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Aripiprazole has Functionally Selective Actions at Dopamine D_2 Receptor-Mediated Signaling Pathways

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Aripiprazole is a unique atypical antipsychotic drug with an excellent side-effect profile presumed, in part, to be due to lack of typical D_2 dopamine receptor antagonist properties. Whether aripiprazole is a typical D_2 partial agonist, or a functionally selective D_2 ligand, remains controversial (eg D_2-mediated inhibition of adenylate cyclase is system dependent; aripiprazole antagonizes D_2 receptor-mediated G-protein-coupled inwardly rectifying potassium channels and guanosine triphosphate nucleotide (GTP)γS coupling). The current study examined the D_{2L} receptor binding properties of aripiprazole, as well as the effects of the drug on three downstream D_2 receptor-mediated functional effectors: mitogen-activated protein kinase (MAPK) phosphorylation, potentiation of arachidonic acid (AA) release, and D_2 receptor internalization. Unlike quinpirole (a full D_2 agonist) or (−)3PPP (*S*(−)-3-(3-hydroxyphenyl)-*N*-propylpiperidine hydrochloride, a D_2 partial agonist), the apparent D_2 affinity of aripiprazole was not decreased significantly by GTP. Moreover, full or partial agonists are expected to have Hill slopes <1.0, yet that of aripiprazole was significantly >1.0. Whereas aripiprazole partially activated both the MAPK and AA pathways, its potency *vs* MAPK phosphorylation was much lower relative to potencies in assays either of AA release or inhibition of cyclic adenosine 3′,5′-cyclic monophosphate accumulation. In addition, unlike typical agonists, neither aripiprazole nor (−)3PPP produced significant internalization of the D_{2L} receptor. These data are clear evidence that aripiprazole affects D_{2L}-mediated signaling pathways in a differential manner. The results are consistent with the hypothesis that aripiprazole is a functionally selective D_2 ligand rather than a simple partial agonist. Such data may be useful in understanding the novel clinical actions of this drug. *Neuropsychopharmacology* (2007) **32,** 67–77. doi:10.1038/sj.npp.1301071; published online 22 March 2006

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INTRODUCTION

Traditional pharmacology posits that a single compound acting through a single receptor will cause a single type of functional response (either full, partial or inverse agonism, or antagonism) for all effector pathways associated with that receptor and its milieu. Accordingly, compounds have been categorized by their 'intrinsic efficacy', often defined by the ability of a ligand to modulate receptor-mediated adenylate cyclase (AC) activity. There is increasing evidence, however, that many ligands do not conform to such a rigid definition of function. In fact, recent observations have led to a growing acceptance of the idea that one ligand, while acting on a specific receptor subtype, can have multiple intrinsic activities depending upon the effectors being examined and the model being employed (Mailman and Gay, 2004; Simmons, 2005).

For example, we have shown previously that a number of dopamine D_2 ligands exhibit functionally selective profiles (Kilts *et al*, 2002; Mottola *et al*, 2002; Gay *et al*, 2004). Further evidence for the ability of ligands to activate G-protein-coupled receptor (GPCR)-mediated effectors differentially has been illustrated in serotonin 5-HT_{2A} and 5-HT_{2C} (Berg *et al*, 1998; Kurrasch-Orbaugh *et al*, 2003), α_{2A}-adrenergic (Brink *et al*, 2000; Kukkonen *et al*, 2001), β_2-adrenergic (Ghanouni *et al*, 2001), cannabinoid CB1 (Glass and Northup, 1999), μ-opioid (Allouche *et al*, 1999), and the oxytocin (Reversi *et al*, 2005) receptor expression systems, among others, effectively illustrating functional selectivity as a universal mechanism of GPCR effector regulation. It is also important to point out that functional selectivity is not an epiphenomenon of a specific receptor expression system, as all of the above observations were made in a number of different physiological models. This mechanism may be relevant to topical issues in neuropsychopharmacology.

Dysfunctional dopaminergic neurotransmission is considered a primary mechanism of schizophrenic

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symptomology (Kapur and Remington, 2001), indeed, the early pharmacotherapy for schizophrenia was based on serendipitous discovery of drugs that turned out to be dopamine receptor antagonists (Carlsson, 1964). Until recently, all of the antipsychotic drugs (APDs), whether 'typical' or 'atypical', or whether of high or low affinity, have been functional D_2 antagonists (Miyamoto *et al*, 2000; Davies *et al*, 2004; Maudsley *et al*, 2005). A clear exception to this, however, is the recently approved compound aripiprazole (Abilify). The unique pharmacology of this compound first was demonstrated in models that showed aripiprazole-activated effectors associated with presynaptic D_2 autoreceptors, whereas it antagonized D_2 postsynaptic receptor-mediated effects (Kikuchi *et al*, 1995). Aripiprazole partially activated D_2 receptor-mediated inhibition of cyclic adenosine 3′,5′-cyclic monophosphate (cAMP) accumulation, although this action was system specific (Lawler *et al*, 1999; Shapiro *et al*, 2003). It was suggested that aripiprazole appeared to activate D_2 receptor-mediated effectors differentially, and could be termed a D_2 functionally selective drug (Lawler *et al*, 1999; Shapiro *et al*, 2003). Conversely, Burris *et al* (2002) have proposed that the unique properties of aripiprazole result solely from its partial agonist properties.

The potential of partial D_2 agonists as a novel treatment of schizophrenia was based in large part on data showing that apomorphine, a high-affinity D_2 dopamine receptor agonist, could preferentially activate D_2 autoreceptors at low doses (Tamminga *et al*, 1978; Roth, 1979). It was hypothesized that a D_2 partial agonist might attenuate the activity of hyperactive mesolimbic neurons, and possibly increase neurotransmission in neurons where there was a deficit of activity (ie mesocortical neurons related to working memory). Presumably such compounds also would induce minimal extrapyramidal side effects (and tardive dyskinesia) that are associated with the receptor blockade caused by typical APDs. The potent D_2-like receptor-selective agonist *N*-propylnorapomorphine (Tamminga *et al*, 1986) and (−)3PPP (*S*(−)-3-(3-hydroxyphenyl)-*N*-propylpiperidine hydrochloride, a D_2 receptor partial agonist) thus seemed to have antipsychotic potential, although patients quickly lost beneficial effects of these agonists rather than having increased efficacy with time as with APDs (Clark *et al*, 1982; Tamminga *et al*, 1992; Lahti *et al*, 1998). For these reasons, there was a great deal of interest in aripiprazole based on the suggestion that it was the long-anticipated D_2 partial agonist (Lawler *et al*, 1999; Burris *et al*, 2002; Shapiro *et al*, 2003). In the process, the idea of functional selectivity (Lawler *et al*, 1999), despite additional support for this mechanism (Shapiro *et al*, 2003), has been dismissed by many authoritative sources (Stahl, 2001; Tamminga, 2002; Lieberman, 2004).

