Molecular Pharmacology Fast Forward. Published on February 14, 2012 as doi:10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

Ligand-Specific Roles for Transmembrane 5 Serine Residues in the Binding and Efficacy of Dopamine D_1 Receptor Catechol Agonists

Benjamin R. Chemel, Lisa A. Bonner, Val J. Watts, David E. Nichols

Department of Medicinal Chemistry and Molecular Pharmacology (B.R.C., V.J.W., D.E.N.), College of Pharmacy, Purdue University, West Lafayette, IN 47907

Department of Chemistry (L.A.B.), Saint Anselm College, Manchester, NH 03102

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: D_1 dopamine receptor TM5 serines differentially influence ligand pharmacology

*Corresponding author, David E. Nichols
Purdue University
Dept. of Medicinal Chemistry and Molecular Pharmacology
575 Stadium Mall Dr., RHPH 210
West Lafayette, IN 47907-2091
Tel. 765-494-1461
Fax. (765) 494-1414
drdave@purdue.edu

42 pages of text

6 Figures

4 Tables

40 References

245 words in abstract

689 words in introduction

1,927 words in discussion

Abbreviations: SCH 23390 (R(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine), SKF 38393 ((±)-1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol), SKF 81297 ((±)-6-Chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine), SKF 82958 ((±)-6-Chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine), SKF 83959 (6-Chloro-7,8-dihydroxy-3-methyl-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine)

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

To refine further the structure-activity relationships of D_1 dopamine receptor agonists, we investigated the roles of three conserved serine residues (Ser198(5.42), Ser199(5.43), and Ser202(5.46)) in agonist binding and receptor activation. These transmembrane domain 5 (TM5) residues are believed to engage catechol ligands through polar interactions. We stably expressed wild-type or mutant (S198A, S199A, and S202A) D_1 receptors in HEK cells. These receptors were expressed at similar levels (approximately 2000 fmol/mg) and bound the radioligand [^{3}H]SCH 23390, although S198A and S199A displayed significant losses of affinity compared to wild-type. The endogenous agonist, dopamine, suffered losses of potency at each of the mutant receptors. We tested cyclohexyl-substituted isochroman, carbocyclic, and chroman bicyclic dopamine analogues and found that the mutations affected the chroman to a lesser extent than the other compounds. These results support our hypothesis that the decreased D_1 activity of chroman analogues results from a ligand intramolecular hydrogen bond that impairs the catechol's ability to engage the receptor. Sensitivities of these rigid catechol agonists to the effects of the serine mutations were dependent on ligand geometry, particularly with respect to the rotameric conformation of the ethylamine side chain and the distance between the amino group and each catechol hydroxyl. Functional experiments in striatal tissue suggest that the ability to engage TM5 serines is largely correlated with agonist efficacy for cAMP stimulation. These results provide a new understanding of the complexities of D_1-ligand recognition and agonist activation, and have implications for the design of rigid catechol ligands.

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

Dopamine (DA) is an important neurotransmitter that plays numerous roles in the central and peripheral nervous systems (Missale et al., 1998). The receptors for dopamine are members of the Class A (rhodopsin-like) group of seven-transmembrane domain G-protein coupled receptors (GPCRs). To date, five distinct subtypes of dopamine receptors have been identified (Civelli et al., 1993). The D1-like subclass of DA receptors includes D_1 and D_5, which couple to $G\alpha_s$ and stimulate the production of cyclic adenosine monophosphate (cAMP) through the activation of adenylyl cyclases (Clark and White, 1987). The D2-like receptors, D_2, D_3, and D_4, couple to $G\alpha_i$ thereby inhibiting the production of cAMP (Neve et al., 2004). Of the five receptor subtypes, D_1 and D_2 have arguably received the most scientific attention.

Dopamine has been implicated in a number of neuropsychiatric conditions including addiction, schizophrenia, Parkinson's disease, and attention-deficit hyperactivity disorder (Kienast and Heinz, 2006). Deficient D_1 receptor expression or signaling is thought to be an important component of the pathology of cognitive deficits and motor dysfunctions associated with aging, Huntingtons's disease, Alzheimer's disease, and Parkinson's disease. Thus, understanding the molecular requirements of D_1 receptor binding and activation may aid in the development of novel therapeutics for these disorders.

Early attempts to define the topography of monoamine neurotransmitter binding pockets used site-directed mutagenesis to probe adrenergic receptors (Liapakis et al., 2000; Strader et al., 1989; Wang et al., 1991). Those studies demonstrated that primary ligand-contact sites are in the third and fifth transmembrane domains (TM3 and TM5). In

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

particular, Asp3.32 in TM3 is important for coordinating the amino functionality, and serine residues in TM5 interact with the catechol moiety. Strader et al. (1989) demonstrated that Ser5.43 and Ser5.46 of the β_2-adrenergic receptor interact with the *meta*- and *para*- hydroxyl groups of catecholamine ligands, respectively. It was later demonstrated by Liapakis et al. (2000) that the *meta*-OH also interacts with Ser5.42, possibly in a bifurcated fashion.

Previous mutagenesis studies exploring the TM5 serines in D_1 receptors have been somewhat limited in their choice of ligands (O'Dowd et al., 2005; Pollock et al., 1992; Tomic et al., 1993). Pollock et al. (1992) individually mutated Ser198(5.42), Ser199(5.43), and Ser202(5.46) to alanine and examined the effects on ligand binding and potency. That study, however, used relatively few test ligands, and found no detectable radioligand binding with S198A. They concluded that S202A has profound effects on the affinity and potency of dopamine, and little to no effect on the phenylbenzazepines (SCH 23390, SKF 38393, and SKF 82958). In contrast, S199A adversely affected the affinity and potency of all test compounds. Despite the lack of demonstrable radioligand binding by the S198A mutant, northern blot analysis revealed that it was expressed at levels similar to the other mutant receptors. Furthermore, functional assays (cAMP accumulation) demonstrated that S198A was functional but had profoundly disrupted ligand potency. Tomic et al. (1993) created the S199V/S202A double mutant, which drastically decreased the affinity of dopamine, and to a lesser extent, SCH 23390. Finally, O'Dowd et al. (2000) used the S198A/S199A double mutant, which bound (+)-butaclamol, but not SCH 23390 or dopamine, to study dopamine receptor oligomerization.

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

The present study aims to broaden these earlier studies by utilizing many structurally-diverse agonist ligands to provide a greater understanding of the molecular interactions of the TM5 serines of D_1 receptors. The ligands used for this study are illustrated in Figure 1. We stably expressed wild-type and mutant D_1 dopamine receptors in HEK cells and employed competitive binding and cAMP accumulation assays to evaluate the effects of the S198A, S199A, and S202A mutations on agonist affinity and potency. We found that the effects of these mutations were structure-specific, suggesting that the engagement of these residues in the wild-type receptor is determined by ligand structure. Furthermore, measurements of agonist efficacy for striatal D_1-like receptors suggest that the trans-β conformation of the ethylamine side-chain is optimal for full efficacy and that inability of the catechol moiety to engage one or more TM5 serine residues may result in partial agonism. The results of this study demonstrate that these TM5 serine residues of the D_1 dopamine receptor play critical, ligand-specific roles in agonist binding and receptor activation.

METHODS

Materials

[^{3}H]SCH 23390 was purchased from Amersham Biosciences (Piscataway, NJ) and PerkinElmer Life and Analytical Sciences (Waltham, MA). [^{3}H]cAMP, [^{3}H]methylspiperone, and Microscint-O were purchased from PerkinElmer. (±)-SKF 38393 HCl, (±)-SKF 82958 HBr, R(+)-SCH 23390 HCl, (+)-butaclamol HCl, R(-)-apomorphine, ketanserin tartrate, and dopamine HCl were purchased from Sigma-Aldrich (St. Louis, MO). (±)-SKF 81297 HBr, and (±)-SKF 83959 HBr were purchased from

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

Tocris Bioscience (Ellisville, MO). All Isochroman compounds (as racemic HCl salts) were kindly provided by Abbott Laboratories (Abbott Park, IL). All other test compounds (as racemates) were synthesized in our own laboratory and verified for identity and purity by TLC, melting point, NMR, mass spectroscopy, and elemental analyses. All compounds synthesized by our laboratory were prepared as racemic HCl salts, except for the phenyl carbocyclic (HBr). Bovine calf serum and fetal clone 1 serum were obtained from VWR (West Chester, PA). Unless otherwise noted, cell culture reagents, including media and antibiotics, were purchased from Gibco Invitrogen Corporation (Carlsbad, CA). All restriction and polymerase enzymes were obtained from New England Biolabs (Beverly, MA). BCA Protein Assay kits were purchased from Thermo Fisher Scientific (Rocford, IL). 96-well, glass fiber MultiScreen Harvest APFB plates were obtained from Millipore (Billerica, MA).

Creation of D_1 Mutants

Wild-type human D_1 cDNA in the pcDNA3.1/V5-His TOPO vector (Invitrogen) was obtained from Dr. Bryan Roth. XL1-Blue competent cells (Stratagene, La Jolla, CA) and the QIAprep Spin Miniprep and Midiprep Kits (Qiagen, Valencia, CA) were used to transform, amplify, and isolate DNA. Mutagenesis was planned using Vector NTI 9 (Invitrogen) and performed using the Quikchange Site-Directed Mutagenesis Kit (Stratagene). The following primers (and corresponding anti-sense primers) were used according to the Quikchange protocol to generate the mutants (Integrated DNA Technologies, Coralville, IA):

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

S198(5.42)A, CCTCAGCAGGACCTATGCCATCTCAGCCTCTGTAATAAGC;

S199(5.43)A, CCT CAGCAGGACCTATGCCATCTCATCCGCTGTAATAAGC;

S202(5.46)A, CCATCTCATCCTCTGTAATAGCCTTTTACATCCCTGTGGC.

The accuracy of mutant cDNA was validated by sequencing by the Purdue University DNA Sequencing Low Throughput Laboratory (West Lafayette, IN) using the T7 and BGH reverse primers.

