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Ligand-Specific Roles for Transmembrane 5 Serine Residues in the Binding and Efficacy  
of Dopamine D<sub>1</sub> Receptor Catechol Agonists

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Running Title: D<sub>1</sub> dopamine receptor TM5 serines differentially influence ligand pharmacology

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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)

## ABSTRACT

To refine further the structure-activity relationships of D<sub>1</sub> 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<sub>1</sub> receptors in HEK cells. These receptors were expressed at similar levels (approximately 2000 fmol/mg) and bound the radioligand [<sup>3</sup>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<sub>1</sub> 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<sub>1</sub>-ligand recognition and agonist activation, and have implications for the design of rigid catechol ligands.

## 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<sub>1</sub> and D<sub>5</sub>, 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<sub>2</sub>, D<sub>3</sub>, and D<sub>4</sub>, couple to G $\alpha_i$  thereby inhibiting the production of cAMP (Neve et al., 2004). Of the five receptor subtypes, D<sub>1</sub> and D<sub>2</sub> 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<sub>1</sub> 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<sub>1</sub> 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

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  $\beta_2$ -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<sub>1</sub> 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.

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<sub>1</sub> receptors. The ligands used for this study are illustrated in Figure 1. We stably expressed wild-type and mutant D<sub>1</sub> 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<sub>1</sub>-like receptors suggest that the trans- $\beta$  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<sub>1</sub> dopamine receptor play critical, ligand-specific roles in agonist binding and receptor activation.

## METHODS

### Materials

[<sup>3</sup>H]SCH 23390 was purchased from Amersham Biosciences (Piscataway, NJ) and PerkinElmer Life and Analytical Sciences (Waltham, MA). [<sup>3</sup>H]cAMP, [<sup>3</sup>H]methylpiperone, 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

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<sub>1</sub> Mutants**

Wild-type human D<sub>1</sub> 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):

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  $\mu\text{g/ml}$  penicillin, 50  $\mu\text{g/ml}$  streptomycin, and 0.25  $\mu\text{g/ml}$  amphoteroicin B. Cells were grown in a humidified incubator with 5%  $\text{CO}_2$ . HEK cells were stably transfected by combining 3  $\mu\text{g}$  pcDNA3.1/V5-His TOPO hD<sub>1</sub> (WT or mutant) with 15  $\mu\text{l}$  Lipofectamine 2000 (Invitrogen) in OptiMEM I medium according to manufacturer's protocol. This mixture was added dropwise to 10  $\text{cm}^2$  tissue culture plates containing ~70% confluent HEK cells. Twenty four hours later, these cells were split into new 10  $\text{cm}^2$  plates at various seeding densities. The following day, and every three days thereafter, the media was replaced with fresh selection media containing 600  $\mu\text{g/ml}$  G418. After approximately four weeks, when colonies were visible to the naked eye, the entire plate was resuspended and transferred to a new 10  $\text{cm}^2$  plate. These plates were grown to 90% confluency in maintenance media containing 300  $\mu\text{g/ml}$  G418 and further split into additional plates to enable receptor evaluation.



## Membrane Preparation

HEK cells expressing either WT or mutant D<sub>1</sub> receptors were grown to confluency in 15 cm<sup>2</sup> 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 °C for 20 min. The supernatant was discarded, and the pellet was resuspended by mechanical homogenization in 4 ml/15 cm<sup>2</sup> plate receptor binding buffer (50 mM Tris-HCl, pH 7.4, and 4 mM MgCl<sub>2</sub>). 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 °C 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 [<sup>3</sup>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 °C 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

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  $\mu$ M) were added in duplicate to a total volume of 250  $\mu$ l. Total binding was defined in the absence of competing ligand, and non-specific binding was defined by the addition of 5  $\mu$ M (+)-butaclamol. Assays were incubated at 37  $^{\circ}$ 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

$\mu\text{M}$ , were added in duplicate to wells containing approximately 1-3 nM [ $^3\text{H}$ ]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  $\mu\text{l}$  in the presence of that was 500  $\mu\text{M}$  in isobutyl-methylxanthine (IBMX). To facilitate normalization, mutants were assayed in parallel with wild-type, and wells containing vehicle (basal) and 100  $\mu\text{M}$  dopamine (max DA) were included alongside each test drug as controls. Assays were incubated for 15 min at 37  $^{\circ}\text{C}$  in a water bath, and were terminated by decanting followed by the addition of 100  $\mu\text{l}$  ice-cold 3% trichloroacetic acid on ice. Plates were stored at 4  $^{\circ}\text{C}$  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  $\text{MgCl}_2$ , 2 mM EDTA, 1 mM IBMX, 0.01% ascorbic acid, 10  $\mu\text{M}$  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*-

methylglycine disodium salt (phosphocreatine), and 3 U creatine phosphokinase), 1  $\mu$ M Gpp(NH)p, 30  $\mu$ g striatal protein, and the indicated drugs (10  $\mu$ M) in a total volume of 100  $\mu$ l. Propranolol and prazosin (1  $\mu$ M 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  $\mu$ L 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  $\mu$ l 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 [<sup>3</sup>H]cAMP and 100  $\mu$ g of bovine cAMP binding protein in a total volume of 500  $\mu$ l. Assays were incubated at 4 °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

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  $\mu\text{M}$ ) at each receptor and graphed using sigmoidal dose-response curves with a Hill slope fixed to unity to generate  $\text{EC}_{50}$  and intrinsic activity (IA, % maximum DA stimulation) values.  $E_{\text{max}}$  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  $\mu\text{M}$ ) were normalized to percent stimulation by 10  $\mu\text{M}$  dopamine over vehicle levels (1  $\mu\text{M}$  Gpp(NH)p alone).