Previous work has examined functional effects of aripiprazole at D_2-regulated AC and receptor-regulated potassium channels (Lawler *et al*, 1999; Burris *et al*, 2002; Shapiro *et al*, 2003). The current study extended this by examining the binding characteristics of aripiprazole at the low and normal affinity states of the dopamine D_2 receptor, as well as the effects of the drug on D_{2L} receptor-mediated phosphorylation of mitogen-activated protein kinase (MAPK), potentiation of arachidonic acid (AA) release (Missale *et al*, 1998), and ligand-induced D_2 receptor internalization.

MATERIALS AND METHODS

Materials

The following were generously donated to this study: aripiprazole (OPC-14597) from Otsuka America Pharmaceuticals (Rockville, MD); olanzapine from Eli Lilly Inc. (Indianapolis, IN); melperone (from Cilag AG-Switzerland); and amisulpride from Dr Shitij Kapur (University of Toronto, Canada). Quinpirole, (−)3PPP, haloperidol, and clozapine were purchased from Sigma/RBI (Natick, MA), whereas dopamine, mepacrine, staurosporine, melittin, adenosine triphosphate, guanosine triphosphate, EDTA, dithiothreitol, sucrose, pepstatin A, leupeptin, PMSF, and other standard laboratory compounds were purchased from Sigma Chemical Co. (St Louis, MO). Sources of other reagents were as follows: 2′-amino-3′-methoxyflavone (PD98059) was purchased from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA); [^{3}H]N-methylspiperone from Perkin-Elmer Life Sciences Inc. (Boston, MA); [5,6,8,9,11,12,13,14-^{3}H]AA from Amersham Biosciences Inc. (Piscataway, NJ); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) from Research Organics Inc. (Cleveland, OH); Ham's F-12 and DMEM media, penicillin, streptomycin, and geneticin (G418) from Invitrogen Co. (Carlsbad, CA); and primary antibody to phospho-Erk1/2 MAPK and secondary anti-rabbit HRP-conjugated antibody from Cell Signaling Technology Inc. (Beverly, MA). M1 anti-FLAG antibody was purchased from Sigma and Alexa594-conjugated goat anti-mouse was from Jackson ImmunoResearch (Malvern, PA).

Cells and Membranes

The Chinese hamster ovary cells (CHO)-hD_{2L} cells are a stable line originally obtained from Dr Tony Sandrasagra (Aventis, Bridgewater, NJ). CHO-hD_{2L} cells were maintained in Ham's F-12 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 500 μg/ml G418 at 37°C and 5.0% CO_2. CHO-hD_{2L} cells were grown to confluence in 75-cm^2 flasks. A measure of 5 ml of cold phosphate-buffered saline was used per flask to rinse the cells, after which 5 ml of lysis buffer (2 mM HEPES, 2 mM EDTA, 1 mM dithiothreitol, 1 μg/ml pepstatin A, 0.5 μg/ml leupeptin, and 10 μg/ml PMSF, pH 7.4 with HCl) was added to each flask. Human embryonic kidney cells (HEK)-FLAG-D_{2L} cells were also grown and handled in a similar manner as the CHO-hD_{2L} cells. Following 10–20 min of incubation at 4°C, the cells were scraped and collected. The cell suspension was homogenized with three strokes in a Wheaton glass homogenizer and centrifuged at 30 000*g* for 20 min. The resulting supernatant was discarded, the pellet resuspended in storage buffer (50 mM HEPES, 0.32 M sucrose, 1 μg/ml pepstatin A, 0.5 μg/ml leupeptin, and 10 μg/ml PMSF, pH 7.4 with NaOH) at approximately 1 mg protein/ml, homogenized again, and aliquoted into 1-ml microcentrifuge tubes. The cell membranes then were frozen, and stored at −80°C until further use.

Saturation and Competition Binding

Saturation studies were performed to verify the expression level of hD_{2L} receptor in the CHO cells, as well as to

determine the levels of FLAG-tagged D_{2L} receptor expressing in the HEK cells. Membranes were incubated with varying concentrations of [^{3}H]*N*-methylspiperone in binding buffer (50 mM HEPES, 4 mM $MgCl_2$, pH = 7.4, with KOH). Nonspecific binding was determined using 10 μM domperidone. Competition binding studies were carried out using 0.3 nM [^{3}H]*N*-methylspiperone with and without 600 μM guanosine triphosphate nucleotide (GTP) to determine differences in affinity of each D_2 receptor ligand for the high- and low-affinity hD_{2L} receptor states in the CHO cell line. Total binding was determined by the amount of radioligand binding in the absence of competing drug, while nonspecific binding was defined by the amount of radioligand bound in the presence of 10 μM haloperidol. Seven to eight concentrations of each D_2 receptor ligand were used. For both binding paradigms, addition of the tissue to each assay tube initiated the binding. Each drug condition was run in triplicate per experiment in a final volume of 500 μl. Following a 15 min incubation at 37°C, tubes were filtered through a FilterMate 196 Cell Harvester (Perkin-Elmer Life and Analytical Sciences, Boston, MA) and the plates were washed four times with ice-cold buffer. The filters were dried in an oven at 55°C for 30 min, and 35 μl of Packard MicroScint 20 scintillation cocktail was added to each well (Perkin-Elmer). A Packard TopCount NXT (Perkin-Elmer) was used to determine the radioactivity of each sample. Saturation binding data were expressed in fmol of receptor/mg of protein. Competition binding data were expressed as a percentage of specific binding.