Cell Culture and Creation of Pooled Cell Lines

Human Embryonic Kidney 293 (HEK) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal clone serum, 5% bovine calf serum, 0.05 µg/ml penicillin, 50 µg/ml streptomycin, and 0.25 µg/ml amphoterocin B. Cells were grown in a humidified incubator with 5% CO_2. HEK cells were stably transfected by combining 3 µg pcDNA3.1/V5-His TOPO hD_1 (WT or mutant) with 15 µl Lipofectamine 2000 (Invitrogen) in OptiMEM I medium according to manufacturer's protocol. This mixture was added dropwise to 10 cm^2 tissue culture plates containing ~70% confluent HEK cells. Twenty four hours later, these cells were split into new 10 cm^2 plates at various seeding densities. The following day, and every three days thereafter, the media was replaced with fresh selection media containing 600 µg/ml G418. After approximately four weeks, when colonies were visible to the naked eye, the entire plate was resuspended and transferred to a new 10 cm^2 plate. These plates were grown to 90% confluency in maintenance media containing 300 µg/ml G418 and further split into additional plates to enable receptor evaluation.

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

Membrane Preparation

HEK cells expressing either WT or mutant D_1 receptors were grown to confluency in 15 cm^2 plates. Preparation of membrane pellets for radioligand binding assays was performed as previously described (Chemel et al., 2006). Briefly, media was decanted and 10 ml of ice cold lysis buffer (1 mM HEPES, pH 7.4, and 2 mM EDTA) was added. After 10 min, cells were scraped and centrifuged at 30,000xg and 4 ^oC for 20 min. The supernatant was discarded, and the pellet was resuspended by mechanical homogenization in 4 ml/15 cm^2 plate receptor binding buffer (50 mM Tris-HCl, pH 7.4, and 4 mM $MgCl_2$). 1 ml aliquots were transferred to pre-chilled microcentrifuge tubes and centrifuged at 13,000xg for 10 min, followed by aspiration of the supernatant. These pellets were frozen at -80 ^oC until use.

Radioligand Saturation Binding

Membrane preparation pellets were resuspended by trituration and mechanical homogenization in receptor binding buffer (approx. 50 µg protein/100 µl) and added in duplicate to assay tubes containing 0.2-5.0 nM [^{3}H]SCH 23390 and either buffer (total binding) or 5 µM (+)-butaclamol (non-specific binding) in a total volume of 500 µl. Assay tubes were incubated at 37 ^oC for 30 min before termination by harvesting by filtration (MultiScreen Harvest APFB plates, Millipore) using a 96-well Packard FilterMate cell harvester. After adding 10 µl of each radioligand concentration in duplicate to empty wells to determine accurately total radioligand added, filter plates were dried overnight. After addition of 30 µl of Packard Microscint-O scintillation fluid to each well, a Packard TopCount scintillation counter was used to determine counts per

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

minute (CPM) per well. Actual protein concentration for resuspended membranes was calculated using the BCA Protein Assay kit (Thermo Fisher Scientific). These values were used to calculate and plot specific binding (fmol/mg) versus free radioligand concentration.

Homologous Competition (Cold Saturation) Binding

Traditional radioligand saturation binding experiments could not be used to generate affinity (K_d) and expression levels (B_{max}) for the S198A and S199A cell lines due to the dramatic loss of radioligand affinity at these mutant receptors. Therefore, we employed homologous competition, or cold saturation, binding assays that use only one concentration of radioligand and enable the practical determination of K_d and B_{max} when the radioligand is expensive, in short supply, or lacks high affinity. Cells were grown and membranes were prepared as described for radioligand saturation binding. 2-3 nM [^{3}H]SCH 23390 was added to each well, and nine concentrations of cold SCH 23390 (10 pM-10 µM) were added in duplicate to a total volume of 250 µl. Total binding was defined in the absence of competing ligand, and non-specific binding was defined by the addition of 5 µM (+)-butaclamol. Assays were incubated at 37 ^{o}C for 30 min before harvesting and scintillation counting as described above for radioligand saturation assays.

Heterologous Competition Binding

Heterologous competition binding assays were performed to estimate the binding affinity (K_i) values of test compounds in essentially the same manner as described for homologous binding. Nine concentrations of test compounds, ranging from 1 pM to 100

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

µM, were added in duplicate to wells containing approximately 1-3 nM [^{3}H]SCH 23390. Drugs were evaluated at mutant and wild-type receptors in parallel to facilitate normalization.

HEK cAMP Stimulation Assays

When cells reached 100% confluency in 48-well plates, growth media was decanted and plates placed on ice. Ten concentrations of test compounds were made in Earle's balanced salt solution (EBSS) buffer (EBSS with 2% bovine calf serum, 0.025% ascorbic acid, and 15 mM HEPES, pH 7.4) and added in duplicate to a total volume of 200 µl in the presence of that was 500 µM in isobutyl-methylxanthine (IBMX). To facilitate normalization, mutants were assayed in parallel with wild-type, and wells containing vehicle (basal) and 100 µM dopamine (max DA) were included alongside each test drug as controls. Assays were incubated for 15 min at 37 ^{o}C in a water bath, and were terminated by decanting followed by the addition of 100 µl ice-cold 3% trichloroacetic acid on ice. Plates were stored at 4 ^{o}C for at least 1 h before quantification of cAMP.

Striatal cAMP Stimulation Assays.

The striatal adenylate cyclase assay was performed as previously described (Przybyla et al., 2009). Assays were carried out in 96-well assay tubes containing (final concentration) reaction buffer (5 mM $MgCl_2$, 2 mM EDTA, 1 mM IBMX, 0.01% ascorbic acid, 10 µM pargyline, and 15 mM HEPES, pH 7.4), reaction mix (1.25 mM adenosine 5'-triphosphate (ATP), 21.5 mM *N*-[imino(phosphonoamino)methyl]-*N*-

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

methylglycine disodium salt (phosphocreatine), and 3 U creatine phosphokinase), 1 μM Gpp(NH)p, 30 μg striatal protein, and the indicated drugs (10 μM) in a total volume of 100 μl. Propranolol and prazosin (1 μM each) were included to block adrenergic receptors. Triplicate samples for each treatment were incubated in a 30 °C water bath for 15 min. Adenylate cyclase activity was terminated by the addition of 200 μL of 3% trichloroacetic acid. The reaction tubes were covered with Parafilm and stored at 4 °C for at least 1 h before the concentration of cyclic AMP was quantified.

cAMP Quantification

A previously described protocol was followed to quantify levels of cAMP production in each well (Watts and Neve, 1996). Briefly, 10-15 μl of lysate was added in duplicate to assay tubes with cAMP binding buffer (100 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 5 mM EDTA) containing 1 nM [^{3}H]cAMP and 100 μg of bovine cAMP binding protein in a total volume of 500 μl. Assays were incubated at 4 ^{o}C for 2-3 h and were harvested and counted by scintillation as described as above. The concentration of cAMP in each sample was estimated from a standard curve ranging from 0.01 to 300 pmol of cAMP.

Molecular Modeling

Molecules were built and minimized using the software package Spartan '06 (Wavefunction, Inc, Irvine, CA). All molecules were minimized as their protonated forms in a vacuum, using AM1 semiempirical potential functions. If two ring conformations were possible, those were built manually and minimized, and the lowest

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

energy final conformation was used. Minimized structures were overlaid, manually aligned, and measured using MacPyMol (DeLano Scientific, San Carlos, CA).

Data Analysis

GraphPad Prism 4.0 was used to generate curves for saturation, competition, and cAMP experiments. Data from cAMP accumulation assays were normalized to percent maximum dopamine stimulation (100 μM) at each receptor and graphed using sigmoidal dose-response curves with a Hill slope fixed to unity to generate EC_{50} and intrinsic activity (IA, %maximum DA stimulation) values. E_{max} and basal values of cAMP accumulation were generated from the tops and bottoms, respectively, of fixed hill slope sigmoidal dose-response curves of raw dopamine-stimulated cAMP values as defined by Prism. Within each striatal cyclase assay, cAMP levels produced in response to each drug (10 μM) were normalized to percent stimulation by 10 μM dopamine over vehicle levels (1 μM Gpp(NH)p alone).

Saturation binding experiments were analyzed using a one site binding (hyperbola) model to generate values for K_d and B_{max}. For homologous competition binding assays, IC_{50} values, as well as top and bottom values, were determined from one-site, variable slope sigmoidal dose-response curves. K_d values were calculated as follows: $K_d = IC_{50} - [radioligand]$. B_{max} values were determined as follows: $B_{max} = (Top - Bottom)/([radioligand]/(K_d - [radioligand]))$. B_{max} values were then converted from CPM to pmol/mg. Competition binding experiments were analyzed using variable slope, one-site sigmoidal curves to calculate K_i values from IC_{50} values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). The hill slopes (not reported) for the agonists

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

evaluated were mostly <1 at each of the receptors, but these data were not reliably resolved by a two-site model. Therefore, the K_i values reported herein reflect contributions of high and low affinity states. When necessary (for low affinity compounds), the bottom limits of curves were constrained to average non-specific binding values.

Prism also was used to perform one-way ANOVA calculations with Dunnett's post-tests comparing mutant to wild-type values. The significance threshold was $p < 0.05$. Within individual competitive binding and cAMP accumulation experiments, changes in affinity and potency values were calculated for each mutant, relative to wild-type. To aid visualization, mutation-induced changes in binding affinities (K_i) were expressed as changes in the standard Gibbs free energy ($\Delta\Delta G^o$), calculated from K_i values as follows: $\Delta\Delta G^o = \Delta G^o_{mutant} - \Delta G^o_{WT} = -RT\ln(K_{i\text{-}mutant}/K_{i\text{-}WT})$, where R is the gas constant and T is absolute temperature. To enable statistical analysis, changes in $-\log K_i$ were calculated for each mutant relative to wild-type for independent experiments performed in parallel as follows: $\Delta pK_i = pK_{i\text{-}mutant} - pK_{i\text{-}WT} = -\log K_{i\text{-}mutant} - (-\log K_{i\text{-}WT})$. Changes in potency were transformed by calculating the differences of the log EC_{50} values for independent experiments performed in parallel as follows: $\Delta pEC_{50} = pEC_{50\text{-}mutant} - pEC_{50\text{-}WT} = -\log EC_{50\text{-}mutant} - (-\log EC_{50\text{-}WT})$. $\Delta\Delta G^o$, ΔpK_i, and ΔpEC_{50} values, calculated from the corresponding affinity or potency values of each replicate experiment, were used to generate the mean and S.E.M. values displayed in the figures.