Saturation binding experiments were analyzed using a one site binding (hyperbola) model to generate values for  $K_d$  and  $B_{\text{max}}$ . For homologous competition binding assays,  $\text{IC}_{50}$  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:  $K_d = \text{IC}_{50} - [\text{radioligand}]$ .  $B_{\text{max}}$  values were determined as follows:  $B_{\text{max}} = (\text{Top} - \text{Bottom}) / ([\text{radioligand}] / (K_d - [\text{radioligand}]))$ .  $B_{\text{max}}$  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  $\text{IC}_{50}$  values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). The hill slopes (not reported) for the agonists

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 ( $\Delta\Delta G^\circ$ ), calculated from  $K_i$  values as follows:  $\Delta\Delta G^\circ = \Delta G^\circ_{\text{mutant}} - \Delta G^\circ_{\text{WT}} = -RT\ln(K_{i\text{-mutant}}/K_{i\text{-WT}})$ , 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:  $\Delta pEC_{50} = pEC_{50\text{-mutant}} - pEC_{50\text{-WT}} = -\log EC_{50\text{-mutant}} - (-\log EC_{50\text{-WT}})$ .  $\Delta\Delta G^\circ$ ,  $\Delta pK_i$ , and  $\Delta pEC_{50}$  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

D<sub>1</sub> 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<sub>max</sub>) levels and their affinities for [<sup>3</sup>H]SCH 23390 (Table 1). The wild-type D<sub>1</sub> cell line displayed saturable radioligand binding with mean values of 1.2 nM and 1840 fmol/mg for K<sub>d</sub> and B<sub>max</sub>, 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 K<sub>d</sub>/K<sub>i</sub> 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<sub>1</sub> receptors were evaluated using the endogenous agonist, dopamine, by measuring cAMP accumulation in response to D<sub>1</sub>-stimulated G<sub>αs</sub> 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<sub>50</sub> value for dopamine at the wild-type D<sub>1</sub> receptor was 22 nM. In contrast, dopamine was dramatically less potent at all three mutant receptors. The S199A

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<sub>1</sub> 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<sub>1</sub> 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 [<sup>3</sup>H]SCH 23390 were used to evaluate the binding affinities (K<sub>i</sub>) of structurally-diverse catechol agonists for wild-type and mutant D<sub>1</sub> 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<sub>i</sub> 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).



$K_i$  values from independent experiments were converted to changes (from wild-type) in standard Gibbs free energy ( $\Delta\Delta G^\circ$ ) 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  $\sim 0.5$  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:  $S198A \leq S199A < S202A$ ). 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  $\delta$ -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 ( $S202A < S199A \approx S198A$ ), 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

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<sub>50</sub> 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<sub>50</sub> 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  $\Delta$ pEC<sub>50</sub> values (Figure 4A, B). Like dopamine, they were less affected by S199A than by S198A and S202A, (magnitude of potency loss: S199A < S198A  $\approx$  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

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<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> receptors

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 [<sup>3</sup>H]SCH 23390. The S202A and wild-type cell lines displayed similar K<sub>i</sub> values for [<sup>3</sup>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<sub>i</sub> and B<sub>max</sub> 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<sub>1</sub> 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<sub>1</sub>-like receptors (DeNinno et al., 1991). A variety of hydrophobic substituents at the C3 position of the isochromans increase D<sub>1</sub>-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<sub>1</sub> receptor-selective agonists (Nichols, 2010). The active enantiomer of the

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<sub>1</sub> 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<sub>1</sub> affinity and selectivity. These data suggest that the poor D<sub>1</sub> 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<sub>1</sub> receptors, assuming that negative effects reflect the loss of favorable interactions between the ligand and receptor. The EC<sub>50</sub> and K<sub>i</sub> values of these compounds in the WT and mutant receptor cell lines paralleled their D<sub>1</sub>-like binding affinities in native tissues (isochroman < carbocyclic < chroman). Comparing the magnitudes of mutation-induced changes in binding affinity ( $\Delta pK_i$ ) and potency

( $\Delta pEC_{50}$ ) 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 intramolecular hydrogen bond.

This finding and the loss of D<sub>1</sub>-like receptor selectivity for the chroman among the unsubstituted bicyclic compounds (Bonner et al., 2011) underscore important differences between the structural requirements of D<sub>1</sub>-like and D<sub>2</sub>-like receptors. Unlike D<sub>1</sub> receptor ligands, many of the prototypical D<sub>2</sub>-like receptor full agonists are non-catechol (e.g., quinpirole), indicating that the catechol hydrogen bond requirements of D<sub>2</sub> receptors are less demanding. Further, the loss of D<sub>2</sub> affinity and reciprocal increase of D<sub>1</sub> affinity upon hydrophobic substitution of the bicyclic ligands highlights the absence of an accessory binding region in the D<sub>2</sub> binding site.

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

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<sub>1</sub> receptors by these compounds. Consistent with their apparent similar modes of interaction within the D<sub>1</sub> binding site, these ligands all behaved as full agonists in native tissues (Figure 5).

All the phenylbenzazepine agonists were similarly affected by the D<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> 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.

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



ligand binding, which barely exceeded the threshold for hydrogen bonding (Figure 3).

The minimized structure of apomorphine demonstrates that its trans- $\alpha$  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<sub>1</sub> 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

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<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> 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.

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## **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.

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## FOOTNOTES

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Chemel BR, Bonner LA, Watts VJ and Nichols DE (2008) D<sub>1</sub> versus D<sub>2</sub> dopamine receptor selectivity is determined by intramolecular hydrogen bonding patterns in catechol-containing novel dopamine analogues. *Experimental Biology*, San Diego, CA, 1125.1.

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## FIGURE LEGENDS

**Figure 1.** Structures of D<sub>1</sub> 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  $\Delta pK_i$  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<sub>1</sub> receptor TM5 serine mutations on catechol agonist binding affinity.  $\Delta\Delta G^\circ$  values for the S198A (white), S199A (grey), and S202A (black) D<sub>1</sub> 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.  $\Delta pEC_{50}$  values of cAMP accumulation for the S198A, S199A, and S202A D<sub>1</sub> receptor mutants, relative to wild-type, were calculated from independent experiments performed in



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)**  $\Delta pEC_{50}$  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)**  $\Delta pEC_{50}$  values at each mutant, relative to wild-type, for dopamine, apomorphine, and DHX. **(C)**  $\Delta pEC_{50}$  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  $\mu$ M 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.

## TABLES

**Table 1.** Characterization of radioligand affinity and expression for D<sub>1</sub> WT, S198A, S199A, and S202A cell lines. Experiments were performed with [<sup>3</sup>H]SCH 23390 at D<sub>1</sub> receptors stably expressed in HEK cells. Values for K<sub>d</sub> and B<sub>max</sub> are expressed as mean ± S.E.M. as calculated from at least seven independent experiments.

