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Efficacy of hybrid tetrahydrobenzo[d]thiazole based aryl piperazines D-264 and D-301 at D₂ and D₃ receptors

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Abstract

In elucidating the role of pharmacodynamic efficacy at D₃ receptors in therapeutic effectiveness of dopamine receptor agonists, the influence of study system must be understood. Here two compounds with D₃ over D₂ selectivity developed in our earlier work, D-264 and D-301, are compared in dopamine receptor-mediated G-protein activation in striatal regions of wild-type and D₂ receptor knockout mice and in CHO cells expressing D₂ or D₃ receptors. In caudate-putamen of D₂ knockout mice, D-301 was ~ 3-fold more efficacious than D-264 in activating G-proteins as assessed by [³⁵S]GTP S binding; in nucleus accumbens, D-301 stimulated G-protein activation whereas D-264 did not. In contrast, the two ligands exerted similar efficacy in both regions of wild-type mice, suggesting both ligands activate D₂ receptors with similar efficacy. In D₂ and D₃ receptor-expressing CHO cells, D-264 and D-301 appeared to act in the [³⁵S]GTP S assay as full agonists because they produced maximal stimulation equal to dopamine. Competition for [³H]spiperone binding was then performed to determine K_i/EC₅₀ ratios as an index of receptor reserve for each ligand. Action of D-301, but not D-264, showed receptor reserve in D₃ but not in D₂ receptor-expressing cells, whereas dopamine showed receptor reserve in both cell lines. G_o1 is highly expressed in brain and is important in D₂-like receptor-G protein coupling. Transfection of G_o1 in D₃- but not D₂-expressing CHO cells led to receptor reserve for D-264 without altering receptor expression levels. D-301 and dopamine exhibited receptor reserve in D₃-expressing cells

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Compliance with Ethical Standards

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both with and without transfection of G_{o1} . Altogether, these results indicate that D-301 has greater intrinsic efficacy to activate D_3 receptors than D-264, whereas the two compounds act on D_2 receptors with similar intrinsic efficacy. These findings also suggest caution in interpreting E_{max} values from functional assays in receptor-transfected cell models without accounting for receptor reserve.

Keywords

dopamine receptors; D_2 ; D_3 ; intrinsic efficacy; G Protein; rat tissue; [^{35}S]GTP S binding assay; G_{o1}

Introduction

The D_2 -like family of dopamine receptors is a major drug target for treatment of motor and neuropsychiatric disorders [1,2]. The D_3 member of this family in particular, is associated with regulation of motivation/reward, mood, cognition and motor function, and is expressed throughout the limbic forebrain and basal ganglia, albeit at lower levels than D_2 receptors [3-5]. D_3 receptors are promising targets for treatment of drug abuse, schizophrenia, attention deficit hyperactivity disorders and motor diseases such as Parkinson's disease [1,6-8]. Despite the promise of therapeutic targeting of this receptor, development of ligands with high selectivity for D_3 over other D_2 -like receptors and which possess a range of intrinsic efficacies, has been challenging. In order to elucidate the role of pharmacodynamic efficacy at D_3 receptors in therapeutic effectiveness, the influence of study system, such as cell lines heterologously expressing the receptor versus native systems (e.g., striatum), must be understood.

D_3 receptors are coupled to the activation of mainly $G_{i/o}$ family G proteins [9,10]. G protein activation by receptors promotes exchange of GDP for GTP, which can be quantified in the GTP ^{35}S binding assay [11,9]. It is well known that G protein-coupled receptor (GPCR) ligands can display partial agonism in this assay with brain tissue when the receptor number is low compared with available G proteins, whereas the same ligands can become full agonists in cells overexpressing GPCRs such that the receptor number is high compared with available G protein [11-17]. Even though most high efficacy agonists maximally stimulate GTP ^{35}S binding in these cell lines, their intrinsic efficacy may vary as shown by the varying levels of receptor reserve depending on the activating ligand [12,17].

The work described here examined the intrinsic efficacy of two synthetic agonists at D_2 and D_3 dopamine receptors. These compounds are two related hybrid tetrahydrobenzo[d]thiazole based arylpiperazines developed in our earlier work [18,19]: D-264 and D-301. Both compounds contain the tetrahydrobenzo[d]thiazole moiety, connected through a (propyl substituted)N-ethyl linker with a piperazine biphenyl moiety (D-264) or a piperazine isoquinolin moiety (D-301) (Fig. 1). The tetrahydrobenzo[d]thiazole portion is thought to bind to the primary binding pocket of the dopamine receptor, whereas the aryl piperazine moiety likely binds an accessory binding site(s) [20]. Both compounds appeared to be full agonists relative to DA. We chose D-264 as one of the compounds for study since it not only displayed preferential D_3 potency but also exhibited efficacious in vivo activity including

potent neuroprotection [19,21]. D-301 was selected because the compound exhibited high potency and very high selectivity for D₃ receptors [18]. Another reason for selecting these two compounds is their structural diversity in the substitution of piperazine ring of the compounds such that D-301 has an isoquinoline moiety as opposed to presence of biphenyl substitution in the case of D-264. This might potentially impact interaction at the accessory binding sites of the receptors which might lead to production of differential receptors activation.