RESULTS

Characterization of cell lines

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

D_1 WT, S198A, S199A, and S202A stable cell lines were constructed as described in the Methods section. Radioligand saturation assays were performed on these cell lines to evaluate their receptor expression (B_{max}) levels and their affinities for [^{3}H]SCH 23390 (Table 1). The wild-type D_1 cell line displayed saturable radioligand binding with mean values of 1.2 nM and 1840 fmol/mg for K_d and B_{max}, respectively. The S202A mutant exhibited values of radioligand affinity and expression that were very similar to wild-type (1.1 nM and 1890 fmol/mg, respectively). By contrast, despite substantial specific binding displayed by the S198A and S199A mutants, we were unable to generate affinity and expression data using radioligand saturation analysis because the specific binding was not saturable. Thus, we used homologous competitive binding (cold saturation binding) to measure these values. Table 1 demonstrates that SCH 23390 possessed significantly lower affinity for S198A and S199A (52 and 28 nM, respectively). The use of this approach was supported by the observations that the Kd/Ki values generated by homologous competition experiments for wild-type and S202A were identical to those generated through radioligand saturation binding (data not shown). These experiments confirmed that all four cell lines express similar receptor levels (1600-2000 fmol/mg).

The functional properties of the D_1 receptors were evaluated using the endogenous agonist, dopamine, by measuring cAMP accumulation in response to D_1-stimulated $G\alpha_s$ activation of adenylyl cyclase (Table 2). Dopamine dose-dependently increased cAMP accumulation in each cell line, but not in mock-transfected cells (data not shown). The EC_{50} value for dopamine at the wild-type D_1 receptor was 22 nM. In contrast, dopamine was dramatically less potent at all three mutant receptors. The S199A

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

mutation resulted in the smallest loss of potency (~100-fold). S198A and S202A led to greater than 300- and 500-fold losses in potency, respectively. Consistent with previous reports (Tiberi and Caron, 1994), the wild-type D_1 receptor did not display appreciable levels of basal activity. Mean basal levels of cAMP for the mutant cell lines also were less than 5 pmol/well, indicating that these mutations did not lead to increased constitutive activity. In addition, the inverse agonists (+)-butaclamol, chlorpromazine, and haloperidol (Cai et al., 1999; Kozell et al., 1994) had no effect on basal levels of WT D_1 receptor activity (data not shown). Dopamine receptor stimulation resulted in similar maximum levels of cAMP in the wild-type, S198A, and S199A cell lines (134, 117, and 111 pmol/well, respectively). In addition to yielding the greatest loss of potency for dopamine, S202A displayed significantly reduced levels of maximum dopamine-stimulated cAMP (55 pmol/well).

TM5 serine to alanine mutations differentially disrupt the binding of catechol agonists

Competitive binding experiments with [^{3}H]SCH 23390 were used to evaluate the binding affinities (K_i) of structurally-diverse catechol agonists for wild-type and mutant D_1 receptors (Figure 1, Table 3). To compare the extent of affinity loss caused by each mutation on the cyclohexyl-substituted bicyclic compounds, we calculated changes in pK_i values, relative to wild-type, from matched experiments (Figure 2). These data demonstrate that each of the mutations affected the cyclohexyl-substituted isochroman and carbocyclic compounds to significantly greater extents than the cyclohexyl chroman ($p < 0.05$, one-way ANOVA with Dunnett's post-test).

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

K_i values from independent experiments were converted to changes (from wild-type) in standard Gibbs free energy ($\Delta\Delta G^o$) of binding to illustrate graphically the effects of these mutations (Figure 3), where the energetic threshold for the disruption of a hydrogen bond is equivalent to ~0.5 kcal/mol (Fersht, 1988). S202A produced the largest loss of affinity for dopamine, the cyclohexyl-substituted bicyclic (isochroman, chroman, and carbocyclic), and the tetracyclic (apomorphine, DNS, DHX, and DOX) compounds (magnitude of affinity loss: S198A ≤ S199A < S202A). The cyclohexyl chroman and apomorphine were somewhat unique in that they were relatively weakly affected by S198A and S199A. Furthermore, the change in affinity of the cyclohexyl chroman caused by S198A did not exceed the energetic threshold of a disrupted hydrogen bond. Although S202A caused the greatest relative losses of affinity for the δ-cyclohexyl and tetracyclic compounds, this mutation caused comparatively minor reductions in affinities for the phenylbenzazepine agonists SKF 38393, SKF 81297, SKF 82958, and SKF 83959. The effects of S202A on these compounds were substantially less than those caused by S198A and S199A (S202A < S199A ≈ S198A), but all were above the threshold for the loss of a hydrogen bond.

TM5 serine-to-alanine mutations differentially disrupt the functional properties of catechol agonists

Table 4 lists the results of cAMP functional assays performed on wild-type and mutant cell lines with structurally-diverse catechol D_1 agonists. SKF 38393 and SKF 83959 displayed partial agonism at wild-type D_1 receptors. The relatively high intrinsic activity of SKF 38393, which is a well-known partial agonist at D_1 receptors (Andersen

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

and Jansen, 1990), likely reflects receptor reserve due to high levels of receptor expression (Watts et al., 1995). Interestingly, each serine mutation reduced the intrinsic activity of this partial agonist. The intrinsic activities of the other test ligands were not drastically altered by S199A, and many were modestly enhanced by S198A. S202A, however, produced ligand-dependent effects on intrinsic activity. Although S202A significantly reduced the maximum levels of cAMP produced by dopamine (Table 2), a number of compounds exhibited very high levels of intrinsic activity (> 200%). This result likely reflects the reduced efficacy of dopamine, but highlights the fact that the cyclohexyl isochroman, SKF 81297, and SKF 82958 were resistant to the negative impact of this mutant on efficacy.

The EC_{50} values reported in Table 4 for each compound at the mutant receptors were significantly different from wild-type ($p < 0.05$, one-way ANOVA with Dunnett's post-test). Changes in pEC_{50} values, relative to wild-type, were calculated from independent matched experiments to illustrate the effects of each mutant (Figure 4). For most compounds, the disruption of potency caused by each serine mutation was similar to the effect on binding affinity, with a few noted exceptions. Interestingly, S198A caused a relatively greater disruption of these compounds' potencies than it did their affinities. The cyclohexyl-substituted bicyclics and the tetracyclic compounds displayed similar trends in ΔpEC_{50} values (Figure 4A, B). Like dopamine, they were less affected by S199A than by S198A and S202A, (magnitude of potency loss: S199A < S198A ≈ S202A). Similar to the results obtained from binding assays, we demonstrated that each mutant disrupted the potency of the cyclohexyl chroman to a significantly lesser extent than the isochroman or carbocyclic compounds (Figure 4A). The phenylbenzazepine agonists exhibited only

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

minor potency losses in the S202A cell line (Figure 4C). In contrast to the binding results, S198A caused a greater loss of potency for these compounds than S199A (S202A < S199A < S198A).

Intrinsic activities of agonists at striatal D_1 dopamine receptors

Partial agonists often behave as full agonists in recombinant cell lines with high levels of receptor expression due to receptor reserve (Watts et al., 1995). To provide a better understanding of the efficacies of test ligands at wild-type D_1 dopamine receptors, we evaluated cAMP production in porcine striatal homogenates (Figure 5). Saturating concentrations (10 μM) of all agonists were used and data were normalized to percent dopamine. These studies revealed that the bicyclic and the trans-β tetracyclic ligands were full agonists with efficacies that were statistically indistinguishable from dopamine. Apomorphine and the phenylbenzazepines behaved as partial agonists with varying degrees of efficacy.

DISCUSSION

Few prior studies have been carried out to investigate the interactions between structurally-diverse catechol agonists and serine residues S198(5.42), S199(5.43), and S202(5.46) in TM5 of D_1 receptors (O'Dowd et al., 2005; Pollock et al., 1992; Tomic et al., 1993). These residues are largely conserved in catecholamine-binding GPCRs. Early studies with adrenergic receptors suggested that these residues (S5.42, S5.43, and S5.46) are involved in important hydrogen bond interactions with the hydroxyls of catecholamine ligands (Liapakis et al., 2000; Strader et al., 1989). Because D_1 receptors

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

remain attractive, but elusive, therapeutic targets (Lewis et al., 2006; Przybyla et al., 2009; Zhang et al., 2009), exploring these molecular interactions may aid in the development of novel, subtype-selective, and bioavailable compounds.

The amino acid substitution of alanine for serine was chosen under the assumption that it ablates the potential for specific polar ligand-receptor interactions without disrupting global protein structure (Fersht et al., 1987). Our findings gave results in agreement with the findings of Pollock et al. (1992), where S199A and S198A, but not S202A, severely disrupted the affinity of [^{3}H]SCH 23390. The S202A and wild-type cell lines displayed similar K_i values for [^{3}H]SCH 23390 (~1 nM), consistent with previous reports (Manik et al., 1988; Ryman-Rasmussen et al., 2007). Homologous competition binding was employed to estimate the K_i and B_{max} for S198A and S199A (Table 1), which produced 50- and 25-fold losses of affinity, respectively. These results strongly suggest that in the wild-type D_1 receptor, the phenolic OH of SCH 23390 interacts with both Ser198 and Ser199, but unlike catechol agonists, SCH23390 does not engage Ser202.