Cell-Based ELISA for the Measurement of Mitogen-Activated Protein Kinase (MAPK) Activation

Measurements of phosphorylated MAPK were made using a published protocol (Versteeg *et al*, 2000). CHO cells (both wild type and those transfected with the human D_{2L} receptor) were seeded in 96-well plates in Ham's F-12 media (10% FBS) at 50 000 cells/cm^2 and allowed to grow at 37°C and 5% CO_2 for 48 h. Cells were serum starved for 6 h prior to stimulation, after which appropriate drugs were added to each well at a volume of 100 μl for 10 min. The reaction was terminated and cells fixed by aspirating each well and adding 100 μl of 4% formaldehyde PBS solution for 20 min. Cells were washed three times with 100 μl wash buffer (0.1% Triton X-100/PBS solution), followed by a 20 min incubation with 0.6% H_2O_2 Triton/PBS solution to quench endogenous peroxidases. After washing the cells three times again with wash buffer, and after a 1 h incubation with 10% BSA in Triton/PBS solution (to block nonspecific antibody binding), cells were incubated overnight (about 12 h) with a 1:250 dilution of PhosphoPlus® p44/42 1° antibody in the Triton/PBS solution (100 μl) containing 5% BSA at 4°C. Cells were washed three times with wash buffer for 5 min and incubated with 100 μl HRP-conjugated goat anti-rabbit 2° antibody (1:100 dilution) with 5% BSA at room temperature for 1 h. Again, cells were washed three times with wash buffer for 5 min, and then twice with PBS. Cells were then incubated with 50 μl of an *o*-phenylenediamine (OPD) solution (0.4 mg/ml OPD, 17.8 mg/ml $Na_2HPO_4 \cdot 7H_2O$, 7.3 mg/ml citric acid and 0.015% H_2O_2) for 15 min at room temperature in the dark. The reaction was terminated by the addition of 25 μl of 1 M H_2SO_4, and the well-solution analyzed spectrophotometrically (using the Vmax Kinetic Microplate Reader from Molecular Devices) at absorbance wavelengths A_{490}–A_{650}.

AA Release Assay

Measurements of phospholipase A_2 (PLA_2) AA release were made by modifying a protocol described by Berg *et al* (1996). CHO-K1 cells (both wild type and those transfected with the human D_{2L} receptor) were seeded in 24-well plates in Ham's F-12 media (10% FBS) at 50 000 cells/well and allowed to grow at 37°C and 5% CO_2 for 24 h. A measure of 500 μl of serum-free Ham's complete media containing 0.5 μCi/ml [5,6,8,9,11,12,14,15-^{3}H]AA was added to wells, after which cells were preincubated for 5 h. Cells were then washed three times for 5 min with Hank's balanced salt solution (HBSS) containing 0.5% fatty acid-free BSA and appropriate enzyme inhibitors and antagonists (500 μl/well/wash). Following the washes, the cells were incubated for 15 min with appropriate agonists with or without ATP dissolved in the HBSS/BSA (1 ml/well). Three 200 μl sample aliquots were taken from each well and the radioactivity of the samples was counted using liquid scintillation spectrometer techniques.

Epifluorescence Microscopy

HEK293 cells had been stably transfected with FLAG epitope-tagged D_{2L} receptors, and their functional integrity confirmed previously (Vickery and von Zastrow, 1999). As described previously (Vargas and von Zastrow, 2004), these cells were plated on glass coverslips and the surface receptors specifically labeled using 3 μg/ml anti-FLAG M1 monoclonal antibody. Cells were exposed to the indicated ligands at 37°C for 30 min, fixed using 4% formaldehyde dissolved in PBS, and then labeled receptors were detected by secondary incubation with Cy3-conjugated goat anti-mouse antibody (1:1000 dilution). Specimens were visualized by confocal fluorescence microscopy using a Zeiss LSM510 microscope fitted with a Zeiss 63XNA1.4 objective operated in single photon mode with standard filter sets and standard (1Airy disc) pinhole. Metamorph software (Molecular Devices) was employed to count internalized vesicles in 20 random cells per condition per coverslip, with three to six coverslips representing each condition.

Data Analysis

Except where noted, data are expressed as means ± SEM. Data from receptor saturation isotherms was analyzed using nonlinear regression with a one-site hyperbolic model, and the data converted to K_D and B_{max}. The receptor competition data were analyzed by nonlinear regression using a sigmoidal model with variable slope, yielding IC_{50} (concentration inhibiting 50% of total binding IC_{50}) and Hill slopes values. The IC_{50}'s were corrected for radioligand concentration and converted to concentration corrected IC_{50} (apparent affinity constant) when Hill coefficient (n_H) ≠ 1.0 ($K_{0.5}$) values using the Cheng–Prusoff formula for a bimolecular competition model (Cheng and Prusoff,

1973). Changes in affinity in the competition assays were tested for significance by performing a one-way analysis of variance (ANOVA), followed by a Tukey–Kramer multiple comparisons *post hoc* analysis. Functional dose–response curves also were analyzed by nonlinear regression using Prism 4.0's sigmoidal equation with variable slope in order to determine estimates of intrinsic activity and apparent potency. Differences in potency values between the MAPK and AA release effectors for each agonist was analyzed with an unpaired two-sided *t*-test, whereas differences among the drug groups were assessed by ANOVA. Significant ANOVA results were followed by the appropriate (Bonferonni's or Dunnett's) *post hoc* analysis, again performed using Prism 4.0.

RESULTS

[^{3}H]*N*-Methylspiperone Binding

The saturation data for [^{3}H]*N*-methylspiperone binding to this receptor fit a one-site binding model in both cell lines, and yielded the following parameters. (1) CHO-hD_{2L}: $K_D = 0.39 \pm 0.11$ nM, $B_{max} = 6.57 \pm 0.63$ pmol/mg protein; and (2) HEK293-FLAG-D_{2L}: $K_D = 0.34 \pm 0.07$ nM, $B_{max} = 1.5 \pm 0.26$ pmol/mg protein ($N = 3$ for both lines).