Two approaches were taken in this work to study potential differences in intrinsic efficacy of D-264 and D-301. First, the ability of the compounds to stimulate GTP ³⁵S binding was determined in assays with brain tissue from either wild-type (WT) or D₂ dopamine receptor knock-out (KO) mice [22]. In WT mice, both D₂ and D₃ receptors can contribute to G protein activation, whereas in D₂ KO mice, the D₂ component has been removed. In this scenario, a difference in maximal activation between the two compounds suggests a difference in intrinsic efficacy at the level of D₃ receptor-mediated G protein activation. In a second approach, cell lines expressing only D₂ or D₃ receptors were used to determine intrinsic efficacy by assessing ligand-stimulated GTP ³⁵S binding and competition for [³H]spiperone binding under identical conditions. In this system, the ratio of binding K_i to G protein activation EC₅₀ values, combined with maximal activation values (E_{max}), was used to evaluate the intrinsic efficacy of ligands [12,23]. Because of the reportedly greater efficiency of D₃ receptor coupling to G_o relative to G_i [24] and the fact that G_o is highly expressed in the brain [25], experiments were conducted in dopamine receptor-expressing cells both in the absence and presence of transiently expressed G_{o1}. The results from the two approaches combined indicate that D-301 has a greater intrinsic efficacy at D₃ receptors than D-264, whereas the two compounds acted on D₂ receptors with similar intrinsic efficacy.

Materials and Methods

Materials and animals

[³H]spiperone (15 Ci/mmol) and [³⁵S]GTP S (1250 Ci/mmol) were purchased from PerkinElmer (Waltham, Massachusetts). Compounds D-264 and D301 were synthesized by us as described previously ([18], in which compound 24c is D-301, and [19], in which compound (–)-34 is D-264). Plasmid DNA (pcDNA3.1) expressing human G_{o1} was obtained from the cDNA Resource Center (www.cdna.org). Other chemicals were obtained from commercial sources such as Fisher (Pittsburg, PA, USA) or Sigma (St. Louis, MO, USA). Female D₂R WT and KO mice on a C57/Bl6 background were bred at the Oregon Health and Science University.

Cell culture and transient transfection of G_o—CHO cells stably expressing D₂ or D₃ human DA receptors (CHO-hD₂ and CHO-hD₃ cells) were the same as used in our previous work [26,27]. CHO-hD₂ was cultured in Dulbecco's Modified Eagle Medium/nutrient mixture F12 and CHO-hD₃ in Dulbecco's Modified Eagle Medium, in both cases supplemented with 10% bovine calf serum, and 2 mM glutamine, at 37°C and 5% CO₂. Geneticin (200 µg/mL G418) was always present in order to maintain selection pressure.

Where indicated in the text, transient transfection with G_{o1} was achieved in both cell lines with Lipofectamine[®] 2000 (Qiagen, Germantown, MD, USA). In order to assess expression of G_{o1}, 36 hr after transfection cells were scraped and resuspended in NP40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton, pH 8.5, containing protease inhibitor cocktail [Sigma-Aldrich] and incubated on ice for a total of 30 min with 10-sec vortexing every 10 min. After centrifugation at 20,000 g for 15 min at 4°C, the supernatant was collected and protein concentration was determined by a Bio-Rad DC protein assay kit. For western blot analysis, equal amounts of protein (30–60 µg per lane) were loaded on 10% Tris-glycine polyacrylamide gel. Membranes were probed with rabbit anti-G_{o1} antibody (GNAO1, Proteintech Group, Chicago, IL) and rabbit anti-β-actin (Sigma-Aldrich). The target protein bands were visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Buckinghamshire, England) and quantified using Image-J (downloaded from website of National Institute of Health). The integrated density value (IDV) for the protein G_{o1} was normalized with that for loading control β-actin. In each experiment the ratio of normalized IDV for G_{o1} protein with transient transfection over that without transient transfection was calculated.

Cell-free membrane preparation—Confluent cells were gently washed with ice-cold Dulbecco's Phosphate-Buffered Saline (DPBS) (without calcium and magnesium salts) and then lysed with 3 mL ice-cold lysis buffer (50 mM Tris-HCl and 1 mM EDTA, pH = 7.4). Cell lysate was transferred to a Beckman polycarbonate thick-wall centrifuge tube and centrifuged at 35,000 g for 15 minutes at 4°C. The resultant pellet was homogenized with a Brinkmann Polytron (Brinkmann Instruments, Westbury, NY) at setting 6 for 15 seconds in the above lysis buffer and centrifuged again at 35,000 g for 15 minutes at 4°C. The final pellet containing crude cell-free membranes was suspended in the following assay buffer, for CHO-hD₂: 20 mM HEPES (pH = 7.4), 10 mM MgCl₂, 150 mM NaCl, 0.2 mM EGTA, 0.001% BSA, 3 µM GDP; and for CHO-hD₃: 20 mM HEPES (pH = 7.4), 3 mM MgCl₂, 100 mM NaCl, 0.2 mM EGTA, 0.001% BSA, 6 µM GDP. The resuspended membrane preparations were kept in ice.

For mouse tissues, mice were euthanized at Oregon Health and Science University, and brains were frozen at –30°C in 2-methylbutane as previously described. Brains were shipped on dry ice by overnight express to Virginia Commonwealth University, where they were stored at –80°C until use. On the day of each assay, one brain each from D₂ KO and wild-type mice were thawed, and the caudate-putamen (dorsal striatum) and nucleus accumbens (ventral striatum) were dissected on ice. The tissue was homogenized in 50 mM Tris-HCl (pH 7.4), 3 mM MgCl₂ and 1 mM EGTA, pH 7.4, and centrifuged at 48,000 × g for 10 min. The supernatant was discarded, and the pellet resuspended in 50 mM Tris-HCl (pH 7.4), 3 mM MgCl₂, 0.1 mM EGTA and 100 mM NaCl, the volume was normalized to obtain equal protein concentrations between samples, and membranes were pre-incubated for 10 min at 30°C with 30 mU/ml adenosine deaminase to inactivate endogenous adenosine prior to assay.