These experiments were initially designed to explore the unexpected pharmacological profiles exhibited by structurally similar isochroman, chroman, and tetralin dopamine analogues (Bonner et al., 2011). Abbott Laboratories had developed bicyclic isochroman ligands with high affinity and selectivity for D_1-like receptors (DeNinno et al., 1991). A variety of hydrophobic substituents at the C3 position of the isochromans increase D_1-like selectivity, presumably by interacting with the same accessory binding region that is exploited by the β-phenyl moiety that is common to many D_1 receptor-selective agonists (Nichols, 2010). The active enantiomer of the

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

adamantyl isochroman (A-77636) is effective in rodent and primate models of Parkinson's disease (Kebabian et al., 1992). It was later shown, however, that A-77636 rapidly induces tolerance and loses effectiveness in these models (Asin and Wirtshafter, 1993; Blanchet et al., 1996).

Encouraged by the creation of dinoxyline (Grubbs et al., 2004), an oxygen bioisostere of dinapsoline (Ghosh et al., 1996), we created the chroman analogues of the isochromans by repositioning the heterocyclic ring oxygen adjacent to the *m*-OH (Bonner et al., 2011). Surprisingly, this modification severely disrupted D_1 receptor affinity and selectivity of the chroman compounds. To explore this effect further we synthesized carbocyclic analogues, which demonstrated that removal of the heterocyclic oxygen largely rescued D_1 affinity and selectivity. These data suggest that the poor D_1 binding of the chromans is due, at least in part, to a detrimental effect of the heterocyclic oxygen atom when it is adjacent to the catechol ring. As proposed by Bonner et. al (2011), this effect appears likely due to an intramolecular hydrogen bond between the chroman oxygen and the *m*-OH, which may limit the ability of the catechol to interact productively with the TM5 serines.

To test this hypothesis, we evaluated the potencies and affinities of the cyclohexyl-substituted compounds from each of the three bicyclic series at the TM5 serine-to-alanine mutant D_1 receptors, assuming that negative effects reflect the loss of favorable interactions between the ligand and receptor. The EC_{50} and K_i values of these compounds in the WT and mutant receptor cell lines paralleled their D_1-like binding affinities in native tissues (isochroman < carbocyclic < chroman). Comparing the magnitudes of mutation-induced changes in binding affinity (ΔpK_i) and potency

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

(ΔpEC_{50}) for each compound revealed that the chroman was the ligand least affected at each mutant (Figure 2 and Figure 4A, respectively).

The small changes in Gibbs standard free energy of binding ($\leq$ 0.5 kcal/mol) for the chroman at S198A and S199A are consistent with weak or nonexistent hydrogen bond interactions between its *m*-OH and these serines in the native receptor (Fersht, 1988). The effect of S202A on the cyclohexyl chroman was substantially greater, yet was significantly less than the changes observed for the isochroman and carbocyclic compounds at this same mutant. Although we cannot completely rule out the potential contribution of solvation effects, these data support the hypothesis that the chroman oxygen disrupts the catechol interactions with critical TM5 serine residues by altering the orientation of the *m*-OH through an intramolecluar hydrogen bond.

This finding and the loss of D_1-like receptor selectivity for the chroman among the unsubstituted bicyclic compounds (Bonner et al., 2011) underscore important differences between the structural requirements of D_1-like and D_2-like receptors. Unlike D_1 receptor ligands, many of the prototypical D_2-like receptor full agonists are non-catechol (e.g., quinpirole), indicating that the catechol hydrogen bond requirements of D_2 receptors are less demanding. Further, the loss of D_2 affinity and reciprocal increase of D_1 affinity upon hydrophobic substitution of the bicyclic ligands highlights the absence of an accessory binding region in the D_2 binding site.

We evaluated a number of ligands in which the ethylamine side chain is constrained into different orientations (Figure 1). The trans-β (e.g., dihydrexidine) tetracyclic and bicyclic (e.g., isochoman) ligands behaved like dopamine at the mutant receptors (Figures 3 and 4), which suggests that their catechol hydroxyls interact with

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

these residues in a similar fashion and that they adopt similar orientations in the ligand binding pocket. The greater detrimental effect on affinity of S202A suggests that when S198A or S199A is individually mutated, the adjacent residue can interact with the *m*-OH in a compensatory fashion. In addition, S198A produced greater relative disruption of potency than affinity, suggesting that Ser198 plays a unique role in the activation of D_1 receptors by these compounds. Consistent with their apparent similar modes of interaction within the D_1 binding site, these ligands all behaved as full agonists in native tissues (Figure 5).

All the phenylbenzazepine agonists were similarly affected by the D_1 receptor mutations (Figures 3 and 4), but as a whole were affected differently than the bicyclic or tetracyclic ligands. Interestingly, S202A produced the smallest changes in binding and potency for these ligands, compared with the relatively large effects of this mutation on the non-benzazepine agonists. This finding suggests that benzazepine ligands adopt unique orientations in the D_1 receptor binding pocket, perhaps due to the constraint of their ethylamine side chain into a "cis-β-like" orientation. Analyzing the energetically preferred conformations of the benzazepine agonists revealed that, although the azepine ring is somewhat flexible, it constrains the amino group above the plane of the catechol ring. Comparison with DHX illustrates that the cis-β-like orientation of the azepine ring places the nitrogen substantially closer to the catechol ring than the trans orientation shared by most other non-benzazepine D_1 agonists (Figure 6A). The distances between the amino group and the *m*- or *p*-O in SKF 38393 are 0.3 Å and 0.8 Å shorter, respectively, than in DHX. This geometry may limit the ability of these compounds to engage simultaneously Asp103 and the TM5 serines.

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

The basic dopamine pharmacophore has four important interaction points within the dopamine receptor binding site corresponding to: a) the protonated amine with Asp103, b) the *m*-OH with Ser198 and Ser199, c) the *p*-OH with Ser202, and d) the catechol ring with TM6 aromatic residues. The most important interaction for an amine ligand is the salt bridge with Asp103 (Strader et al., 1988), and the relative rigidity of TM3 suggests that the protonated amine of different ligands will occupy approximately the same space when bound. It appears that Ser198/Ser199 is the more important interaction for the catechol moiety because it offers the potential for more hydrogen bond interactions than Ser202 alone. This proposal is supported by an unpublished study that examined the binding affinities of monohydroxy DHX analogues (Jassen et al., 2000). The removal of the *p*-OH of DHX resulted in approximately 20-fold lower affinity, whereas removal of the *m*-OH reduced affinity by more than 200-fold.

As a result of their constrained geometry, the benzazepine ligands are unable to engage both of their catechol hydroxyl groups with TM5 serine residues, and appear preferentially to engage Ser198 and Ser199 with the *m*-OH over Ser202 with the *p*-OH. When the amino groups of DHX and SKF 38393 are aligned (Figure 6A), it is apparent that the *p*-OH of SKF 38393 will be substantially further away from Ser202, perhaps explaining the relatively modest detrimental effects of S202A on the phenylbenzazepine agonists.

To provide additional support for the hypothesis that ligand geometry determines the extent of engagement of TM5 serine residues by the catechol moiety, we aligned the minimized structures of apomorphine and DHX (Figure 6B). Apomorphine was unique among the tetracyclic agonists in that S198A and S199A had only modest effects on

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

ligand binding, which barely exceeded the threshold for hydrogen bonding (Figure 3). The minimized structure of apomorphine demonstrates that its trans-α orientation reduces the distance between its protonated amine and *m*-OH. Thus, in contrast to the benzazepines, the *m*-OH, not the *p*-OH, of apomorphine is, in essence, pulled away from TM5. This increased distance could be expected to reduce the strength of the interaction of apomorphine's *m*-OH with S198A or S199A, but not S202A.

It has been proposed that activation of catecholamine receptors occurs sequentially and that the engagement of TM3 and TM5 by the amine and catechol moieties, respectively, of catechol agonists stabilizes the ligand-receptor complex (Swaminath et al., 2004). Once these primary contacts are established, the top of TM6 is pulled toward the ligand by interactions between aromatic residues and the catechol ring. The conformational constraints imposed by the ring systems used to rigidify the ethylamine side-chains of apomorphine and the benzazepine compounds likely reduce the ability of these ligands to engage Asp103(3.32) and all three TM5 serine residues simultaneously. By reducing the stability of the ligand-receptor complex and impairing the engagement of the catechol ring with TM6 aromatic residues, these limitations may decrease the ability of these ligands to induce an active receptor conformation, and may explain the partial agonism displayed by most phenylbenzazepine ligands and apomorphine (Andersen and Jansen, 1990; Watts et al., 1995). This reasoning suggests that the simultaneous engagement of Ser198/Ser199 and Ser202 is required for full D_1 receptor activation. This conclusion is further supported by the fact that the efficacy of many full agonists (including dopamine and DHX) was decreased in S202A (Table 4). The partial agonist properties of the antagonist SCH 23390 (Tiberi and Caron, 1994) may

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

be due to a relatively weak interaction of Ser202 with its *p*-Cl group. Additionally, the apparent ability of the *m*-Cl to enhance the efficacy of some benzazepines (e.g. SKF 81297), may result from the ability of the chlorine atom to interact with Phe203(5.47), thereby compensating for the decreased ability of the *p*-OH to engage Ser202.

In addition to elucidating the molecular determinants of D_1 receptor agonist activity and providing valuable empirical evidence to help refine future homology models, these studies may have direct therapeutic implications. The S199A mutation in D_1 receptors has recently been identified as a naturally occurring single nucleotide polymorphism in the human population (Al-Fulaij et al., 2008). The ability of this mutation to reduce ligand binding and potency suggests that traditional D_1 receptor drugs will be less effective for people with this polymorphism. The insights gained by these studies can guide the design of drugs that retain their activity at this mutant receptor, and can be used to screen novel ligands.

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

ACKNOWLEDGMENTS

We thank Abbott Laboratories for the generous donation of the isochroman compounds, Dr. Uros Laban for the synthesis of the chroman compounds, and Drs. Mike Braden and Jason Parrish for their valuable counsel. Dr. Bruce Craig, and Jeff Li at the Purdue University Statistical Counseling Service provided helpful statistical guidance.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Chemel, Nichols, Watts.

Conducted experiments: Chemel

Contributed new reagents or analytic tools: Bonner.

Performed data analysis: Chemel.

Wrote or contributed to the writing of the manuscript: Chemel, Bonner, Watts, Nichols.

