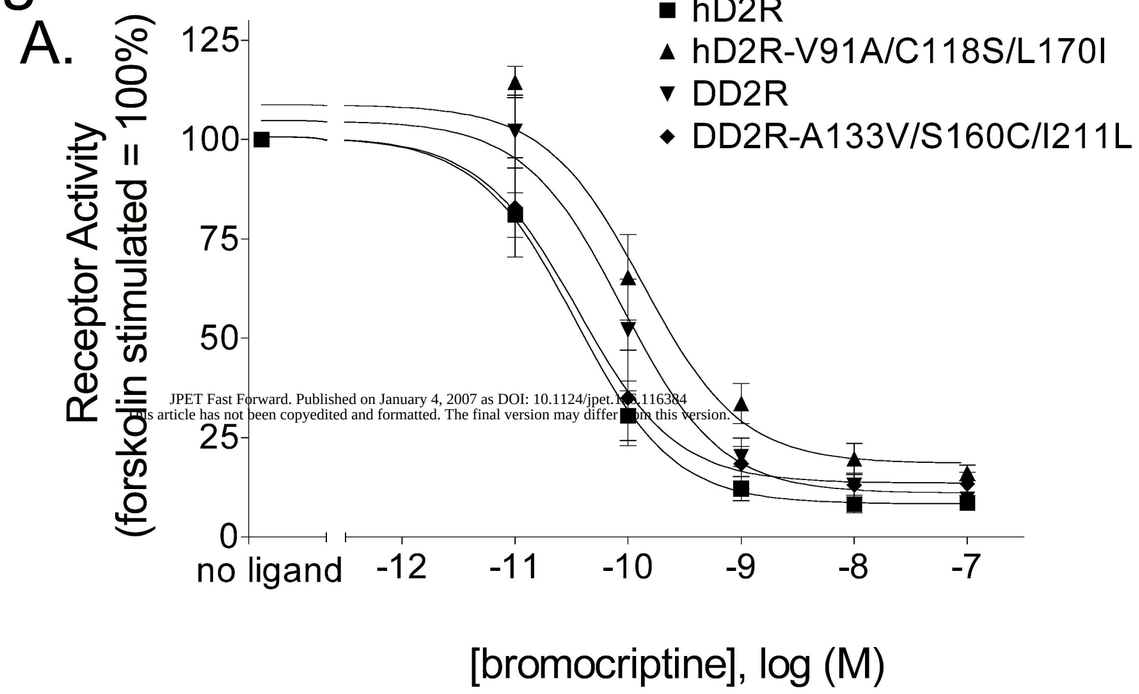
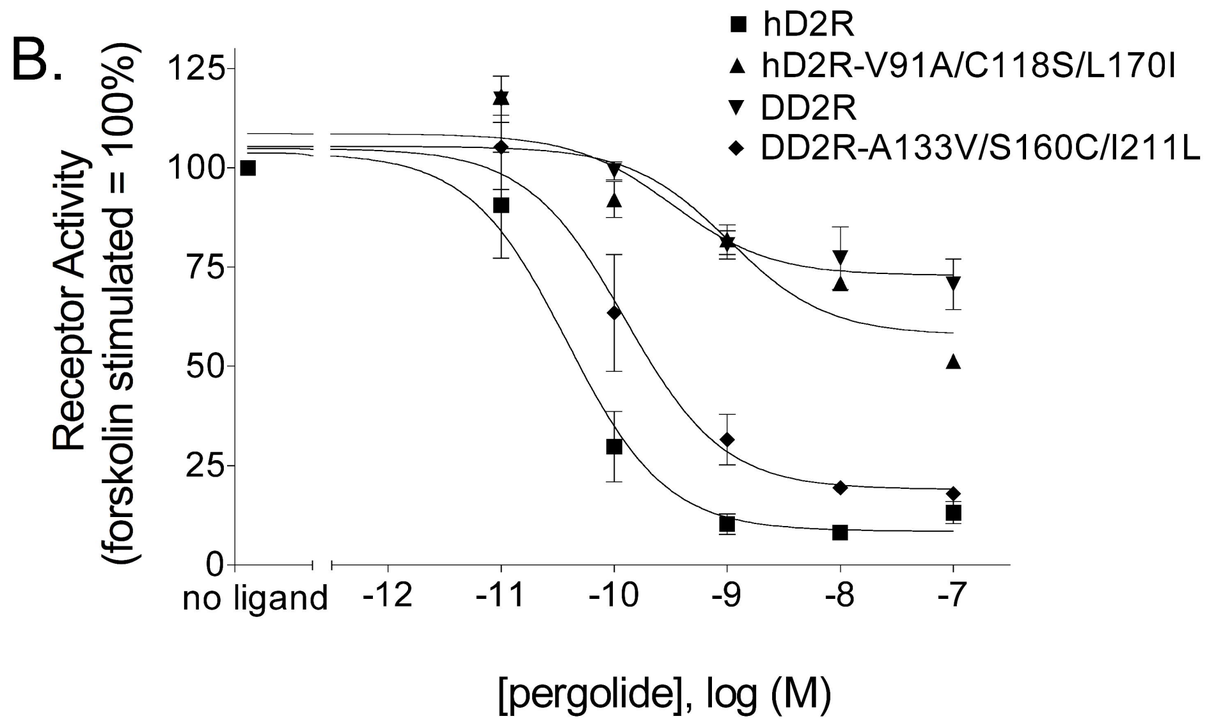