Cell Line	K <sub>d</sub> (nM)	B <sub>max</sub> (fmol/mg)
HEK hD <sub>1</sub> WT <sup>a</sup>	1.2 ± 0.2	1840 ± 120
HEK hD <sub>1</sub> S198A <sup>b</sup>	51.6 ± 7.8**	1610 ± 350
HEK hD <sub>1</sub> S199A <sup>b</sup>	27.5 ± 4.1**	1990 ± 160
HEK hD <sub>1</sub> S202A <sup>a</sup>	1.1 ± 0.1	1890 ± 150

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

<sup>a</sup> indicates that data were generated by radioligand saturation binding. <sup>b</sup> indicates that data were generated by homologous competition binding.

**Table 2.** Effects of D<sub>1</sub> receptor TM5 serine mutations on DA-stimulated cAMP

production. Dopamine dose-response curves were performed in the presence of 500  $\mu$ M IBMX. Experiments were performed in 48-well plates and cAMP levels were calculated for each well (total volume of 100  $\mu$ l). Data represent mean  $\pm$  S.E.M. as calculated from at least six independent experiments.

Cell Line	basal cAMP (pmol/well)	dopamine	
		EC <sub>50</sub> (nM)	E <sub>max</sub> (pmol/well)
HEK hD <sub>1</sub> WT	3.7 $\pm$ 0.7	22 $\pm$ 3.4	134 $\pm$ 10
HEK hD <sub>1</sub> S198A	1.8 $\pm$ 0.6	7800 $\pm$ 570**	117 $\pm$ 10
HEK hD <sub>1</sub> S199A	1.3 $\pm$ 0.3*	2800 $\pm$ 400**	111 $\pm$ 5.9
HEK hD <sub>1</sub> S202A	1.4 $\pm$ 0.4	12000 $\pm$ 370**	55 $\pm$ 3.1**

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

**Table 3.** Binding affinities of catechol agonists for wild-type D<sub>1</sub> and TM5 serine mutants.

Data represent mean ± S.E.M. from at least 4 independent competitive binding experiments performed with [<sup>3</sup>H]SCH 23390. K<sub>i</sub> values were calculated from IC<sub>50</sub> values of one-site sigmoidal curves (with hill slopes < 1) as described in materials and methods. Statistical significance was determined from pK<sub>i</sub> values.

Ligand	hD <sub>1</sub> WT	S198A	S199A	S202A
	K <sub>i</sub> (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<sub>1</sub> and TM5 serine mutants. Data represent mean  $\pm$  S.E.M. of potency (EC<sub>50</sub>) and intrinsic activity (IA, normalized to percent of 100  $\mu$ M dopamine) in response to D<sub>1</sub> receptor-stimulated cAMP accumulation (n  $\geq$  3). Statistical significance was determined from pEC<sub>50</sub> values. The dopamine EC<sub>50</sub> values are taken from Table 2.

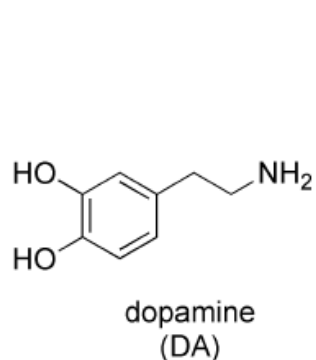
Ligand	hD <sub>1</sub> WT		S198A		S199A		S202A	
	EC <sub>50</sub> (nM)	IA (% DA)	EC <sub>50</sub> (nM)	IA (% DA)	EC <sub>50</sub> (nM)	IA (% DA)	EC <sub>50</sub> (nM)	IA (% DA)
DA <sup>a</sup>	22 $\pm$ 3.4	100	7800 $\pm$ 570**	100	2800 $\pm$ 400**	100	12000 $\pm$ 370**	100
cyc. isochro.	1.6 $\pm$ 0.1	114 $\pm$ 4.2	430 $\pm$ 88**	157 $\pm$ 5.7	75 $\pm$ 12**	119 $\pm$ 6.9	570 $\pm$ 74**	231 $\pm$ 14
cyc. carbo.	120 $\pm$ 7.4	108 $\pm$ 5.2	14000 $\pm$ 1500**	149 $\pm$ 15	7900 $\pm$ 730**	132 $\pm$ 6.7	17000 $\pm$ 1700**	175 $\pm$ 9.8
cyc. chro.	820 $\pm$ 110	125 $\pm$ 2.9	49000 $\pm$ 7500**	76 $\pm$ 5.5	12000 $\pm$ 1900**	124 $\pm$ 5.3	34000 $\pm$ 3900**	48 $\pm$ 1.1
apomorphine	70 $\pm$ 9.4	110 $\pm$ 6.1	14000 $\pm$ 810**	78 $\pm$ 4.2	1200 $\pm$ 310**	124 $\pm$ 6.4	14000 $\pm$ 2200**	108 $\pm$ 8.1
DNS	6.3 $\pm$ 1.0	113 $\pm$ 3.6	1600 $\pm$ 130**	132 $\pm$ 9.7	190 $\pm$ 34**	132 $\pm$ 10	3000 $\pm$ 400**	145 $\pm$ 5.9
DHX	5.2 $\pm$ 0.8	104 $\pm$ 2.9	940 $\pm$ 106**	132 $\pm$ 10	240 $\pm$ 18**	109 $\pm$ 12	750 $\pm$ 140**	101 $\pm$ 3.3
DOX	6.4 $\pm$ 1.2	101 $\pm$ 3.4	470 $\pm$ 80**	125 $\pm$ 16	240 $\pm$ 49**	101 $\pm$ 4.2	240 $\pm$ 33**	95 $\pm$ 4.5
SKF 38393	38 $\pm$ 3.1	92 $\pm$ 3.5	2600 $\pm$ 340**	58 $\pm$ 3.3	1000 $\pm$ 190**	51 $\pm$ 1.7	180 $\pm$ 6.7**	62 $\pm$ 3.5
SKF 81297	2.1 $\pm$ 0.4	107 $\pm$ 5.9	480 $\pm$ 29**	139 $\pm$ 5.4	64 $\pm$ 9.2**	122 $\pm$ 1.9	7.6 $\pm$ 1.2**	223 $\pm$ 16
SKF 82958	2.6 $\pm$ 0.9	115 $\pm$ 1.3	200 $\pm$ 35**	137 $\pm$ 2.8	63 $\pm$ 3.4**	123 $\pm$ 7.4	11 $\pm$ 2.9**	211 $\pm$ 8.8
SKF 83959	1.8 $\pm$ 0.2	82 $\pm$ 3.5	130 $\pm$ 4.3**	90 $\pm$ 4.1	25 $\pm$ 0.5**	74 $\pm$ 6.6	6.5 $\pm$ 2.4**	103 $\pm$ 4.9

<sup>a</sup> The dopamine EC<sub>50</sub> values are taken from Table 2.