Competition of four D_2 ligands *vs* [^{3}H]*N*-methylspiperone binding was carried out using CHO-hD_{2L} cell membranes both in the absence and presence of 600 μM GTP. The resulting competition curves were best fitted to a variable slope binding model, from which their apparent affinities ($K_{0.5}$) and Hill slope values (n_H) were derived (Table 1). ANOVA was utilized to evaluate shifts in affinity in the presence of GTP. Both quinpirole and (−)3PPP demonstrated significant loss of affinity in the presence of GTP, as would be expected of a GPCR agonist, and is illustrated by a rightward shift of their competition curves (Figure 1a and b). They also display Hill slope values typical of agonists ($n_H < 1$) in the absence of GTP. Conversely, aripiprazole failed to demonstrate a significant change in affinity (Table 1 and Figure 1c). It was observed, however, that there was a small, but consistent, shift in every experiment. A more stringent analysis (paired one-tailed *t*-test) was employed,

Table 1 Binding Affinities and Hill Slope Values of Ligands for hD_{2L} Receptors in CHO Cells in the Absence and Presence of 600 μM GTP

	Drug alone		+600 μM GTP	
Compound	$K_{0.5}$	n_H	$K_{0.5}$	n_H
Quinpirole	820 ± 70	−0.79 ± 0.12	2180 ± 160***	−0.89 ± 0.09
(−)-3PPP	1300 ± 170	−0.74 ± 0.13	2360 ± 200***	−0.96 ± 0.13
Aripiprazole	40.3 ± 4.3	−1.4 ± 0.2	52.7 ± 5.7	−1.2 ± 0.2
Haloperidol	2.68 ± 1.02	−1.1 ± 0.2	2.38 ± 0.79	−0.90 ± 0.12

Binding data represent the means ± SEM (in nM) from five to seven independent experiments performed in triplicate. Significant shifts are appropriately marked according to the results of ANOVA and Tukey–Kramer multiple comparison's *post hoc* analyses (***$p < 0.001$).

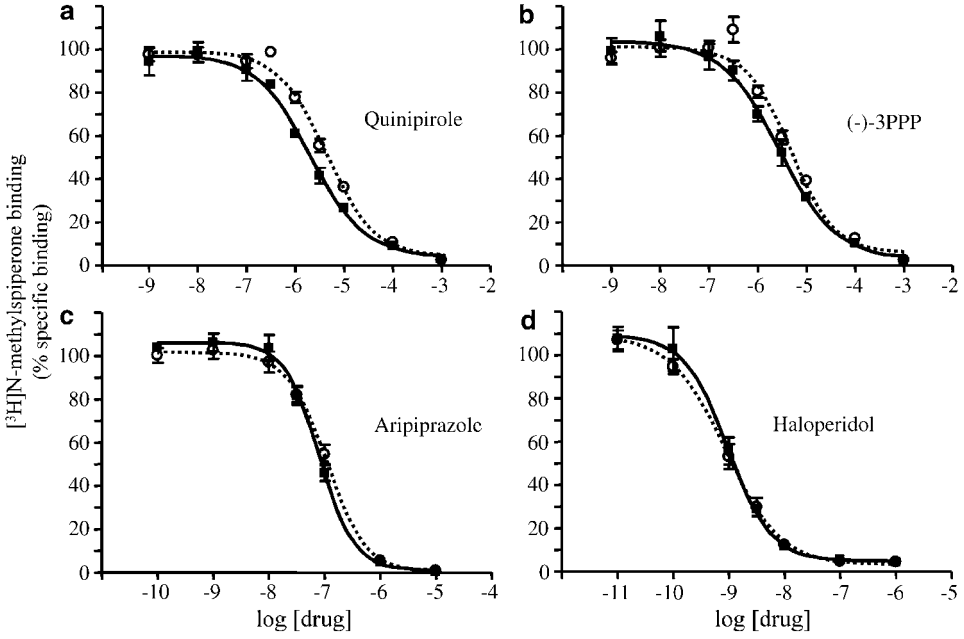

Figure 1 Binding of test ligands to hD_{2L} receptors in the presence or absence of 600 μM GTP. Membranes were prepared from CHO cells stably expressing hD_{2L} receptors. Receptors were labeled with ~0.3 nM [^{3}H]*N*-methylspiperone. Nonspecific binding was defined by 10 μM haloperidol. For each compound, five to seven independent experiments were performed. The curves shown are from representative experiments. Summary of quantitative analysis is shown in Table 1.

and it determined that the slight shifts were indeed significant. It appears, therefore, that the binding of aripiprazole is slightly affected by the G-protein-coupled state of the D_{2L} receptor, and is unique among the D_2 receptor agonists studied. Moreover, the Hill slope for aripiprazole ($n_H > 1$) does not correspond with what would be expected of either an agonist or antagonist following the law of mass action. There appears to be a suggestion of positive cooperativity that affects the binding of aripiprazole to the D_{2L} receptor. As expected, the apparent affinity of the typical APD and known D_2 receptor antagonist, haloperidol, was not affected by GTP (Figure 1d), and its Hill slope value in the absence of GTP corresponds well with what would be expected of an antagonist ($n_H \sim 1$).

Aripiprazole Activation of D_2 Receptor-Mediated MAPK Phosphorylation

To confirm that the D_2 receptor-mediated activation of the MAPK pathway was indeed regulated by MEK, the maximal MAPK phosphorylation by quinpirole was shown to be fully inhibited by 50 μM PD98059, an inhibitor of MEK (data not shown). Quinpirole was found to exhibit slightly higher intrinsic activity than the endogenous agonist dopamine, while (−)3PPP and aripiprazole displayed about 60 and 50% of the activity of quinpirole, respectively (Figure 2). The rank order of potency among the four compounds was dopamine = quinpirole > (−)3PPP > aripiprazole. Haloperidol was able to fully inhibit maximally stimulating concentrations of all four agonists, further indicating that the pathway is D_2 receptor mediated. It was also confirmed that aripiprazole failed to promote the phosphorylation of MAPK in the untransfected CHO-K1 cell line (Figure 3). The other atypical APDs (clozapine, olanzapine, amisulpride, and melperone) showed no significant intrinsic activity for MAPK phosphorylation, and largely inhibited a maximally stimulating concentration (100 nM) of quinpirole (Figure 3). The inability of clozapine, olanzapine, and melperone to inhibit the MAPK effector pathway fully can be attributed to inadequate fractional occupancy at concentrations used rather than low intrinsic partial agonism.