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

REFERENCES

Al-Fulaij MA, Ren Y, Beinborn M and Kopin AS (2008) Pharmacological analysis of human D1 AND D2 dopamine receptor missense variants. *J Mol Neurosci* 34(3):211-223.

Ambrosio C, Molinari P, Cotecchia S and Costa T (2000) Catechol-binding serines of beta(2)-adrenergic receptors control the equilibrium between active and inactive receptor states. *Mol Pharmacol* 57(1):198-210.

Andersen PH and Jansen JA (1990) Dopamine receptor agonists: selectivity and dopamine D1 receptor efficacy. *Eur J Pharmacol* 188(6):335-347.

Asin KE and Wirtshafter D (1993) Effects of repeated dopamine D1 receptor stimulation on rotation and c-fos expression. *Eur J Pharmacol* 235(1):167-168.

Blanchet PJ, Grondin R, Bedard PJ, Shiosaki K and Britton DR (1996) Dopamine D1 receptor desensitization profile in MPTP-lesioned primates. *Eur J Pharmacol* 309(1):13-20.

Bonner LA, Laban U, Chemel BR, Juncosa JI, Lill MA, Watts VJ and Nichols DE (2011) Mapping the catechol binding site in dopamine d(1) receptors: synthesis and evaluation of two parallel series of bicyclic dopamine analogues. *ChemMedChem* 6(6):1024-1040.

Cai G, Gurdal H, Smith C, Wang HY and Friedman E (1999) Inverse agonist properties of dopaminergic antagonists at the D(1A) dopamine receptor: uncoupling of the D(1A) dopamine receptor from G(s) protein. *Mol Pharmacol* 56(5):989-996.

Chemel BR, Roth BL, Armbruster B, Watts VJ and Nichols DE (2006) WAY-100635 is a potent dopamine D4 receptor agonist. *Psychopharmacology (Berl)* 188(2):244-251.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* 22(23):3099-3108.

Civelli O, Bunzow JR and Grandy DK (1993) Molecular diversity of the dopamine receptors. *Annu Rev Pharmacol Toxicol* 33:281-307.

Clark D and White FJ (1987) D1 dopamine receptor--the search for a function: a critical evaluation of the D1/D2 dopamine receptor classification and its functional implications. *Synapse* 1(4):347-388.

Del Carmine R, Molinari P, Sbraccia M, Ambrosio C and Costa T (2004) "Induced-fit" mechanism for catecholamine binding to the beta2-adrenergic receptor. *Mol Pharmacol* 66(2):356-363.

DeNinno MP, Schoenleber R, Perner RJ, Lijewski L, Asin KE, Britton DR, MacKenzie R and Kebabian JW (1991) Synthesis and dopaminergic activity of 3-substituted 1-(aminomethyl)-3,4-dihydro-5,6-dihydroxy-1H-2-benzopyrans: characterization of an auxiliary binding region in the D1 receptor. *J Med Chem* 34(8):2561-2569.

Fersht AR (1988) Relationships between apparent binding energies measured in site-directed mutagenesis experiments and energetics of binding and catalysis. *Biochemistry* 27(5):1577-1580.

Fersht AR, Leatherbarrow RJ and Wells TN (1987) Structure-activity relationships in engineered proteins: analysis of use of binding energy by linear free energy relationships. *Biochemistry* 26(19):6030-6038.

Ghosh D, Snyder SE, Watts VJ, Mailman RB and Nichols DE (1996) 9-Dihydroxy-2,3,7,11b-tetrahydro-1H-naph[1,2,3-de]isoquinoline: a potent full dopamine D1 agonist containing a rigid-beta-phenyldopamine pharmacophore. *J Med Chem* 39(2):549-555.

Grubbs RA, Lewis MM, Owens-Vance C, Gay EA, Jassen AK, Mailman RB and Nichols DE (2004) 8,9-dihydroxy-1,2,3,11b-tetrahydrochromeno[4,3,2,-de]isoquinoline (dinoxyline), a high affinity and potent agonist at all dopamine receptor isoforms. *Bioorg Med Chem* 12(6):1403-1412.

Jassen AK, Lewis MM, Miller DW, Leonard SK, Nicholas RA, Nichols DE, Tropsha A, Suzuki K and Mailman R (2000) Molecular and pharmacological characterization of D1-like dopamine receptors reveals structural requirements for critical amino acid residues. *Society for Neuroscience*, New Orleans, LA, 532.9.

Kebabian JW, Britton DR, DeNinno MP, Perner R, Smith L, Jenner P, Schoenleber R and Williams M (1992) A-77636: a potent and selective dopamine D1 receptor agonist with antiparkinsonian activity in marmosets. *Eur J Pharmacol* 229(2-3):203-209.

Kienast T and Heinz A (2006) Dopamine and the diseased brain. *CNS Neurol Disord Drug Targets* 5(1):109-131.

Kozell LB, Machida CA, Neve RL and Neve KA (1994) Chimeric D1/D2 dopamine receptors. Distinct determinants of selective efficacy, potency, and signal transduction. *J Biol Chem* 269(48):30299-30306.

Lewis MM, Huang X, Nichols DE and Mailman RB (2006) D1 and functionally selective dopamine agonists as neuroprotective agents in Parkinson's disease. *CNS Neurol Disord Drug Targets* 5(3):345-353.

Liapakis G, Ballesteros JA, Papachristou S, Chan WC, Chen X and Javitch JA (2000) The forgotten serine. A critical role for Ser-2035.42 in ligand binding to and activation of the beta 2-adrenergic receptor. *J Biol Chem* 275(48):37779-37788.

Manik CP, Molinoff PB and McGonigle P (1988) Comparison of 125I-SCH 23982 and [3H]SCH 23390 as ligands for the D-1 dopamine receptor. *J Neurochem* 51(2):391-397.

Missale C, Nash SR, Robinson SW, Jaber M and Caron MG (1998) Dopamine receptors: from structure to function. *Physiol Rev* 78(1):189-225.

Neve KA, Seamans JK and Trantham-Davidson H (2004) Dopamine receptor signaling. *J Recept Signal Transduct Res* 24(3):165-205.

Nichols DE (2010) Dopamine receptor subtype-selective drugs: D1-like receptors, in *The dopamine receptors, second edition* (Neve KA ed), Humana Press, New York, NY.

O'Dowd BF, Ji X, Alijaniaram M, Rajaram RD, Kong MM, Rashid A, Nguyen T and George SR (2005) Dopamine receptor oligomerization visualized in living cells. *J Biol Chem* 280(44):37225-37235.

Pollock NJ, Manelli AM, Hutchins CW, Steffey ME, MacKenzie RG and Frail DE (1992) Serine mutations in transmembrane V of the dopamine D1 receptor affect ligand interactions and receptor activation. *J Biol Chem* 267(25):17780-17786.

Przybyla JA, Cueva JP, Chemel BR, Hsu KJ, Riese DJ, 2nd, McCorvy JD, Chester JA, Nichols DE and Watts VJ (2009) Comparison of the enantiomers of (+/-)-doxanthrine, a high efficacy full dopamine D(1) receptor agonist, and a reversal of

enantioselectivity at D(1) versus alpha(2C) adrenergic receptors. *Eur Neuropsychopharmacol* 19(2):138-146.

Ryman-Rasmussen JP, Griffith A, Oloff S, Vaidehi N, Brown JT, Goddard WA, 3rd and Mailman RB (2007) Functional selectivity of dopamine D1 receptor agonists in regulating the fate of internalized receptors. *Neuropharmacology* 52(2):562-575.

Strader CD, Candelore MR, Hill WS, Sigal IS and Dixon RA (1989) Identification of two serine residues involved in agonist activation of the beta-adrenergic receptor. *J Biol Chem* 264(23):13572-13578.

Strader CD, Sigal IS, Candelore MR, Rands E, Hill WS and Dixon RA (1988) Conserved aspartic acid residues 79 and 113 of the beta-adrenergic receptor have different roles in receptor function. *J Biol Chem* 263(21):10267-10271.

Swaminath G, Xiang Y, Lee TW, Steenhuis J, Parnot C and Kobilka BK (2004) Sequential binding of agonists to the beta2 adrenoceptor. Kinetic evidence for intermediate conformational states. *J Biol Chem* 279(1):686-691.

Tiberi M and Caron MG (1994) High agonist-independent activity is a distinguishing feature of the dopamine D1B receptor subtype. *J Biol Chem* 269(45):27925-27931.

Tomic M, Seeman P, George SR and O'Dowd BF (1993) Dopamine D1 receptor mutagenesis: role of amino acids in agonist and antagonist binding. *Biochem Biophys Res Commun* 191(3):1020-1027.

Wang CD, Buck MA and Fraser CM (1991) Site-directed mutagenesis of alpha 2A-adrenergic receptors: identification of amino acids involved in ligand binding and receptor activation by agonists. *Mol Pharmacol* 40(2):168-179.

Watts VJ, Lawler CP, Gonzales AJ, Zhou QY, Civelli O, Nichols DE and Mailman RB (1995) Spare receptors and intrinsic activity: studies with D1 dopamine receptor agonists. *Synapse* 21(2):177-187.

Watts VJ and Neve KA (1996) Sensitization of endogenous and recombinant adenylate cyclase by activation of D2 dopamine receptors. *Mol Pharmacol* 50(4):966-976.

Zhang J, Xiong B, Zhen X and Zhang A (2009) Dopamine D1 receptor ligands: where are we now and where are we going. *Med Res Rev* 29(2):272-294.

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

FOOTNOTES

This work was supported by the National Institute of Mental Health [MH42705 and MH060397] (D.E.N. and V.J.W., respectively). The authors acknowledge support from the Purdue Research Foundation and the Department of Medicinal Chemistry and Molecular Pharmacology.

This work is part of the Purdue University Ph.D. dissertation of Benjamin Chemel (2010) entitled "Exploring the Molecular Determinants of Binding Selectivity and Efficacy for D_1 Dopamine Receptor Agonists."

Parts of this work have been presented as posters at the following meetings:

Chemel BR, Bonner LA, Watts VJ and Nichols DE (2007) D1 versus D2 dopamine receptor selectivity is determined by intramolecular hydrogen bonding patterns in catechol-containing novel dopamine analogues. *Society for Neuroscience*, San Diego, CA, 351.1.