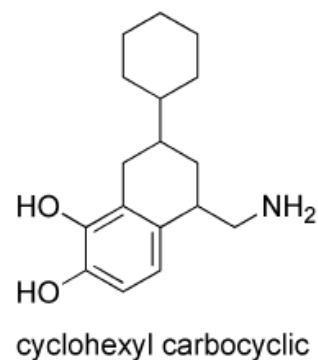
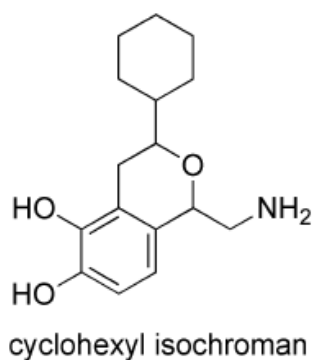
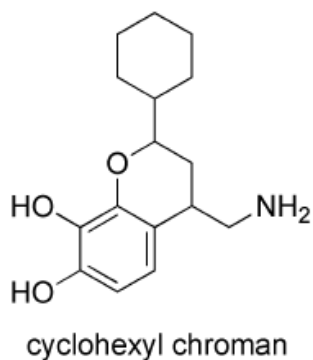
\* Significantly different from wild type (p < 0.05)

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

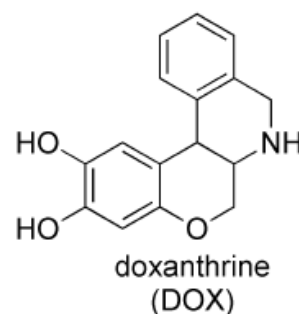
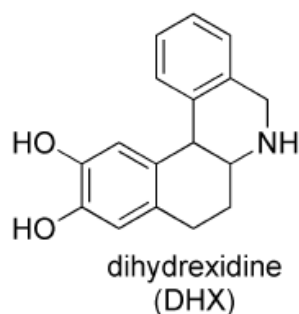
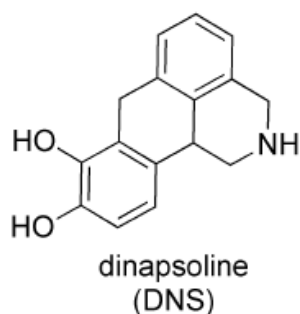
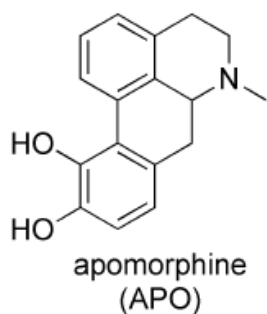
**Figure 1**



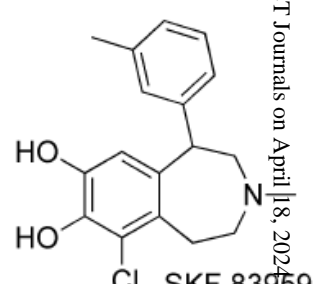
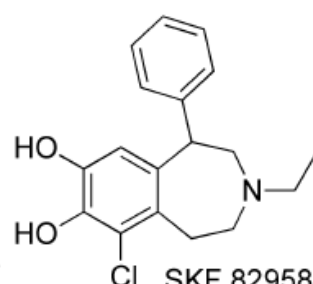
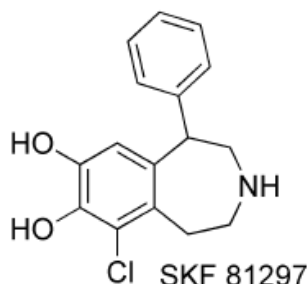
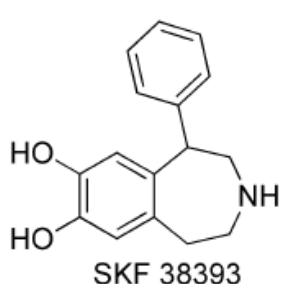
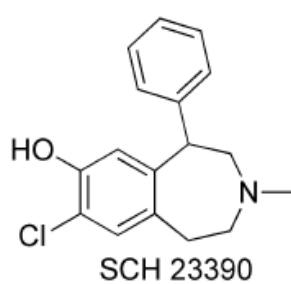
**Bicyclics**