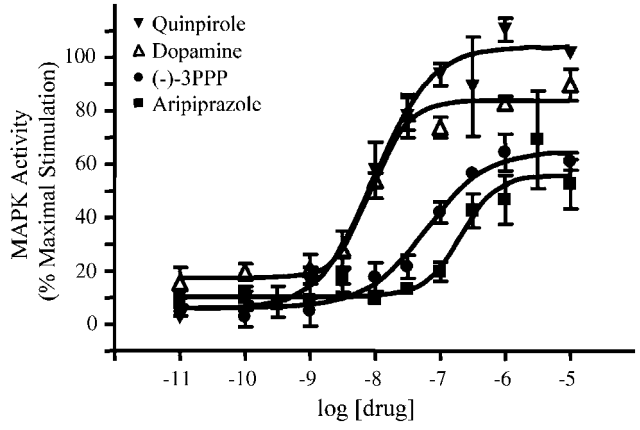

Figure 2 Activation of MAPK phosphorylation. The ability of D_{2L} ligands with known agonist activity to phosphorylate MAPK was assessed in CHO-hD_{2L} cells, and observations were quantified using an enzyme-linked immunosorbent assay. Cells were incubated with increasing concentrations of D_{2L} ligand for 10 min at room temperature (20°C). Both quinpirole and dopamine displayed intrinsic activities that corresponded to full agonists, while aripiprazole and (−)3PPP were partial agonists. The rank order of potency was quinpirole = dopamine > (−)3PPP > aripiprazole. Data are expressed as a percentage of the maximal stimulation of quinpirole over basal phosphorylation. All values represent the mean ± SEM of four to five experiments conducted in quadruplicate.

Aripiprazole Promotes the D_2 Receptor-Mediated Potentiation of AA Release

There has been much debate on the pathway mechanism of GPCR-mediated AA release, and it appears that the mechanism may vary somewhat among different receptors and may be dependent on multiple signaling pathways (Xu *et al*, 2002). We used staurosporine (a pan-kinase inhibitor), PD98059 (an MEK inhibitor), and Ro318220 (a protein kinase C (PKC) inhibitor), in the presence of 10 μM quinpirole. Staurosporine and PD98059 both inhibited AA release (54 and 73%, respectively), but did not cause an additive change when cells were treated with both (53% inhibition). Analysis by ANOVA with Bonferroni's *post hoc* test demonstrated that there was no significant difference among these three inhibitor treatment scenarios (Figure 4). Treatment with Ro318220 failed to affect the intrinsic activity of quinpirole, indicating that the activity of PKC does not appear to influence D_{2L}-meditated potentiation of AA release in this CHO cell line. Our observations indicate that, at least in the CHO cell line, the potentiation of AA release as regulated by hD_{2L} receptor activation is dependent on multiple pathways. Mepacrine (100 μM) fully inhibited the quinpirole potentiation of AA release, whereas melittin was found to stimulate the release of AA (data not

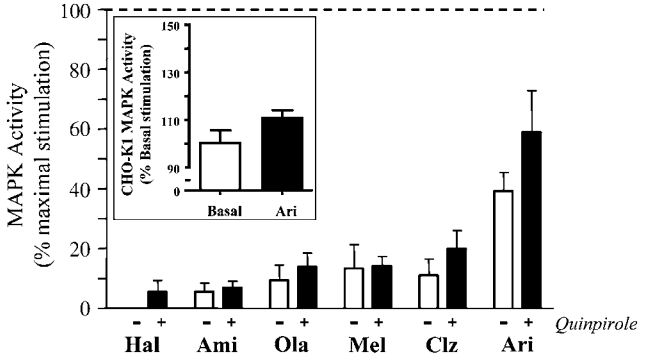

Figure 3 Atypical APD effect on D_{2L}-mediated phosphorylation of MAPK. To evaluate the intrinsic activity of haloperidol (Hal), amisulpiride (Ami), clozapine (Clz), melperone (Mel), olanzapine (Ola), and aripiprazole (Ari), the following design was used. The open bars show the intrinsic activity of each compound alone, where the compounds were used at a concentration of 10 μM. The antagonism study (black bars) also used this same concentration of each potential antagonist *vs* a challenge concentration of quinpirole (100 nM except in the case of aripiprazole where 10 μM was used (see Results)). None of the compounds except aripiprazole caused a significant response alone. All of the atypical APDs except aripiprazole were able to block quinpirole stimulation to a similar degree as haloperidol. *Inset*: The degree of MAPK stimulation elicited by aripiprazole (Ari) (closed bars) relative to basal levels of MAPK activity (open bars) in the untransfected parental CHO-K1 cell line. Data are expressed as a percentage of the maximal stimulation of quinpirole over basal phosphorylation. Each value represents the mean ± SEM of three independent experiments conducted in triplicate.

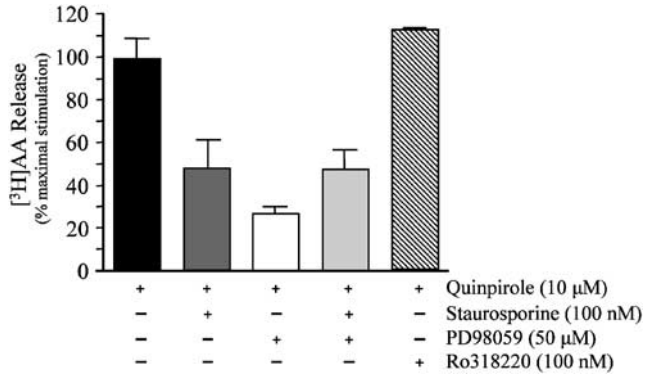

Figure 4 PD98059 and staurosporine inhibition of [^{3}H]AA release. Cells were treated with PD98059 (50 μM) and staurosporine (100 nM) either alone or together in the presence of a maximally stimulating concentration of quinpirole (10 μM). Both inhibitors were able to block significantly quinpirole-stimulated [^{3}H]AA release, but there was no significant difference used alone or together (ANOVA, Bonferroni *post hoc*). In addition, neither alone nor in combination did these compounds cause total inhibition of [^{3}H]AA release. Ro318220 (100 nM) was also used in the presence of quinpirole, but this PKC-specific inhibitor failed to block quinpirole activity. Data are expressed as a percentage of the maximal stimulation of quinpirole over ATP basal [^{3}H]AA release. Each value represents the mean ± SEM of three independent experiments conducted in triplicate.