Chemel BR, Bonner LA, Watts VJ and Nichols DE (2008) D1 versus D2 dopamine receptor selectivity is determined by intramolecular hydrogen bonding patterns in catechol-containing novel dopamine analogues. *Experimental Biology*, San Diego, CA, 1125.1.

Chemel BR, Bonner LA, Watts VJ and Nichols DE (2010) Ligand-specific roses for transmembrane 5 serine residues in D1 dopamine receptor binding and activation. *Experimental Biology*, Anaheim, CA, 584.2.

Reprint requests to:

David E. Nichols
Purdue University
Dept. of Medicinal Chemistry and Molecular Pharmacology
575 Stadium Mall Dr., RHPH 210
West Lafayette, IN 47907-2091
Tel. 765-494-1461
Fax. (765) 494-1414
drdave@purdue.edu

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE LEGENDS

Figure 1. Structures of D_1 dopamine receptor ligands used in this study.

Figure 2. Relative effects of TM5 serine to alanine mutations on binding affinity of cyclohexyl-substituted bicyclic compounds. Data represent ΔpK_i values (mean and S.E.M.) for each mutant, relative to wild-type, of the cyclohexyl-substituted isochroman (white), carbocyclic (grey), and chroman (black) (n=4 matched experiments). * $p < 0.05$, ** $p < 0.01$ significantly different from cyclohexyl chroman (one-way ANOVA with Dunnett's post-test).

Figure 3. Effects of D_1 receptor TM5 serine mutations on catechol agonist binding affinity. $\Delta\Delta G^o$ values for the S198A (white), S199A (grey), and S202A (black) D_1 receptor mutants relative to wild-type were calculated from mutant and wild-type K_i values generated in parallel (See Materials and Methods). Negative values indicate detrimental effects on affinity. The dashed line illustrates the lower energetic limit corresponding to the loss of a hydrogen bond (Fersht, 1988). Data represent mean and S.E.M for at least three matched experiments. The corresponding pK_i values were all significantly different from wild-type ($p < 0.05$, one-way ANOVA with Dunnett's post-test, Table 4).

Figure 4. Effects of TM5 serine mutations on potencies of catechol agonists. ΔpEC_{50} values of cAMP accumulation for the S198A, S199A, and S202A D_1 receptor mutants, relative to wild-type, were calculated from independent experiments performed in

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

parallel. Data represent mean and S.E.M for at least three matched experiments. The corresponding pEC_{50} values were all significantly different from wild-type ($p < 0.05$, one-way ANOVA with Dunnett's post-test). **(A)** ΔpEC_{50} values at each mutant, relative to wild-type, for the cyclohexyl-substituted isochroman (white), chroman (grey), and carbocyclic (black). * $p < 0.05$, ** $p < 0.01$ significantly different from cyclohexyl chroman (one-way ANOVA with Dunnett's post-test). **(B)** ΔpEC_{50} values at each mutant, relative to wild-type, for dopamine, apomorphine, and DHX. **(C)** ΔpEC_{50} values at each mutant, relative to wild-type, for three phenylbenzazepine ligands.

Figure 5. D_1 dopamine receptor agonist efficacy for cAMP production in porcine striatal homogenates. Data represent mean and standard error for cAMP levels produced in response to 10 μM of each test compound, normalized to dopamine ($n = 6$). * $p < 0.05$, ** $p < 0.01$ significantly different from dopamine (100%) (one-way ANOVA with Dunnett's post-test).

Figure 6. Comparison of the low energy conformations of catechol agonists. DHX (blue) and **(A)** SKF 38393 (yellow) or **(B)** apomorphine (pink) were manually aligned with priority for the amino proton that interacts with Asp103. Nonpolar hydrogens have been omitted for clarity. TM5 serines are included to illustrate the proposed interacting partners of the meta-OH and para-OH of the catechol moieties.

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

TABLES

Table 1. Characterization of radioligand affinity and expression for D_1 WT, S198A, S199A, and S202A cell lines. Experiments were performed with [^{3}H]SCH 23390 at D_1 receptors stably expressed in HEK cells. Values for K_d and B_{max} are expressed as mean ± S.E.M. as calculated from at least seven independent experiments.

Cell Line	**K_d (nM)**	**B_{max} (fmol/mg)**
HEK hD_1 WT [a]	1.2 ± 0.2	1840 ± 120
HEK hD_1 S198A [b]	51.6 ± 7.8**	1610 ± 350
HEK hD_1 S199A [b]	27.5 ± 4.1**	1990 ± 160
HEK hD_1 S202A [a]	1.1 ± 0.1	1890 ± 150

** significantly different from wild-type ($p < 0.01$, one-way ANOVA with Dunnett's post-test)

[a] indicates that data were generated by radioligand saturation binding. [b] indicates that data were generated by homologous competition binding.

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2. Effects of D_1 receptor TM5 serine mutations on DA-stimulated cAMP production**.** Dopamine dose-response curves were performed in the presence of 500 µM IBMX. Experiments were performed in 48-well plates and cAMP levels were calculated for each well (total volume of 100 µl). Data represent mean ± S.E.M. as calculated from at least six independent experiments.

		dopamine	
Cell Line	**basal cAMP (pmol/well)**	**EC_{50} (nM)**	**E_{max} (pmol/well)**
HEK hD_1 WT	3.7 ± 0.7	22 ± 3.4	134 ± 10
HEK hD_1 S198A	1.8 ± 0.6	7800 ± 570**	117 ± 10
HEK hD_1 S199A	1.3 ± 0.3*	2800 ± 400**	111 ± 5.9
HEK hD_1 S202A	1.4 ± 0.4	12000 ± 370**	55 ± 3.1**

** significantly different from wild-type ($p < 0.01$, one-way ANOVA with Dunnett's post-test)

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

Table 3. Binding affinities of catechol agonists for wild-type D_1 and TM5 serine mutants. Data represent mean ± S.E.M. from at least 4 independent competitive binding experiments performed with [^{3}H]SCH 23390. K_i values were calculated from IC_{50} values of one-site sigmoidal curves (with hill slopes < 1) as described in materials and methods. Statistical significance was determined from pK_i values.

	hD_1 WT	**S198A**	**S199A**	**S202A**
Ligand	**K_i (nM)**			
DA	1010 ± 230	4700 ± 1200**	13000 ± 3700**	54400 ± 1500**
cyc. isochro.	13.1 ± 2.4	464 ± 140**	402 ± 70**	1200 ± 91**
cyc. carbo.	183 ± 13	2800 ± 690**	3240 ± 690**	7610 ± 620**
cyc. chro.	2110 ± 140	5210 ± 1400*	10500 ± 2400**	27900 ± 2900**
apomorphine	274 ± 52	700 ± 150*	669 ± 99*	4780 ± 360**
DNS	110 ± 16	689 ± 190**	885 ± 180**	3950 ± 420**
DHX	114 ± 11	1370 ± 170**	2680 ± 470**	3430 ± 260**
DOX	238 ± 69	1150 ± 320*	2660 ± 660**	6160 ± 400**
SKF 38393	290 ± 28	2140 ± 600**	1440 ± 310**	860 ± 110*
SKF 81297	18.8 ± 3.6	213 ± 50**	365 ± 29**	68.5 ± 12**
SKF 82958	9.16 ± 2.7	390 ± 190**	173 ± 17**	42.2 ± 9.7*
SKF 83959	1.19 ± 0.3	66.6 ± 15**	29.5 ± 11**	5.23 ± 1.7*

* significantly different from wild-type ($p < 0.05$)

** significantly different from wild-type ($p < 0.01$)

Table 4. Functional properties of catechol agonists for wild-type D_1 and TM5 serine mutants. Data represent mean ± S.E.M. of potency (EC_{50}) and intrinsic activity (IA, normalized to percent of 100 μM dopamine) in response to D_1 receptor-stimulated cAMP accumulation ($n \geq 3$). Statistical significance was determined from pEC_{50} values. The dopamine EC50 values are taken from Table 2.

	hD_1 WT		**S198A**		**S199A**		**S202A**	
Ligand	**EC_{50} (nM)**	**IA (% DA)**	**EC_{50} (nM)**	**IA (% DA)**	**EC_{50} (nM)**	**IA (% DA)**	**EC_{50} (nM)**	**IA (% DA)**
DA[a]	22 ± 3.4	100	7800 ± 570**	100	2800 ± 400**	100	12000 ± 370**	100
cyc. isochro.	1.6 ± 0.1	114 ± 4.2	430 ± 88**	157 ± 5.7	75 ± 12**	119 ± 6.9	570 ± 74**	231 ± 14
cyc. carbo.	120 ± 7.4	108 ± 5.2	14000 ± 1500**	149 ± 15	7900 ± 730**	132 ± 6.7	17000 ± 1700**	175 ± 9.8
cyc. chro.	820± 110	125 ± 2.9	49000 ± 7500**	76 ± 5.5	12000 ± 1900**	124 ± 5.3	34000 ± 3900**	48 ± 1.1
apomorphine	70 ± 9.4	110 ± 6.1	14000 ± 810**	78 ± 4.2	1200 ± 310**	124 ± 6.4	14000 ± 2200**	108 ± 8.1
DNS	6.3 ± 1.0	113 ± 3.6	1600 ± 130**	132 ± 9.7	190 ± 34**	132 ± 10	3000 ± 400**	145 ± 5.9
DHX	5.2 ± 0.8	104 ± 2.9	940 ± 106**	132 ± 10	240 ± 18**	109 ± 12	750 ± 140**	101 ± 3.3
DOX	6.4 ± 1.2	101 ± 3.4	470 ± 80**	125 ± 16	240 ± 49**	101 ± 4.2	240 ± 33**	95 ± 4.5
SKF 38393	38 ± 3.1	92 ± 3.5	2600 ± 340**	58 ± 3.3	1000 ± 190**	51 ± 1.7	180 ± 6.7**	62 ± 3.5
SKF 81297	2.1 ± 0.4	107 ± 5.9	480 ± 29**	139 ± 5.4	64 ± 9.2**	122 ± 1.9	7.6 ± 1.2**	223 ± 16
SKF 82958	2.6 ± 0.9	115 ± 1.3	200 ± 35**	137 ± 2.8	63 ± 3.4**	123 ± 7.4	11 ± 2.9**	211 ± 8.8
SKF 83959	1.8 ± 0.2	82 ± 3.5	130 ± 4.3**	90 ± 4.1	25 ± 0.5**	74 ± 6.6	6.5 ± 2.4**	103 ± 4.9

[a] The dopamine EC50 values are taken from Table 2.