[³⁵S]GTPγS binding to D₂ and D₃ DA receptors in CHO cells—Because GDP is sensitive to temperature and light, GDP was only retrieved and added to the cell suspension

(3 μ M unlabeled GDP for CHO-D₂ and 6 μ M for CHO-D₃ in the assay) just before initiating the binding experiment. Different concentrations of test compounds were first pre-incubated with cell suspension for 15 minutes at 30°C. After addition of [³⁵S]GTP S at 0.1 nM (final concentration), the binding assay was continued for another 45 minutes at 30°C. Nonspecific binding was defined with 10 μ M unlabeled GTP S. Assays were terminated by filtration with ice-cold lysis buffer through a glass fiber filtermat B in the Brandel-96 cell harvester. The filtermat was dried under hot air and, after addition of 10 ml of Betaplate Scint (PerkinElmer), counted for radioactivity in a Microbeta liquid scintillation counter (PerkinElmer).

[³⁵S]GTP S binding in mouse striatum—Membranes were prepared from dissected caudate-putamen (dorsal striatum) and nucleus accumbens (ventral striatum), and ligand-stimulated [³⁵S]GTP S binding was conducted essentially as described[28]. Briefly, membranes (3-4 μ g protein) was incubated with 30 μ M GDP, 0.1 nM [³⁵S]GTP S and varying ligand concentrations in 50 mM Tris-HCl (pH 7.4), 3 mM MgCl₂, 0.1 mM EGTA, 100 mM NaCl and 3 mU/ml adenosine deaminase, 0.5 ml final volume, for 90 min at 30°C. The incubation was terminated by rapid filtration through GF/B glass fiber filters and washed three times with ice-cold 50 mM Tris-HCl (pH 7.4). Bound radioactivity was determined by liquid scintillation spectrophotometry after overnight extraction of the filters in scintillation fluid. Non-specific binding was determined in the presence of 20 μ M unlabeled GTP S, and basal binding was determined in the absence of agonist. All assays were performed in triplicate, and data were analyzed as specific binding.

[³H]spiperone binding to D₂ and D₃ DA receptors in CHO cells—In order to have conditions identical to those used in the above functional [³⁵S]GTP S assay, we had the same final concentrations of GDP, 3 μ M for D₂ and 6 μ M for D₃, and 0.1 nM GTP S, present in the [³H]spiperone binding assays. Various concentrations of test compounds, cell-free membranes, and [³H]spiperone (2 nM final) were incubated together for 1 h at 30°C in the assay buffer. Two μ M (+)-butaclamol was used to define nonspecific binding. Assays were terminated by filtration with ice-cold 0.9% saline through a glass fiber filtermat B in the Brandel-96 cell harvester. Avoiding ligand depletion is especially critical for ultra-high affinity ligands such as [³H]spiperone in the case of D_{2/3} receptors. Thus, receptor concentration should be lower than the K_d of radioligand or K_i of unlabeled compound; otherwise the affinity of ligand binding to receptors would be under-estimated [29,30]. Here we use the optimized protocol of [³H]spiperone binding to DA receptors, consisting of raising the concentration of [³H]spiperone (2 nM in the current study) instead of the assay volume [30], allowing the determination of the affinity of test ligand as low as 0.01 nM.

D₄ DA receptor affinity—Broad-spectrum *in vitro* receptor binding data for analogue D-264 were kindly provided by Dr. Bryan L. Roth (University of North Carolina at Chapel Hill, NC) as part of the National Institute of Mental Health's (NIMH) Psychoactive Drug Screening Program (PDSP), and these data included the binding affinity of D-264 for D₄ DA receptors. In brief, the PDSP assesses binding of novel compounds at a wide array of cloned human G-protein coupled receptors, membrane transporters and other proteins known to be psychoactive drug targets. For detailed description of the PDSP experimental protocols,

please refer to the PDSP Web site at <http://pdsp.med.unc.edu/> and select the “Binding Assay” link.

Data calculations and statistical analysis—[³⁵S]GTP S data were transformed to % stimulation (where net stimulated [³⁵S]GTP S binding (fmol/mg) = ligand-stimulated – basal [³⁵S]GTP S binding (fmol/mg) and basal [³⁵S]GTP S binding was measured in the absence of agonist. Results from the mouse striatum experiments were analyzed with GraphPad Prism and fit to the 3-parameter logistic equation: $E = (E_{\max} / (1 + 10^{[(\log EC_{50} - \log L) \times n_H]}))$, where $[\log L]$ = the log10 of the ligand concentration and n_H = the Hill coefficient (slope). IC₅₀ values in [³H]spiperone binding were fit to the one-site competitive binding model (logistic equation) with the computer program Origin (Microcal Software, Northampton, MA, USA) and converted to inhibition constants (K_i) by the Cheng–Prusoff equation [31]. The logistic equation in Origin was also used for fitting the activation of [³⁵S]GTP S binding to CHO cells by dopamine, and is the same as the approach used above. The intrinsic efficacy was calculated from relative $E_{\max}$ ($E_{\max}$ of test ligand/ $E_{\max}$ of full agonist, i.e., dopamine in the current study) and EC₅₀ obtained from concentration-effect curves in the [³⁵S]GTP S binding assay, and K_i obtained in the [³H]spiperone binding assay, as follows:

$$\text{Intrinsic Efficacy} = \left(\frac{E_{\max, \text{drug}}}{E_{\max, \text{dopamine}}} \right) \times \left(\frac{K_i}{EC_{50}} + 1 \right) \times \frac{1}{2}$$

[12,23].