**Tetracyclics**



**Phenylbenzazepines**



**Rotameric conformations of dopamine**

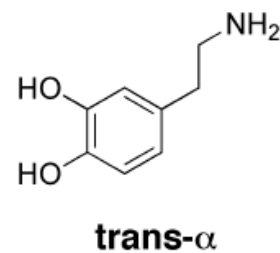
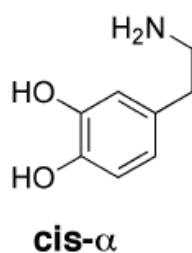
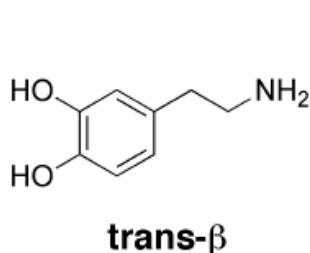
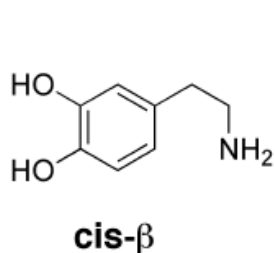
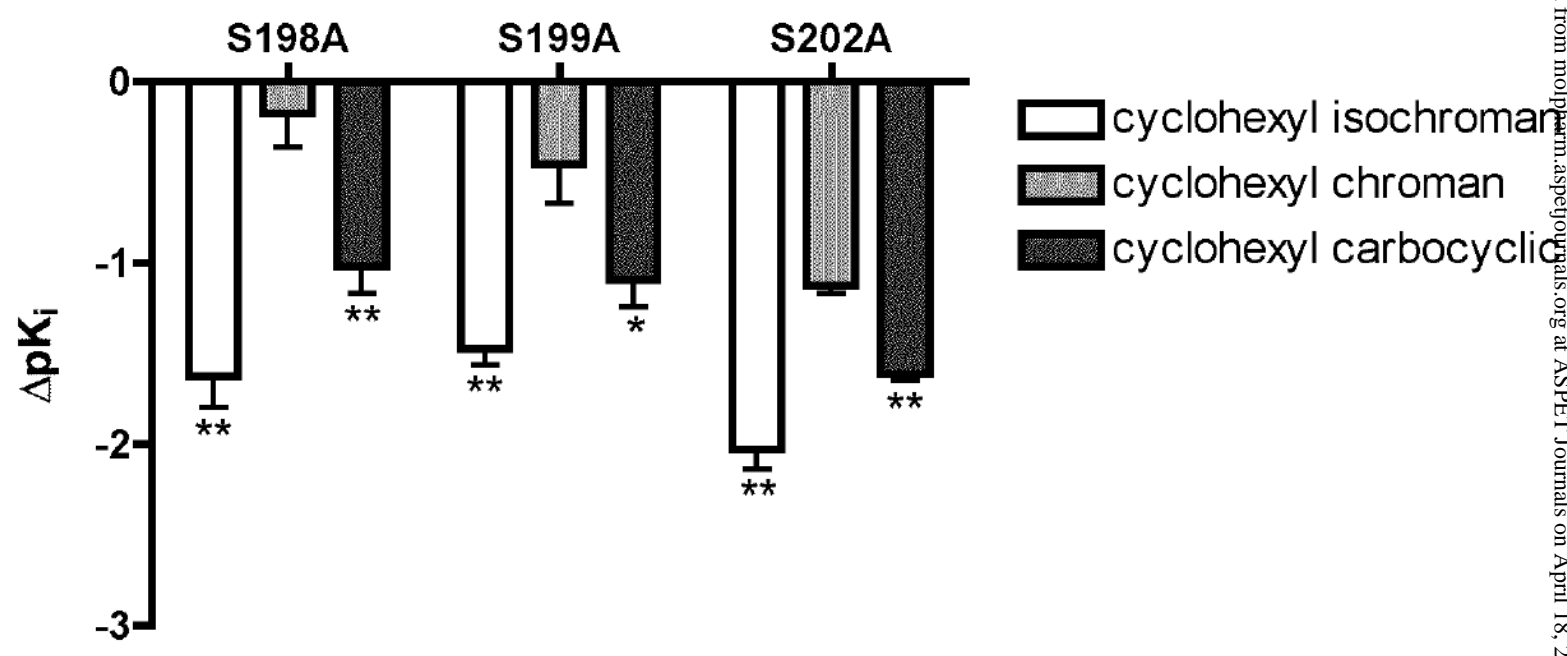
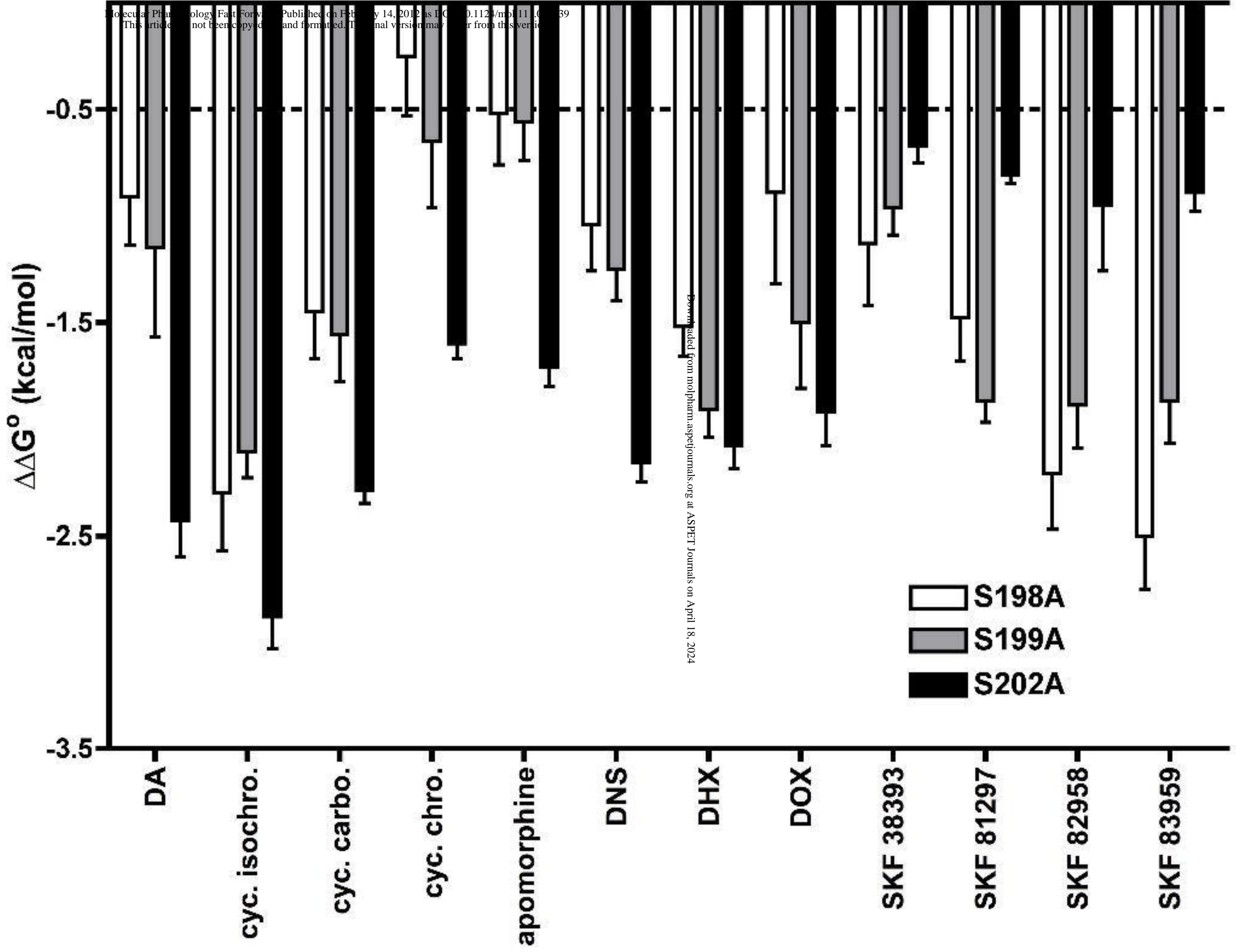