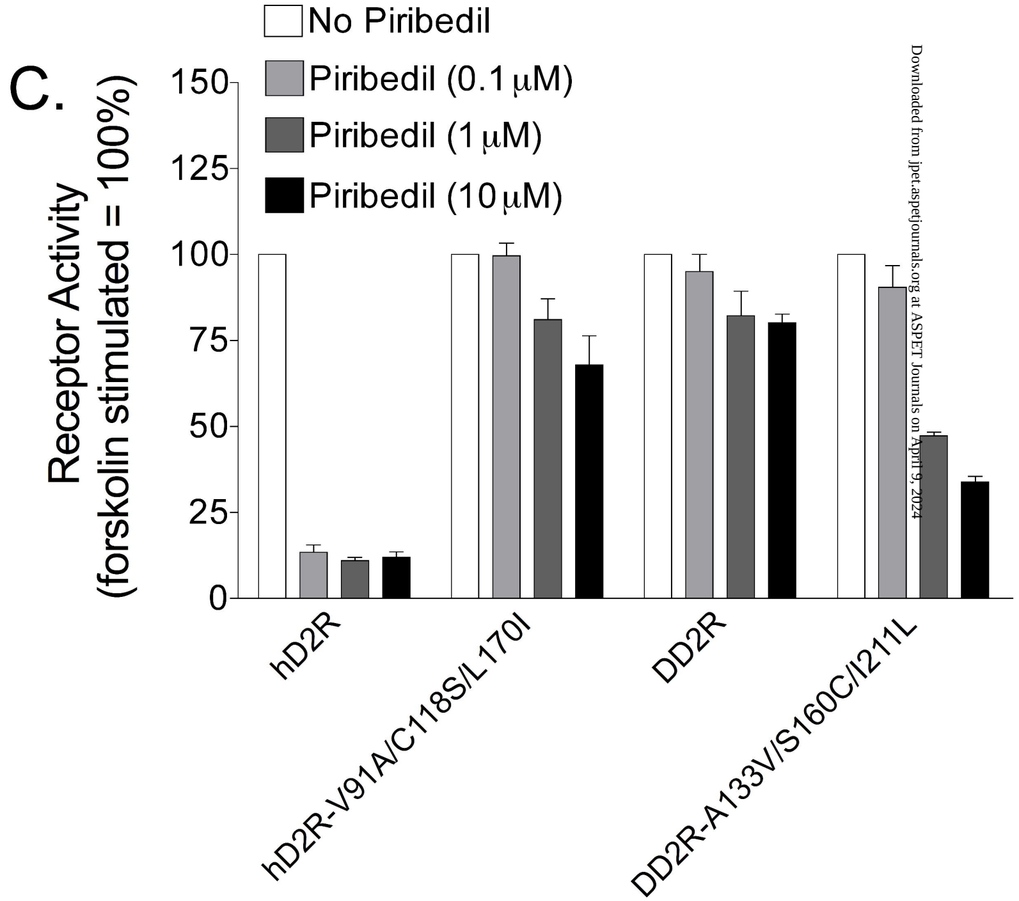