The error in the K_i/EC_{50} ratio was computed from the propagation of the errors in the K_i and EC₅₀ ([32], equation 1a therein); likewise these errors along with that in $E_{\max, \text{drug}}$ were propagated for calculating the error in intrinsic efficacy. Statistical tests involving values for the K_i/EC_{50} ratio and intrinsic efficacy were performed by inputting the mean, SEM, and smallest group size (conservative approach) in the given data set, into the InStat program which is part of the Graphpad software; the smallest n varied between 3 and 7 depending on the data set. The accepted level of significance was 0.05. Tests were two-tailed except in the case of determining whether the K_i/EC_{50} ratio or intrinsic efficacy was different from unity, because receptor reserve would drive such values only in the direction of > 1 (i.e., representing a one-tailed case).

Results

D₂ KO mice

To determine the contribution of D₃ receptors to agonist-stimulated G-protein activation in mouse striatum by D-264 and D-301, stimulation of [³⁵S]GTP S binding was examined in membranes prepared from caudate-putamen (Figure 2A, C) and nucleus accumbens (Figure 1B, D) of D₂ receptor KO compared to WT mice. The concentration-effect curves were analyzed by non-linear regression to generate maximal stimulation ($E_{\max}$) and EC₅₀ values, as well as Hill coefficients (Table 1). Both agonists produced similar maximal stimulation of [³⁵S]GTP S binding in caudate-putamen (58-64% stimulation) and nucleus accumbens

(31-41% stimulation) of WT mice (Table 1). In D₂ KO mice, however, the D-301 E_{max} value was significantly greater than that of D-264 (approximately 17% versus 6% stimulation, respectively) in caudate-putamen. In nucleus accumbens, only D-301 significantly stimulated [³⁵S]GTP S binding in D₂ KO mice (E_{max} = 11.5%), whereas D-264 did not stimulate [³⁵S]GTP S binding above basal levels. The EC₅₀ values of D-301 were significantly greater than those of D-264 in both regions of WT mice, although the EC₅₀ value of D-301 was not significantly different from that of D-264 in the caudate-putamen of D₂ KO mice. These results suggest that despite its generally lower potency, D-301 activates D₃ receptors more efficaciously than D-264. In agreement with this interpretation, the Hill coefficients of the D-301 concentration-effect curves were significantly less than unity in both regions of WT mice, whereas those of D-264 were not, suggesting that two different binding sites (presumably D₂ and D₃ receptors) were contributing to the stimulation by this ligand. Nonetheless, Hill coefficients for both D-264 and D-301 were significantly greater in the caudate-putamen of D₂ KO compared to WT mice, suggesting that D₃ receptors also contribute slightly to the stimulation by D-264 in this region. Moreover, the Hill coefficients of D-301 in both regions of D₂ KO mice were not significantly lower than unity, in accordance with the response being purely D₃-mediated. Altogether, these results suggest that in WT mice, D₃ receptor-mediated G-protein activation had a greater influence on the concentration-effect curves of the higher efficacy D₃ ligand, D-301, than on those of D-264, which appeared to be a lower efficacy ligand at D₃ receptors.

Cells heterologously expressing D₂ or D₃ receptors

In the GTP ³⁵S binding assay, D-264 and D-301 appeared to act as full agonists at D₂ and D₃ receptors expressed in CHO-cells with relative E_{max} values close to 100% of the maximal activation by DA (top half of Tables 2a and 2b). As observed previously [18,19,27], both compounds were D₃-selective, with EC₅₀ values more than an order of magnitude lower at D₃ than D₂ receptors (please note that in above [18] D-301 = compound 24c). Such selectivity was also observed for the competition of these compounds for [³H]spiperone binding (see K_i in top half of Tables 2a and 2b), as observed previously under somewhat different assay conditions [18,27]. Hill coefficients for binding and concentration-effect curves with all three agonists, in cells expressing either D₂ or D₃ receptors, were generally not different from unity (not shown). The one exception was the D-264 competition-binding curve in D₂ receptor-expressing cells, which had a Hill coefficient of 0.66 ± 0.12.

Because the conditions for the GTP ³⁵S and [³H]spiperone binding assays were identical in the current experiments, the ratio K_i/EC₅₀ can be used as an estimate of receptor reserve, with ratios > 1 indicating receptor reserve [12,23]. The K_i/EC₅₀ ratio is proportional to intrinsic efficacy when E_{max} of a ligand / E_{max} full agonist is ~ 1, as seen in formula (1) (Methods). For the agonists D-264 and D-301 studied here, only D-301 showed a K_i/EC₅₀ ratio statistically greater than unity (as did DA itself) at D₃ but not at D₂ receptors, with intrinsic efficacies statistically greater than unity in the same cases (top halves of Tables 2a and 2b).