Figure 2



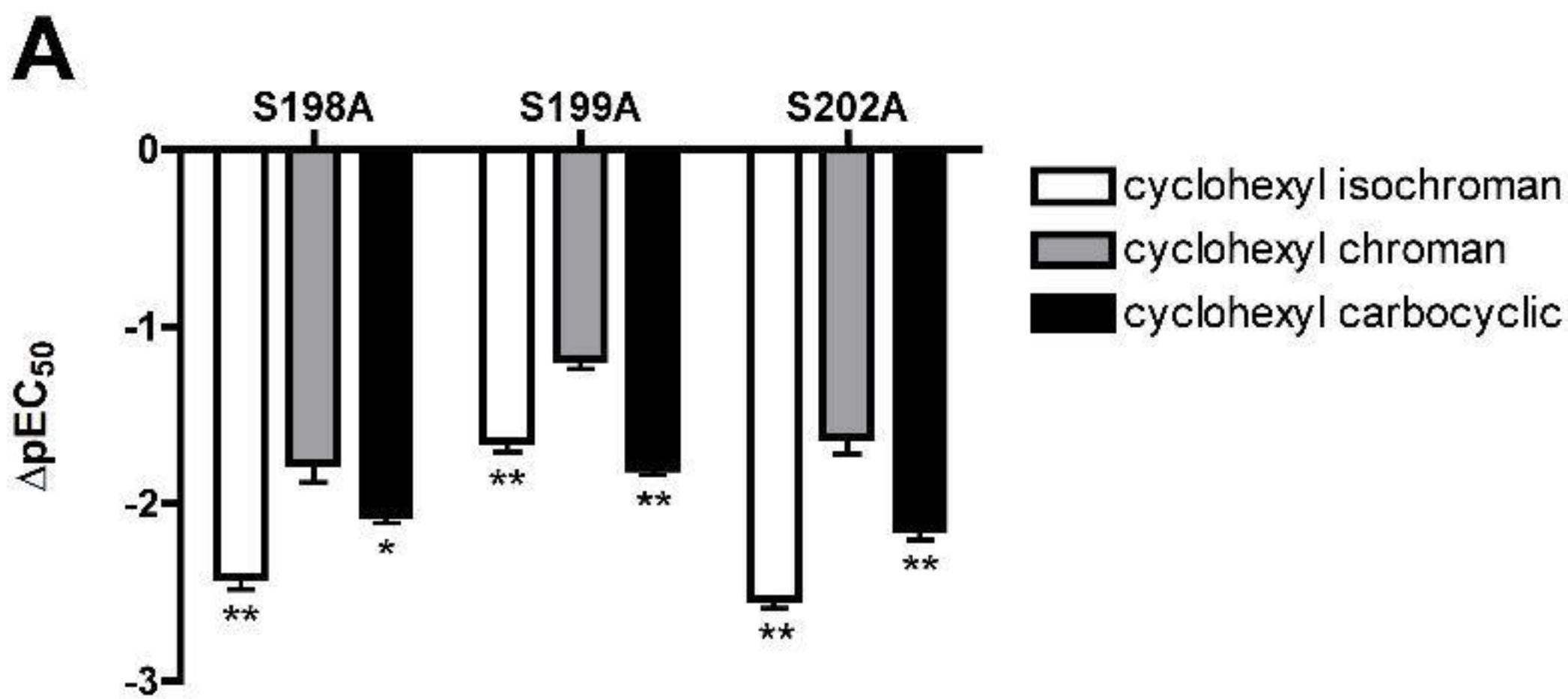
**Figure 3**



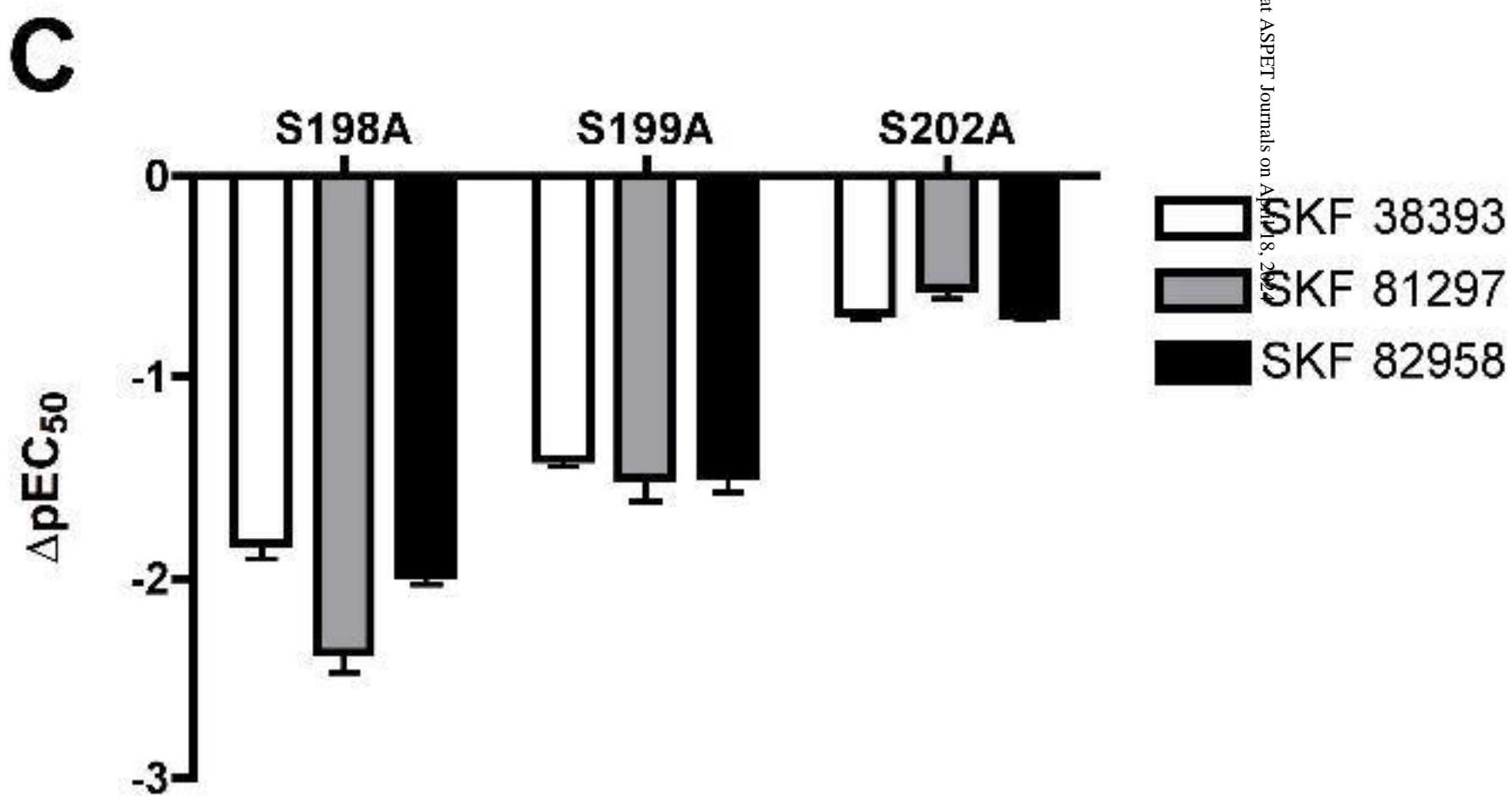