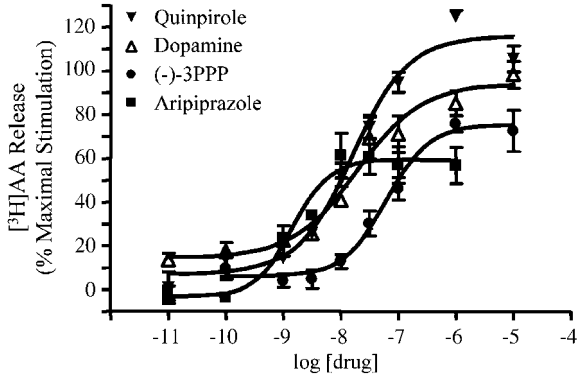

Figure 5 Potentiation of [^{3}H]AA release. The ability of D_{2L} ligands with known agonist activity to potentiate the release of [^{3}H]AA was assessed in CHO-hD_{2L} cells that were incubated with 0.5 μCi/ml [^{3}H]AA-supplemented media for 5 h. Cells were then exposed to varying concentrations of ligand at 37°C for 15 min dopamine stimulated AA release with similar intrinsic activity to quinpirole, while both (−)3PPP and aripiprazole were partial agonists. The rank order of potency was aripiprazole > quinpirole = dopamine(−)33PPP. Data are expressed as a percentage of the maximal stimulation of quinpirole over ATP basal [^{3}H]AA release. All values represent the mean ± SEM of three independent experiments conducted in triplicate.

shown), suggesting the role of PLA_2 in the hD_{2L} receptor-mediated potentiation of the AA release signaling pathway.

Similar to the MAPK effector studies, all four of the D_2 ligands were able to potentiate the release of AA to varying degrees. Both quinpirole and dopamine fully stimulated AA release, while aripiprazole and (−)3PPP demonstrated partial activity of the effector pathway (Figure 5). The rank order of potency among the four compounds was aripiprazole > dopamine = quinpirole > (−)3PPP. Of interest, however, were the potencies that characterized the activities of the compounds for the pathway (Table 2). While the three typical agonists had potencies for AA release fairly congruent with those observed for the MAPK pathway, aripiprazole illustrated a 20-fold higher potency for the D_2-mediated potentiation of AA release than MAPK phosphorylation. Further analysis of the potency differences between MAPK and AA release experiments for each drug using ANOVA (followed by a Dunnett's *post hoc* multiple comparisons test) illustrated that only aripiprazole exhibited a significant potency shift relative to that of quinpirole ($p < 0.01$). Incidentally, the EC_{50} of aripiprazole for AA release is similar to the potency of aripiprazole reported for D_2 receptor-mediated inhibition of AC (Lawler *et al*, 1999; Burris *et al*, 2002; Shapiro *et al*, 2003). As with the MAPK studies, haloperidol was able to fully inhibit the ability of all four compounds to potentiate AA release, verifying D_{2L} receptor activation as the means by which these ligands were able to exact their influence on the pathway. It was also observed that aripiprazole was unable to promote the release of AA in the parental CHO-K1 cell line, confirming that the activity of aripiprazole at this effector is specific to the transfected D_{2L} receptor. Finally, none of the other atypical APDs examined illustrated agonist activity for the AA release pathway (Figure 6).

Aripiprazole Fails to Stimulate Internalization of the FLAG-Tagged hD_{2L} Receptor

It was originally thought a compound that displayed an agonist functional profile would induce some degree of receptor endocytosis, and that competitive antagonists, by their very nature, would be unable to provoke such a response (Ferguson, 2001). More recent evidence indicates, however, that certain antagonists can internalize certain receptors (Berry *et al*, 1996; Roettger *et al*, 1997), and that the internalization process can be agonist-specific depending on the cellular milieu specific to that receptor expression system (Ryman-Rasmussen *et al*, 2005). These observations complement the fundamentals of functional selectivity, which state that the intrinsic activity of a ligand

Table 2 Functional Potencies of Agonists for hD_{2L} Receptors in CHO Cells

Compound	Quinpirole	Dopamine	(−)-3PPP	Aripiprazole
MAPK phosphorylation	13.4 ± 3.3	8.8 ± 0.9	92.8 ± 12.5	170 ± 35**
[^{3}H]AA release	17.3 ± 1.5	31.9 ± 11.8	71.1 ± 12.9	1.53 ± 0.39

Functional data represent the means ± SEM (in nM) of three to five independent experiments. Significant potency shifts between the functional end points among the compounds was determined by ANOVA followed by a Dunnett's multiple comparisons *post hoc* analysis with each compound's mean shift difference compared to that of quinpirole (**$p < 0.01$, with all other comparisons having $p > 0.05$). See Materials and methods for further details.