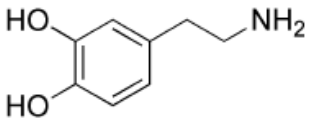
* Significantly different from wild type ($p < 0.05$)

** Significantly different from wild type ($P < 0.01$)

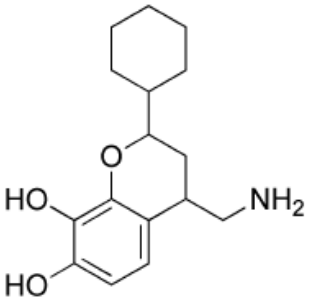
Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 1

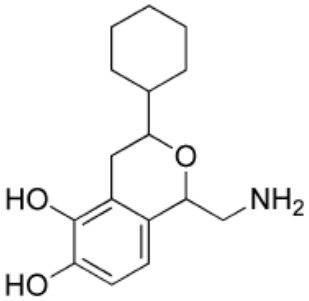
Bicyclics



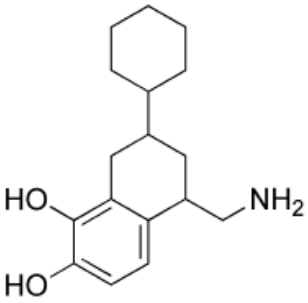
dopamine
(DA)



cyclohexyl chroman

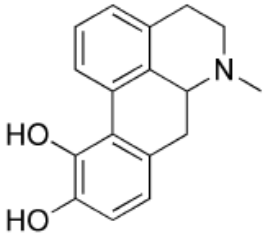


cyclohexyl isochroman

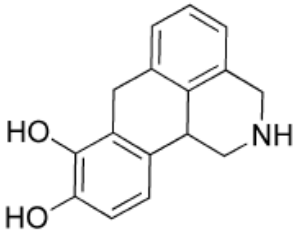


cyclohexyl carbocyclic

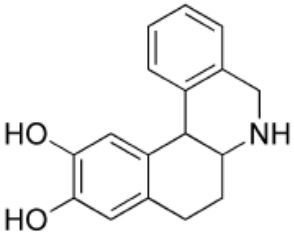
Tetracyclics



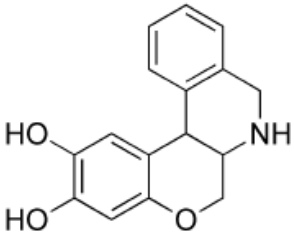
apomorphine
(APO)



dinapsoline
(DNS)

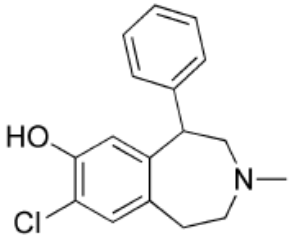


dihydrexidine
(DHX)

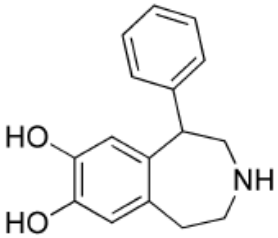


doxanthrine
(DOX)