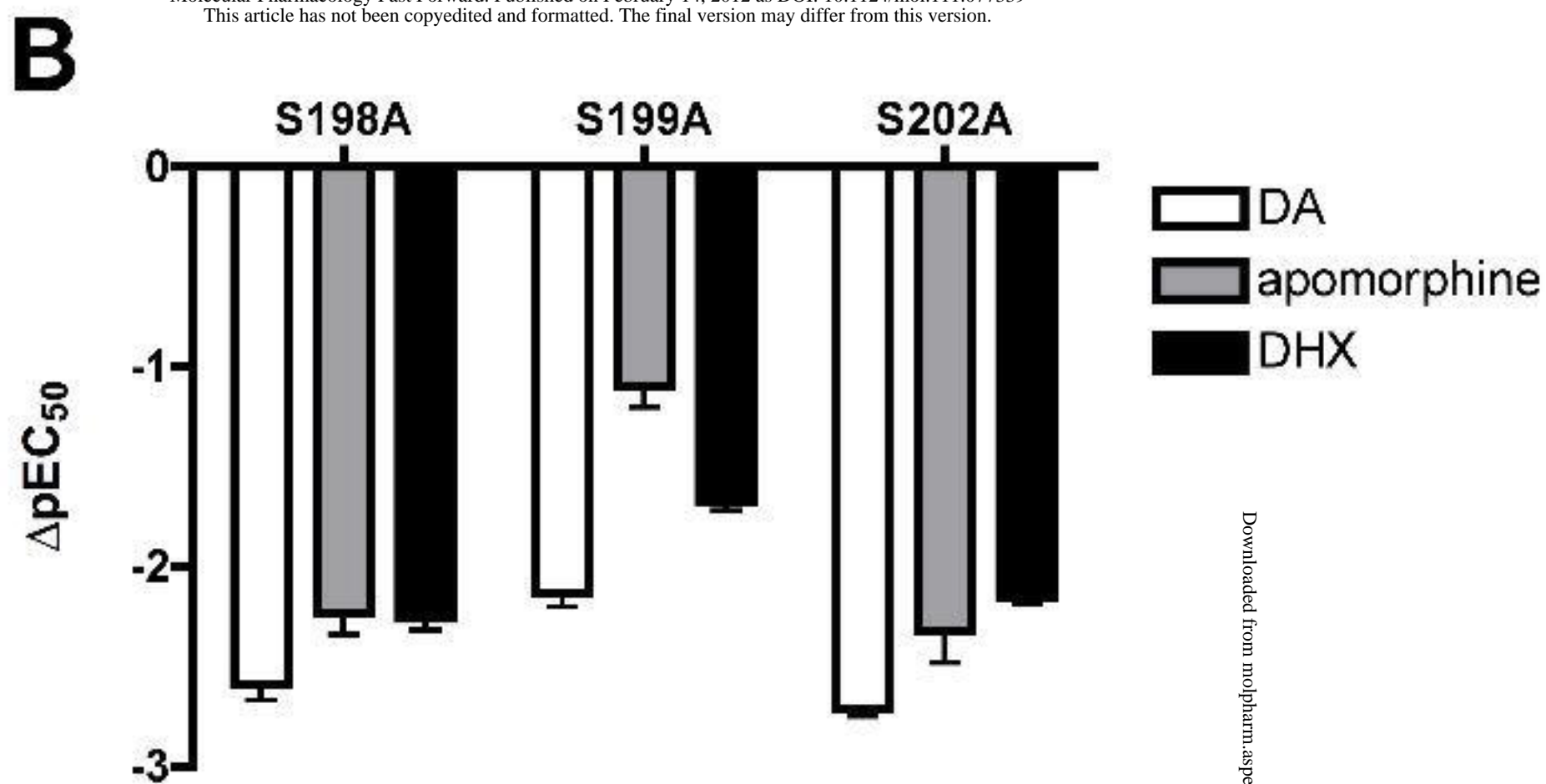
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Figure 4