Because G_o is highly expressed in the brain [25], but not CHO cells [33], and previous reports emphasize the importance of G_o in D_3 receptor-G-protein coupling [9,34-37], G_{o1} was transiently co-expressed in CHO cells expressing either D_2 or D_3 . Although our CHO cell lines were found to express endogenous G_{o1} , detectable with GNAO1 antibody (Fig. 3 bottom panel), transient transfection with exogenous G_{o1} approximately doubled the total G_{o1} expression (Fig. 3 top panel). The presence of exogenous G_{o1} did not affect the observed K_D or B_{max} values of [3H]spiperone binding (Table 3). In the case of D_2 , exogenous G_{o1} affected some values of functional and binding parameters, but this did not result in significant changes in intrinsic efficacy as compared with the absence of exogenous G_o (Table 2a). Although the intrinsic efficacy values for both D-264 and D-301 with G_{o1} appeared slightly elevated above unity (~1.4-fold), there was no statistically significant elevation over unity at D_2 . In contrast, the changes observed at D_3 with G_o transfection led to an intrinsic efficacy significantly higher than unity for D-301 (2.48) but not D-264 (1.53) (Table 2b). Dopamine itself had a higher than unity intrinsic activity both in the absence and presence of exogenous G_{o1} at D_3 . Hill coefficients (not shown) were not different from unity in any of the binding or concentration-effect curves with all three ligands in either D_2 or D_3 receptor-expressing cells after co-transfection of G_o .

Discussion

The present study revealed intrinsic efficacy differences for D_3 receptor-mediated G protein activation by two synthetic D_3 -selective ligands, D-264 and D-301, that were both previously reported to be full agonists relative to the endogenous ligand dopamine in cell lines heterologously expressing D_2 and D_3 receptors [18,19]. Results in D_2 KO mice revealed differences in E_{max} values of ligand-stimulated [^{35}S]GTP S binding in caudate-putamen, such that D-301 was nearly three-fold more efficacious to activate G proteins in this region than D-264. In nucleus accumbens of D_2 KO mice, D-301 produced significant G protein activation with an E_{max} value that was similar to the maximal stimulation by this ligand in caudate-putamen, whereas no significant stimulation of [^{35}S]GTP S binding was detected with D-264 in nucleus accumbens. In contrast, E_{max} values of the two ligands did not differ significantly in either region of WT mice. These results indicate that D-301 possesses greater intrinsic efficacy to activate D_3 receptors than D-264 in mouse striatum, whereas the two ligands have similar efficacy to activate D_2 receptors. In agreement, Hill coefficients of the D-301 concentration-effect curves were statistically less than unity in both striatal regions of WT but not D_2 KO mice, suggesting the contribution of both D_2 and D_3 receptors to the overall D_2 -like receptor-mediated G protein signal with this ligand. In contrast, Hill coefficients for D-264 were not statistically different from unity in either region, suggesting that G protein activation by this ligand was mediated predominantly by a single receptor type, presumably the D_2 receptor. In the case of D-264 in caudate-putamen, the contribution of the D_3 receptor was apparently too low to reduce the Hill number significantly below unity.

The finding that D-264 produced small but significant stimulation of [^{35}S]GTP S binding in caudate-putamen but not in nucleus accumbens was somewhat unexpected, because D_3 receptor levels are reportedly greater in the latter region in rats [4]. It is possible that our dissections also included portions of the Islands of Calleja, where D_3 receptors are also

highly expressed [4]. Moreover, autoradiographic studies in post-mortem human brain also showed relatively high D₃ receptor levels in the more ventral aspects of both the caudate and putamen [5]. It is also possible that D₃ receptors are more efficiently coupled to G protein activation in the caudate-putamen than nucleus accumbens, despite their lower expression level in the former region. Finally, it is possible that G_{i/o} protein-coupled D₄ receptors contributed to the non-D₂ mediated [³⁵S]GTP S signal in both caudate-putamen and nucleus accumbens, as D₄ receptor expression has been reported in the range of 20-25% of that of D₂ receptors in these striatal regions, with even distribution between caudate-putamen and nucleus accumbens [38,39]. D-264 has a K_i value of 49 nM at D₄ receptors (National Institute of Mental Health's Psychoactive Drug Screening Program [PDSP], data not shown), well within the concentration range examined, but D264 had very minimal effect on G-protein activity in the striatum of D₂R KO mice. D4 affinity of D-301 is unknown, however, we have observed no significant effect of the D₄-selective antagonist LY745870 on D-301-stimulated [³⁵S]GTP S binding in ICR mouse striatum at antagonist concentrations up to 1 μM (J.R. Secor McVoy and D.E. Selley, unpublished data), which is approximately 1,000 times greater than its K_i value at D₄ receptors [40]. Future studies could be conducted to determine the relative efficacy of these ligands to stimulate D₃ receptor-mediated G protein activation in striatal regions of D₂ and D₄ double KO mice.