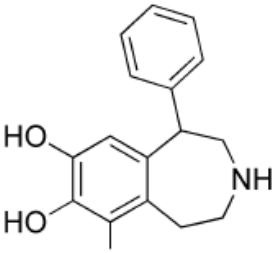
Phenylbenzazepines



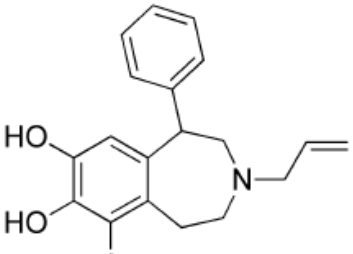
SCH 23390



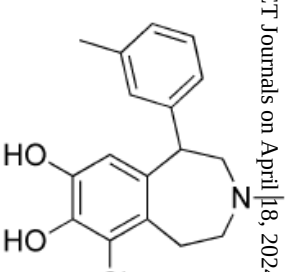
SKF 38393



SKF 81297

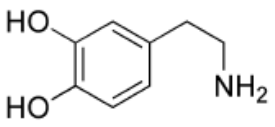


SKF 82958

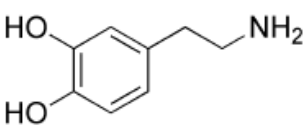


SKF 83959

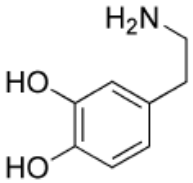
Rotameric conformations of dopamine



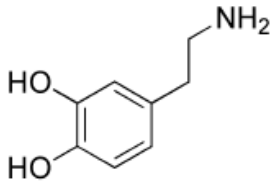
cis-β



trans-β



cis-α



trans-α

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2

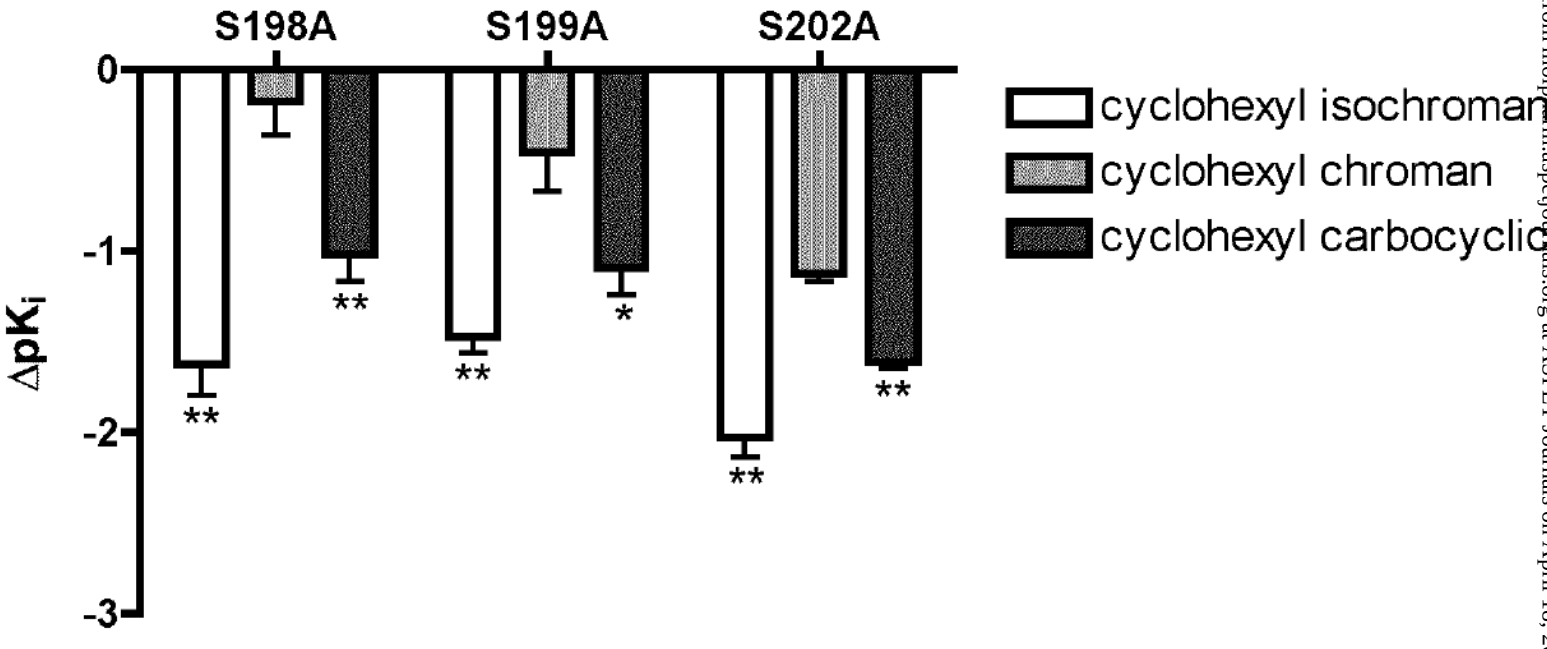


Figure 3

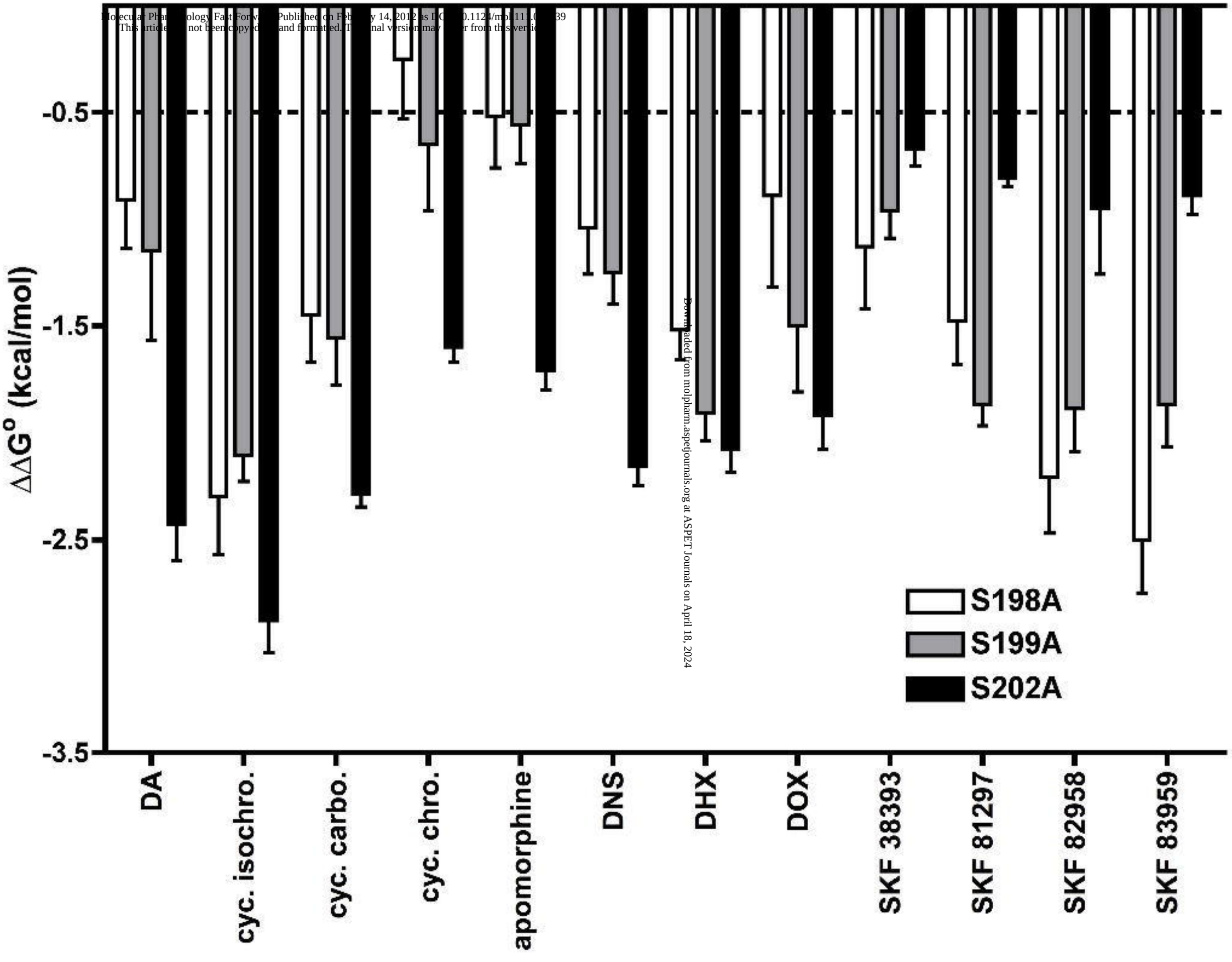
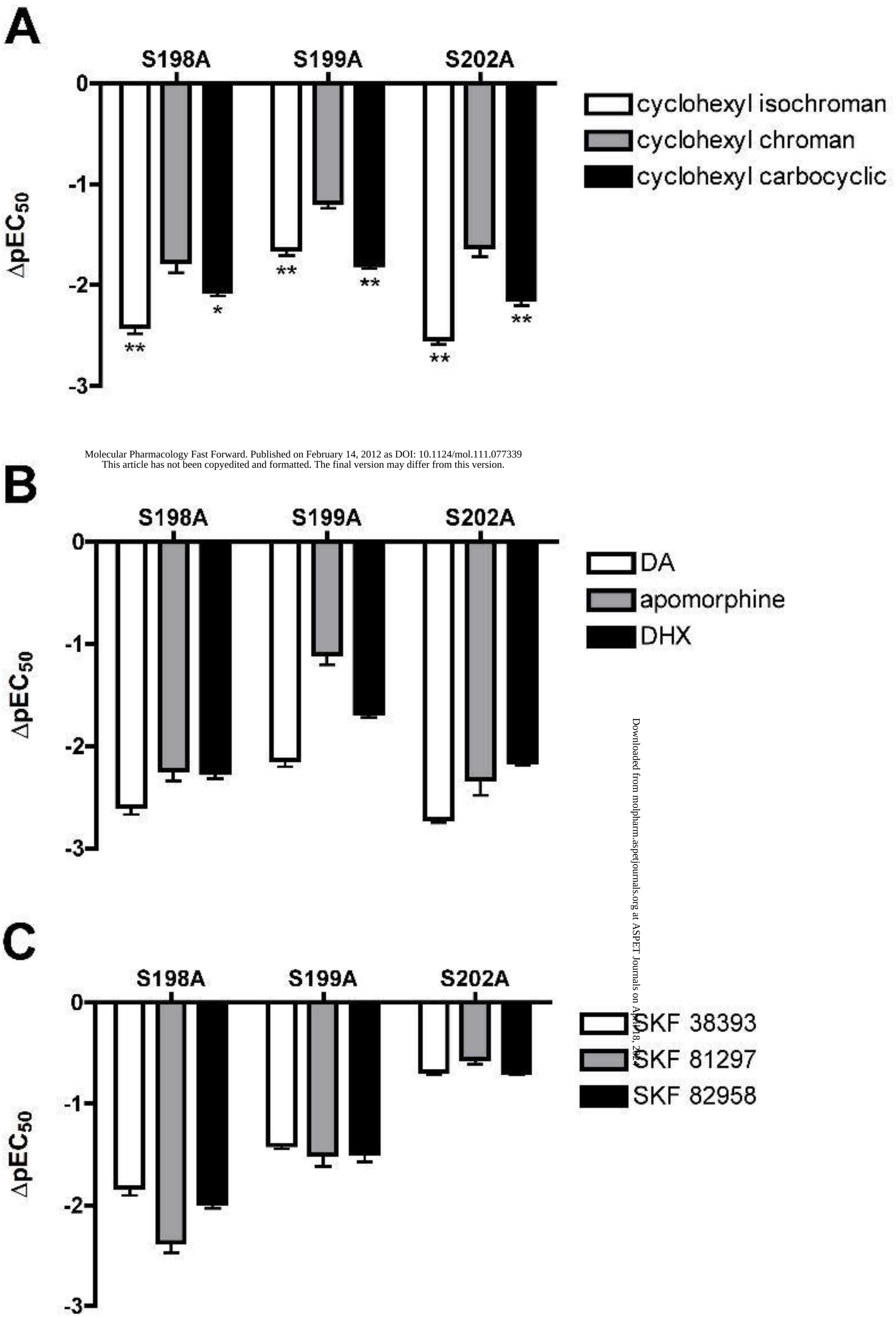


Figure 4



Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5

Molecular Pharmacology Fast Forward. Published on February 14, 2012 as DOI: 10.1124/mol.111.077339
This article has not been copyedited and formatted. The final version may differ from this version.

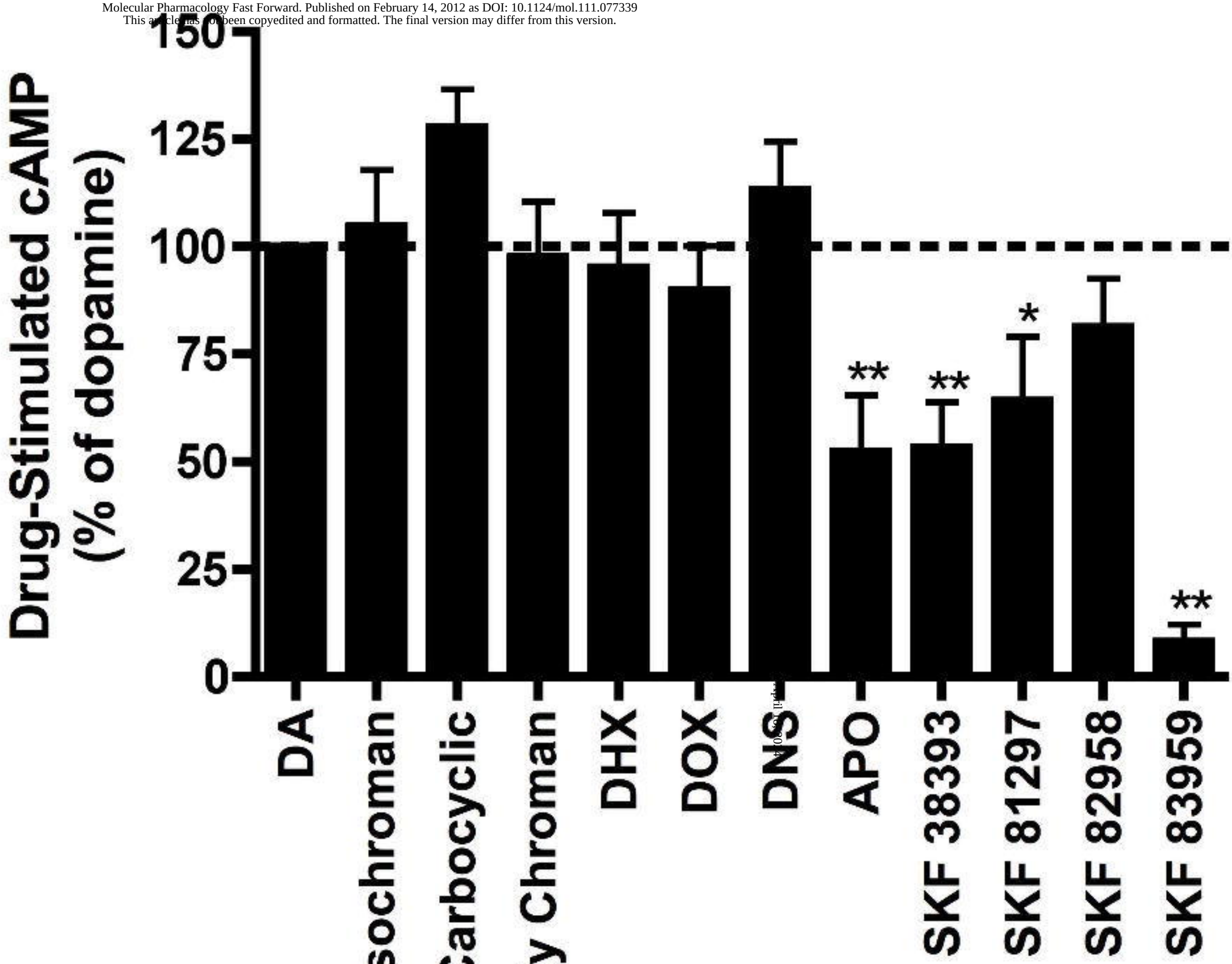


Figure 6