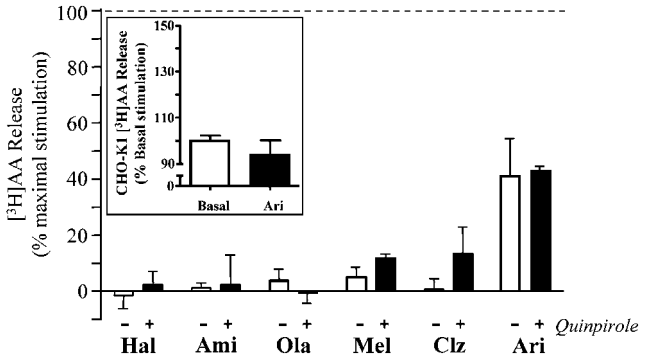

Figure 6 Atypical APD effect on D_{2L} receptor potentiation of [^{3}H]AA release. To evaluate the intrinsic activity of haloperidol (Hal), amisulpiride (Ami), clozapine (Clz), melperone (Mel), olanzapine (Ola), and aripiprazole (Ari), the following design was used. The open bars show the intrinsic activity of each compound alone, where the compounds were used at a concentration of 10 μM. The antagonism study (black bars) also used this same concentration of each potential antagonist *vs* a challenge concentration of quinpirole (100 nM except in the case of aripiprazole where 10 μM was used (see Results)). None of the compounds except aripiprazole caused a significant response alone. All of the atypical APDs except aripiprazole were able to block quinpirole stimulation to a similar degree as haloperidol. *Inset*: The degree of MAPK stimulation elicited by aripiprazole (closed bars) relative to basal levels of MAPK activity (open bars) in the untransfected parental CHO-K1 cell line. Data are expressed as a percentage of the maximal stimulation of quinpirole over ATP basal [^{3}H]AA release. Each value represents the mean ± SEM of two to three independent experiments conducted in triplicate.

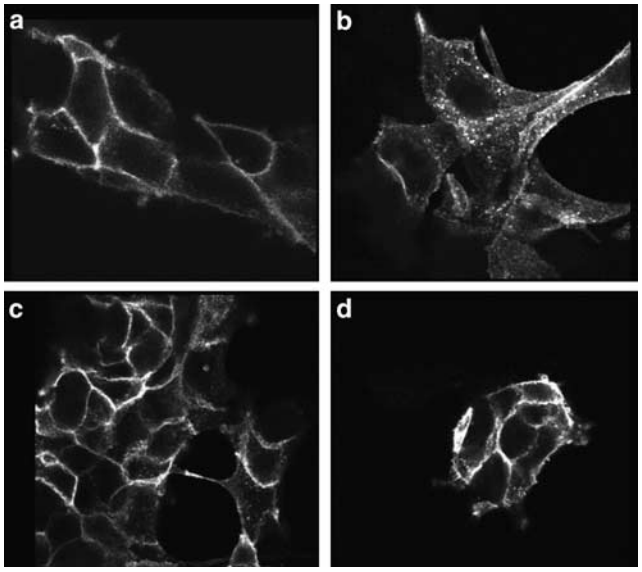

Figure 7 Ligand effects on receptor internalization. FLAG-tagged D_{2L} receptors expressed stably in HEK293 cells were specifically labeled at 37°C with M1mAb as described in Materials and methods. Fluorescence microscopy was used to visualize the localization of antibody-labeled receptor in cells incubated for 30 min in: (a) No drugs; (b) 10 μM dopamine; (c), 10 μM aripiprazole; or (d) 10 μM (−)3PPP. Representative micrographs of each condition are shown.

is dependent on the pathway being examined and the model around which the investigation revolves. Thus, the study of the ability of a ligand to stimulate receptor internalization has been utilized to understand this aspect of the functional profile of many GPCR ligands. Utilizing HEK293-fD_{2L} cells ($B_{max} = 1.50 \pm 0.26$ pmol receptor/mg protein) and epifluorescent microscopy techniques, we were able to observe a pronounced dopamine-induced increase in the internalization of the FLAG-tagged D_{2L} receptors relative to basal levels (Figure 7a and b). Neither aripiprazole, nor the D_2 partial agonist (−)3PPP, however, were able to elicit a detectable endocytotic response relative to basal levels (Figure 7c and d). Quantification of these results is shown in Figure 8.

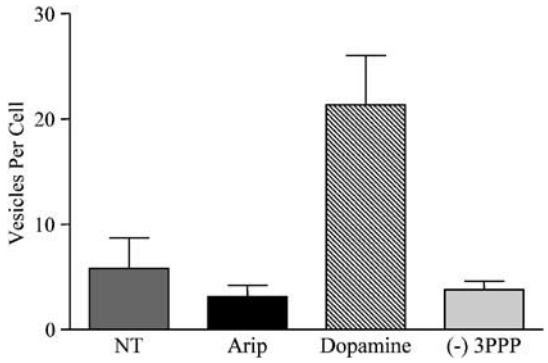

Figure 8 Quantification of ligand-mediated differences in dopamine receptor internalization in stably transfected D_{2L} cells. Ligand-dependent dopamine receptor internalization was assayed as described in the Materials and methods. A significant number of endocytic vesicles containing endocytosed M1 antibody were observed in untreated cells (NT), consistent with the constitutive internalization that has been observed for this receptor. There was, however, a large increase in antibody uptake induced by dopamine (10 μM for 30 min). In contrast, neither aripiprazole (Arip) or (−)3PPP-mediated significant endocytosis of monoclonal antibody. The bars represent the mean number of antibody-positive vesicles (± SE) detected by MetaMorph (Molecular Devices) analysis of a region of interest that outlined the entire intracellular area in 20 cells.

DISCUSSION

Pharmacotherapy for the treatment of schizophrenia has utilized several strategies over the past fives decades, although the pharmacological mechanism common to all successful drugs is the antagonism of dopamine D_2 receptors (Kapur and Mamo, 2003). The unique pharmacology of aripiprazole, a drug having both partial agonist and antagonist activity at D_2 receptor functions depending on the end point under study, suggests that functionally selective ligands may provide a new arena for the development of novel therapeutics for psychoses and other disorders (Miyamoto *et al*, 2000; Davies *et al*, 2004; Maudsley *et al*, 2005). The current study was designed to shed further light on the question of whether the D_2 activity of aripiprazole is simple partial agonism or a case of ligand-induced differential signaling. To further investigate this issue, the D_2 receptor-mediated signaling profile of aripiprazole was compared to several other atypical APDs and to both a full and partial agonist, by investigating effectors that, to date, had been used infrequently to characterize APD pharmacology.