Our findings with D_{2/3} receptor-mediated G protein activity in mouse striatum have implications for the exclusive use of heterologously transfected cell lines to determine dopaminergic ligand efficacy, because both D-301 and D-264 produced similar E_{max} values that were not different from DA in CHO cells expressing either D₂ or D₃ receptors. Similar results have been obtained with several mu opiate ligands that appeared to be full agonists in cell lines heterologously expressing the mu opioid receptor but partial agonists in rat thalamus, based on E_{max} values of ligand-stimulated [³⁵S]GTP S binding [11,12,14,41]. However, heterologous receptor expression is generally driven by efficient viral promoters and is therefore often higher than in native tissues. The relationship between receptor expression levels and ligand efficacy determinations are well established; in the mu opioid receptor example we previously observed that MOR expression levels were approximately five-fold greater in transfected CHO cells than in rat thalamus [11,12]. In the current study, D₂ and D₃ receptor B_{max} values were approximately 2 to 4-fold and 135-fold, respectively, greater than those reported in rodent striatum [22,42,43]. Although receptor B_{max} comparisons between brain and cell lines must be viewed with caution because presumably not every cell type in a dissected brain region expresses the receptor of interest, it seems likely that these receptors, especially D₃, are over-expressed in our CHO cell models relative to naturally occurring levels in the striatum. This is particularly true when taking into account the ratio of receptors to activated G_{i/o} proteins, because activated G_{i/o} protein levels tend to be much greater in the brain [11]. One should, however, keep in mind that compensatory changes in dopamine receptor expression – especially ‘D2 high’ receptor expression- are well documented in dopamine receptor knockout animals [44]. All considerations taken together, one expects receptor reserve for G protein activation by high efficacy ligands to be more likely present in transfected cell models. Therefore, we determined the intrinsic efficacy of ligand-stimulated G protein activation by determining receptor binding K_i to G protein activation EC₅₀ values [12,23], which revealed significantly

greater intrinsic efficacy of DA than either D-264 or D-301 in cells expressing either D₂ or D₃ receptors. Strikingly, the intrinsic efficacy of the synthetic ligands relative to DA was only approximately 23-28% at D₂ and 6.4% (D-264) to 10.5% (D-301) at D₃ receptors. Accordingly, DA produced K_i/EC₅₀ ratios greater than unity in both D₂ and D₃ receptor-expressing cells, indicative of receptor reserve, whereas only D-301 in D₃ receptor-expressing cells and neither synthetic ligand in D₂ receptor-expressing cells had a K_i/EC₅₀ ratio significantly greater than unity. Moreover, the intrinsic efficacies of D-264 and D-301 were approximately equal at D₂ receptors, whereas that of D-301 was 1.65-fold greater than D-264 at D₃ receptors. Although these results are in general agreement with those obtained in mouse striatum, the efficacy of D-301 relative to D-264 for D₃ receptor-mediated G-protein activation seemed to be lower in the cell model compared to striatum.

Another major difference between the CHO cell models and mouse striatum, besides D₃ receptor expression levels, is the high expression of G_{o1} in striatum [25] compared to its reported absence in CHO cells [33]. We therefore examined the relative intrinsic efficacies of D-264 and D-301 in the same cell lines with co-expression of G_{o1}. Unexpectedly, endogenous G_{o1} protein was detectable in our CHO cell lines, and transfection with exogenous G_{o1} only led to an approximate doubling of G_{o1} (Fig. 3). It is therefore not surprising that in most cases the effects of transfected exogenous G_{o1} were quite moderate, as were the observed DA-induced increases in GTPγS binding (exogenous G_{o1} increased the % baseline response to standard, maximally effective concentration of DA from 223% to 244% in D₂ cells and from 134% to 160% in D₃ cells as measured for each receptor subtype in two independent transfection experiments assayed in sextuplicate). Expression of exogenous G_{o1} did not affect receptor expression levels of either receptor type, and minimally affected the intrinsic efficacy of DA at D₂ receptors. Perhaps surprisingly, the intrinsic efficacy of DA at D₃ receptors was significantly reduced by exogenous G_{o1} expression, and this reduction appeared to mainly result from a significant decrease in binding K_i value, suggesting that DA might form more stable high affinity D₃ receptor complexes with G_{o1} than with the G_i isoforms present in CHO cells. However, the lack of a corresponding decrease in EC₅₀ value suggests that this enhanced high affinity complex did not result in increased efficiency to induce guanine nucleotide exchange.

Studies suggest that both D₂ and D₃ receptors are more efficiently coupled to G_o than G_{i2} or 3 [24,45], which are the major G_i isoforms in CHO cells[46]. Moreover, differential activation of G_o versus G_i isoforms by D₂-like receptors can also be ligand-dependent [47,48]. In the present study, the intrinsic efficacies of both D-264 and D-301 were not significantly affected by exogenous G_{o1} expression in D₂-expressing cells. Exogenous G_{o1}-expression appeared to enhance the intrinsic efficacy of D-264 and D-301 (by ~50%) in D₃-expressing cells, such that D-264 gained receptor reserve indicated by a K_i/EC₅₀ value greater than unity. However, there was not a statistically significant increase in the calculated intrinsic efficacy of either ligand and only D-301 exhibited an intrinsic efficacy value greater than unity. Nonetheless, only in the presence of exogenous G_{o1} did the intrinsic efficacy of D-301 at D₃ receptors approach that of DA, whereas this ligand retained very low intrinsic efficacy in D₂-expressing cells regardless of the presence or absence of exogenous G_{o1}. Examination of the receptor-binding data suggests that the major explanation for this convergence of intrinsic efficacy values between D-301 and DA was

that the binding K_i values were differentially affected by exogenous G_{o1} expression. The D-301 affinity decreased in exogenous G_{o1} expressing cells whereas the DA affinity increased, and the EC_{50} values of both ligands were unaffected. Although the potency of D-264 to activate G-proteins was significantly increased by exogenous G_{o1} expression in D_3 -expressing cells, the E_{max} value was moderately decreased so there was no significant effect of exogenous G_{o1} expression on intrinsic efficacy of this ligand. Thus, the major effect of exogenous G_{o1} expression in D_3 -expressing cells was enhancement of the binding affinity of DA but not of the synthetic ligands, such that the intrinsic efficacy values became more similar between DA and the synthetic ligands (especially D-301) in exogenous G_{o1} -expressing cells.