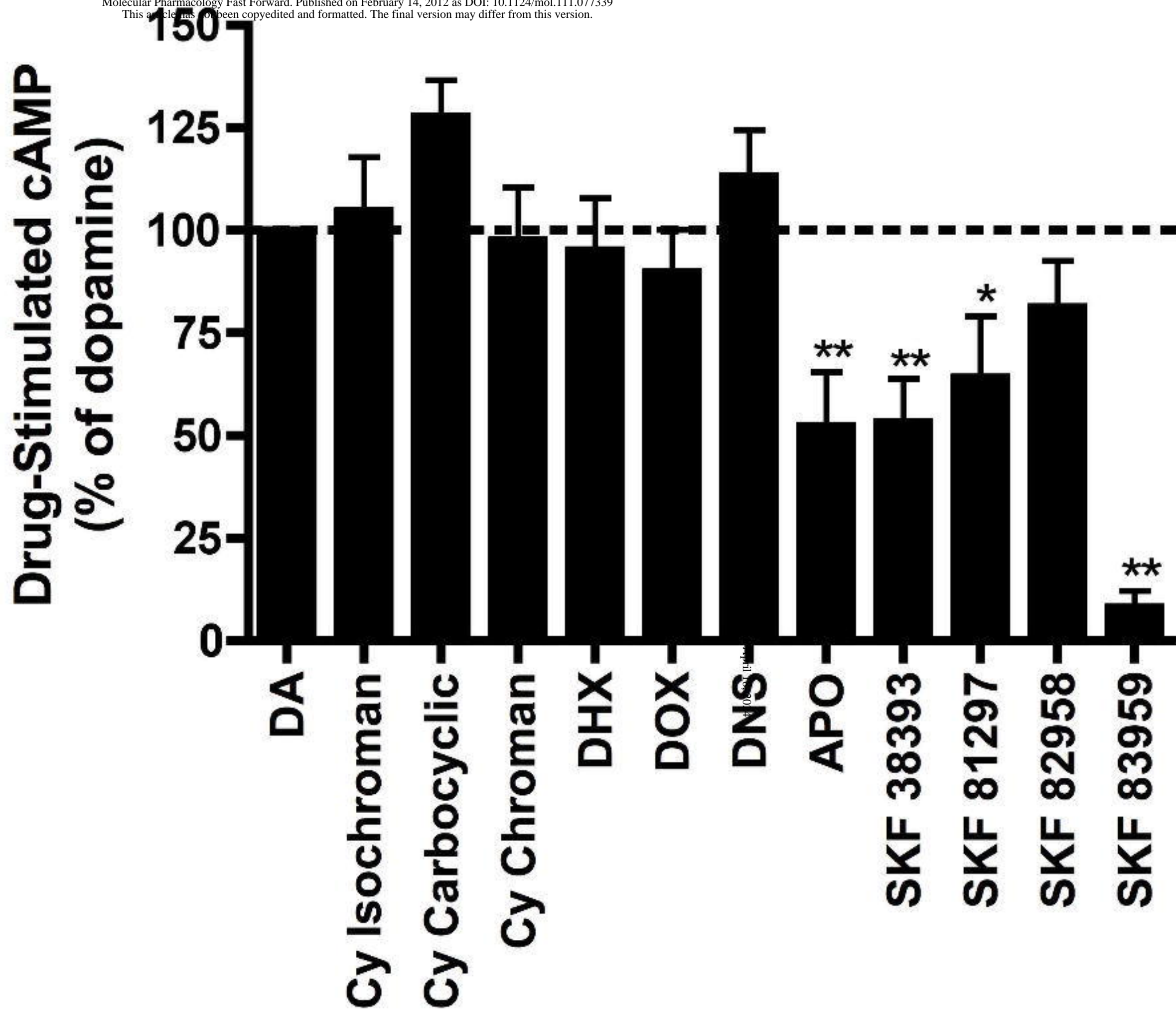


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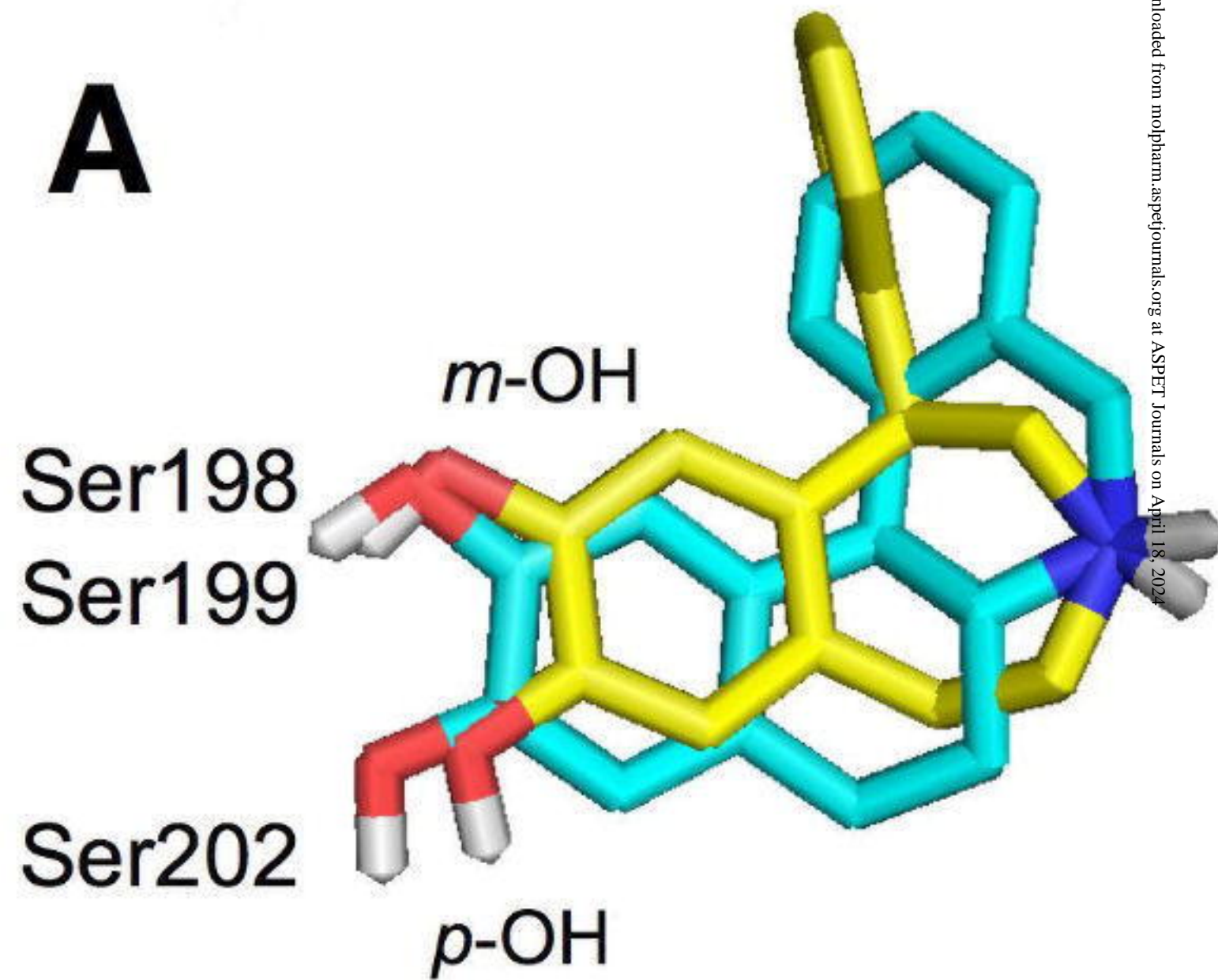
**Figure 5**

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**Figure 6**

**A**



**B**