Figure 6

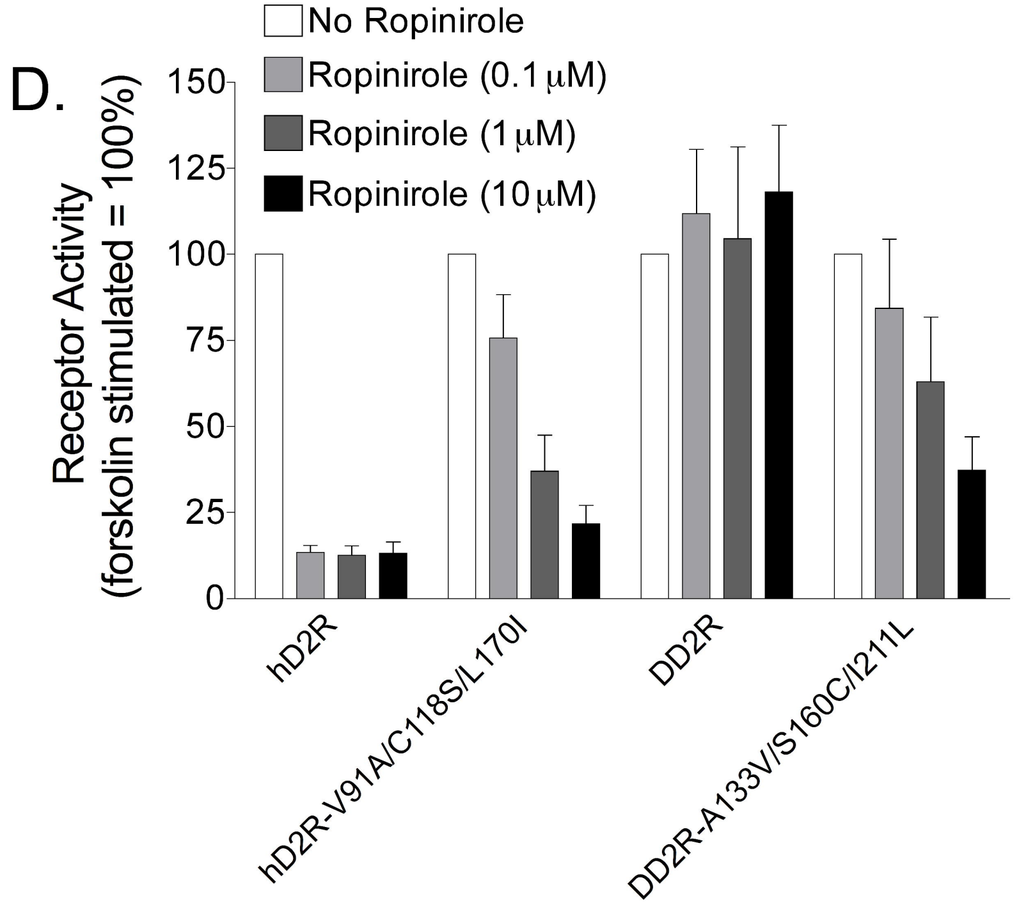






# Figure 7