While the precise mechanisms underlying the relative intrinsic efficacy differences between D-301 and D-264 at D_3 receptors remains to be elucidated, one relevant conclusion from these CHO cell studies is that exogenous G_{o1} expression did not affect the ratio of intrinsic efficacies of D-301:D-264 at D_3 receptors, which was approximately 1.65 regardless of exogenous G_{o1} expression. Thus, the major difference between the D-301:D-264 efficacy ratio in D_3 -CHO cells compared to mouse striatum appears to be D_3 receptor expression level, which could be addressed in future studies by progressively reducing D_3 receptor expression in the CHO cell model using an irreversible antagonist. This treatment would be expected to disproportionately suppress the efficacy of D-264 relative to that of D-301. It is known that decreases in receptor expression levels disproportionately suppress the relative efficacy of low compared to high efficacy ligands, as has been seen with mu opioid [12] and other GPCRs [49-51]. Nonetheless, because results in both the striatum of D_3 KO mice and transfected CHO cells (when taking into account the intrinsic efficacy calculation) indicated that D301 is more efficacious than D264 to activate G-proteins via D_3 receptors, we can therefore reject the null hypothesis that D301 and D264 are equally efficacious as D_3 agonists.

Considering the important role of receptor expression density in the determination of ligand efficacy, it is interesting that maximal stimulation of [35 S]GTP S binding in absolute terms was similar between cells expressing D_2 and D_3 receptors with B_{max} values of <1 and >7 pmol/mg, respectively. This discrepancy between receptor density and G protein activation magnitude probably derives from the low efficiency of D_3 receptor-G-protein coupling, as previously reported [9,34,52]. The low efficiency of D_3 -mediated G protein activation could be related to its reportedly rigid conformation, which also imparts lesser sensitivity of agonist binding affinity to guanine nucleotides [53-55]. Interestingly, despite the reported low efficiency of D_3 receptor-mediated activation of $G_{i/o}$ proteins, this receptor seems to be more promiscuously coupled to activation of multiple G families, including G_s [56,57] and G_q [9], relative to other D_2 -like receptor family members. It will be of interest in future studies to determine whether the efficacy relationship between D301 and D264 is similar for activation of these other G types.

In conclusion, the present study revealed differential intrinsic efficacies for D_3 receptor-mediated G protein activation between two chemically similar synthetic ligands, with D-301 showing greater efficacy than D-264. In contrast, these two ligands showed similar efficacy to activate D_2 receptors. This conclusion is supported by data showing greater E_{max} values

of D-301 than D-264 in striatal regions from D₂ KO mice, and greater intrinsic efficacy of D-301 than D-264 in D₃-expressing CHO cells based on a calculation that accounts for receptor reserve in this model. The higher intrinsic efficacy of D-301 relative to D-264 was not significantly altered by co-expression of exogenous G_{o1}, suggesting that higher D₃ receptor expression and not differential expression of G subtypes is the predominant factor underlying the differential relative E_{max} values for G protein activation between the CHO cell model and mouse striatum. These findings suggest caution in interpretation of ligand efficacy determinations from a single model system, particularly when potential receptor reserve for the response is not directly determined.

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Fig. 1.
Chemical structures of D264 and D301.

Fig. 2.

Stimulation by D264 and D301 of [³⁵S]GTP γ S binding to membranes prepared from mouse -WT (■) and D₂R-KO (□) -caudate-putamen and Nucleus accumbens. Points are shown as means and standard error.

Fig. 3.

Expression of $G_{\alpha 1}$ protein after transient transfection with exogenous $G_{\alpha 1}$ of CHO cells stably expressing D₂ and D₃. Cell lysates of CHO-D₂ and CHO-D₃ with and without transient transfection were resolved on reducing 10% SDS-PAGE gels and subjected to western analysis. Data were averaged from 3 independent experiments and variation is shown as SEM. Increases upon transfection were 2.31 ± 0.32 -fold and 1.80 ± 0.23 -fold for D₂ and D₃ cells, respectively. **, $P < 0.005$, *, $P < 0.01$ (compared with unity, one sample Student's t-test).

Table 1

$E_{\max}$ and EC_{50} values from agonist-stimulated [35 S]GTP S binding in caudate-putamen (CPu) and nucleus accumbens (NAc) of D_2R WT and KO mice

	D-264			D-301		
CPu	$E_{\max}$ (%)	EC_{50} (nM)	n_H	$E_{\max}$ (%)	EC_{50} (nM)	n_H
D_2R WT	58.2 ± 4.6	11.9 ± 4.9	0.73 ± 0.12	63.6 ± 6.0	$39.1 \pm 9.9^{\$}$	$0.56 \pm 0.09^{\#}$
D_2R KO	$5.7 \pm 0.2^{**}$	3.8 ± 1.8	$1.23 \pm 0.14^*$	$16.8 \pm 1.4^{**\$}$	50.5 ± 19.9	$1.36 \pm 0.29^*$

NAc	$E_{\max}$ (%)	EC_{50} (nM)	n_H	$E_{\max}$ (%)	EC_{50} (nM)	n_H
D_2R WT	30.7 ± 5.1	39.4 ± 22.3	0.94 ± 0.12	41.0 ± 5.8	$369.5 \pm 157.6^{\$}$	$0.66 \pm 0.04^{\#}$
D_2R KO	N.D.	N.D.	N.D.	$11.5 \pm 2.8^*$	42.6 ± 25.8	0.85 ± 0.10

Data are mean values $\pm$ SEM (n = 3-4). N.D., not determined because there was no significant stimulation by this agonist.

* $p < 0.05$

** $p < 0.01$ different from the corresponding value in WT mice.

$\$$ $p < 0.05$ different from the D-264 value within the same genotype.

$\#$ $p < 0.05$ different from unity.

Table 2

Functional parameters ($E_{\max}$ and EC_{50}) obtained in [35 S] GTP S assay and binding parameter (K_i) in [3 H]spiperone assay in CHO-D₂ (a) and CHO-D₃ (b) without and with G_o present from transient transfection. Results are shown as mean $\pm$ S.E. averaged from 3-8 independent experiments. Ratios of K_i over EC_{50} and intrinsic efficacies were computed as described in Materials and Methods.

<i>Table 2a</i>					
CHO-D₂					
<i>Compound</i>	<i>E_{max} (% DA)</i>	<i>EC₅₀ (nM)</i>	<i>K_i (nM)</i>	<i>K_i/EC₅₀</i>	<i>Intr. Effic.</i>
<i>D-264</i>	104 $\pm$ 1.5	39.0 $\pm$ 4.6	44.7 $\pm$ 6.4	1.14 $\pm$ 0.21	1.11 $\pm$ 0.11
<i>D-301</i>	97.7 $\pm$ 1.6	62.6 $\pm$ 7.8	108 $\pm$ 13	1.73 $\pm$ 0.30	1.33 $\pm$ 0.15
<i>DA</i>	100	363 $\pm$ 32	3,074 $\pm$ 340	8.47 $\pm$ 1.20 ^{\$}	4.73 $\pm$ 0.60 ^{\$}
<i>CHO-D₂ with exogenous G_{o1} transient transfection</i>					
<i>D-264</i>	92.4 $\pm$ 9.8	56 $\pm$ 14.5	125 $\pm$ 27.6 [*]	2.23 $\pm$ 0.76	1.49 $\pm$ 0.39
<i>D-301</i>	101 $\pm$ 6	31.9 $\pm$ 3.6 [*]	52.9 $\pm$ 11.8 [*]	1.66 $\pm$ 0.41	1.34 $\pm$ 0.22
<i>DA</i>	100	199 $\pm$ 53	1922 $\pm$ 439	9.66 $\pm$ 3.39	5.33 $\pm$ 1.69

<i>Table 2b</i>					
CHO-D₃					
<i>Compound</i>	<i>E_{max} (% DA)</i>	<i>EC₅₀ (nM)</i>	<i>K_i (nM)</i>	<i>K_i/EC₅₀</i>	<i>Intr. Effic.</i>
<i>D-264</i>	93.0 $\pm$ 4.2	2.34 $\pm$ 0.34	2.82 $\pm$ 0.62	1.21 $\pm$ 0.32	1.03 $\pm$ 0.15
<i>D-301</i>	107 $\pm$ 4.4	1.01 $\pm$ 0.23	2.19 $\pm$ 0.33	2.17 $\pm$ 0.59 ^{\$}	1.70 $\pm$ 0.32 ^{\$}
<i>DA</i>	100	7.51 $\pm$ 0.29	235 $\pm$ 20	31.3 $\pm$ 2.9 ^{\$}	16.2 $\pm$ 1.5 ^{\$}
<i>CHO-D₃ with exogenous G_{o1} transient transfection</i>					
<i>D-264</i>	75.7 $\pm$ 5.83 [*]	1.31 $\pm$ 0.08 [*]	4.00 $\pm$ 0.61	3.05 $\pm$ 0.50 ^{*\$}	1.53 $\pm$ 0.22
<i>D-301</i>	102 $\pm$ 2.3	1.22 $\pm$ 0.11	4.72 $\pm$ 0.77 [*]	3.87 $\pm$ 0.72 ^{\$}	2.48 $\pm$ 0.37 ^{\$}
<i>DA</i>	100	11.6 $\pm$ 1.78	70.4 $\pm$ 3.41 [*]	6.07 $\pm$ 0.98 ^{*\$}	3.53 $\pm$ 0.49 ^{*\$}

^{*} P<0.05 compared with absence of G_{o1} in same cell line (Student's t-test);

^{\$} P<0.05 compared with unity (one-sample Student's t-test).;

Table 3

Saturation analysis of [³H]spiperone binding (K_d and B_{max}) in CHO-D₂/CHO-D₃ with and without transient transfection of G α_{o1} . Results are shown as mean $\pm$ SE from 4 independent experiments.

<i>CHO-D₂</i>				<i>CHO-D₃</i>			
<i>Without exogenous Gα_{o1}</i>		<i>with exogenous Gα_{o1}</i>		<i>Without exogenous Gα_{o1}</i>		<i>with exogenous Gα_{o1}</i>	
<i>K_D, nM</i>	<i>B_{max}, pmoles/mg</i>	<i>K_D, nM</i>	<i>B_{max}, pmoles/mg</i>	<i>K_D, nM</i>	<i>B_{max}, pmoles/mg</i>	<i>K_D, nM</i>	<i>B_{max}, pmoles/mg</i>
0.038 $\pm$ 0.004	0.640 $\pm$ 0.13	0.040 $\pm$ 0.005	0.930 $\pm$ 0.26	0.277 $\pm$ 0.03	6.76 $\pm$ 1.32	0.321 $\pm$ 0.02	7.33 $\pm$ 0.55