First, the apparent affinity of the D_2 receptor ligands was determined in CHO cells stably expressing the hD_{2L} receptor. The rank order of apparent affinities was similar

to what has been reported previously (haloperidol > aripiprazole > quinpirole > (−)3PPP). Agonists for GPCR receptors are expected to produce competition isotherms that shift to the right in the presence of guanine nucleotides (Lefkowitz *et al*, 1978), a phenomenon clearly illustrated by the current quinpirole and (−)3PPP binding data. Although GTP caused a consistent shift in the aripiprazole competition curves, this effect was very small, and was statistically significant only when utilizing a one-sided paired *t*-test. Conversely, quinpirole and (−)3PPP, but not haloperidol, caused a larger shift in affinity and Hill slope (see Table 1). These data with aripiprazole may reconcile with the previously published GTPγS binding data in which aripiprazole was found to be a pure antagonist of D_2 receptor-mediated GTPγS release in CHO-hD_{2L} membranes (Shapiro *et al*, 2003).

Also of interest were Hill slopes (n_H) for aripiprazole that were greater than 1.0. A Hill slope less than one is often considered a reflection of the ability of a ligand to bind to G protein-precoupled receptors with a higher affinity than uncoupled receptors. Thus, antagonists tend to have Hill slope values close to one since they fail to differentiate between the precoupled and uncoupled receptor populations, thereby obeying the general law of mass action for single site competition. As expected, both the typical full and partial agonists quinpirole and (−)3PPP had different affinities for these populations of hD_{2L} receptors based on the changes in competition curves seen in response to GTP. The competition isotherm of haloperidol fits the binding profile of a typical antagonist (ie it both lacks a GTP shift and has an n_H value close to one). The steep slope for aripiprazole, as well as a small but significant GTP effect, suggests that additional mechanisms are involved. One hypothesis for such observations is that certain ligands may promote positive cooperativity of dimerized or oligomerized receptor (Lavoie and Hebert, 2003), or by allosteric interaction with a secondary domain of the receptor or another protein present in the microdomain (eg a scaffolding or accessory/chaperone protein). It is plausible that the receptor–receptor or receptor–protein interaction that imparts this positive cooperativity only occurs upon the induction of specific receptor conformations. Whatever the case, the unique receptor binding characteristic of aripiprazole is not exclusive to the CHO-hD_{2L} stable cell model, as similar observation was made in brain tissue (Lawler *et al*, 1999). Further study is necessary to understand the underlying mechanism.

It is known that D_2 receptor agonists can stimulate the MAPK pathway in stable C6 and CHO-transfected cells (Luo *et al*, 1998; Choi *et al*, 1999). By modifying an ELISA high-throughput assay (Versteeg *et al*, 2000), we were able to determine intrinsic activities and potencies for the D_2 ligands we studied. The intrinsic activity of aripiprazole correlates well with its reported activity for the D_{2L}-mediated inhibition of cAMP accumulation (Lawler *et al*, 1999; Burris *et al*, 2002; Shapiro *et al*, 2003). The major point of interest, however, lies in the low potency of aripiprazole for the MAPK effector pathway. With the exception of aripiprazole, prior cAMP inhibition EC_{50} data for all compounds correspond fairly well with the MAPK potencies reported in this study (Wilson *et al*, 2001; Burris *et al*, 2002; Gay *et al*, 2004).

The biosynthesis and regulation of prostaglandin-like compounds by catecholamines has been of interest for several years (Levine and Moskowitz, 1979). The ability of the D_2 receptor to mediate the release and metabolism of AA from the intercellular membrane is known (Piomelli *et al*, 1991; Felder *et al*, 1991), and although the exact mechanism is not well understood, there has been much discussion as to how it might be regulated by other signaling effectors, most notably AC, MAPK, and PKC (see Chakraborti, 2003 for a review). There appears to be significant evidence that the GPCR-mediated release of AA, in fact, may be regulated by more than just one pathway, and both the MAPK and PKC effectors appear to play significant roles in AA release (Xu *et al*, 2002). For this study, it was vital to demonstrate that the release of AA was not solely dependent on a D_2 receptor-mediated effector already under study (ie MAPK phosphorylation). Neither staurosporine nor PD98059 was able to inhibit the D_2 receptor-mediated release of AA fully. This demonstrates a significant degree of independence of AA release from modulation by MAPK signaling pathways. In addition, PKC inhibition had no effect on the D_2 receptor regulation of this pathway. This independence of D_{2L}-mediated AA release from PKC indicates that this cell line differs from some others (Xu *et al*, 2002).

The intrinsic activity (E_{max}) of aripiprazole for AA release proved to be similar to its ability to stimulate the phosphorylation of MAPK (Figures 3 and 6), although its potency for the former end point clearly paralleled its reported potency for the D_2 receptor-mediated inhibition of cAMP accumulation (Lawler *et al*, 1999; Burris *et al*, 2002; Shapiro *et al*, 2003). This method of comparison assessment is valid because the intrinsic activity of aripiprazole was similar at both MAPK and AA release, and therefore the direct comparison of ED_{50}'s is equivalent to the direct comparison of ED_{50}/E_{max}. Unlike aripiprazole, the D_2 agonists quinpirole, dopamine, and (−)3PPP all displayed potencies for the release of AA similar to that for the phosphorylation of MAPK. In fact, the difference in the potencies of aripiprazole between the two effectors is over 20-fold (Figure 9). This disparity is especially striking in context with the observation hat other functionally selective D_{2L} ligands (eg DHX, DNS, RNPA) also have demonstrated significant potency differences among D_2 receptor effectors (Gay *et al*, 2004).

The intrinsic activity of aripiprazole for the D_{2L} receptor-mediated phosphorylation of MAPK may not be of significant physiological relevance since the cell system used had a high level of receptor expression ($B_{max} \sim 6$ pmol/mg protein). It is well documented that, in systems with high receptor reserve, compounds with low intrinsic activity can mimic compounds with higher intrinsic activities for that effector system (eg Watts *et al*, 1995). This notion, coupled with preliminary experiments that utilized the nonspecific, irreversible protein binding compound EEDQ to reduce receptor levels (data not shown), suggests that D_2 modulation of MAPK may not be an effector pathway affected by aripiprazole in physiologically relevant systems. It underscores the importance of using, when available, cell lines that mirror as much as feasible those cells expressing the receptor under study *in situ*.

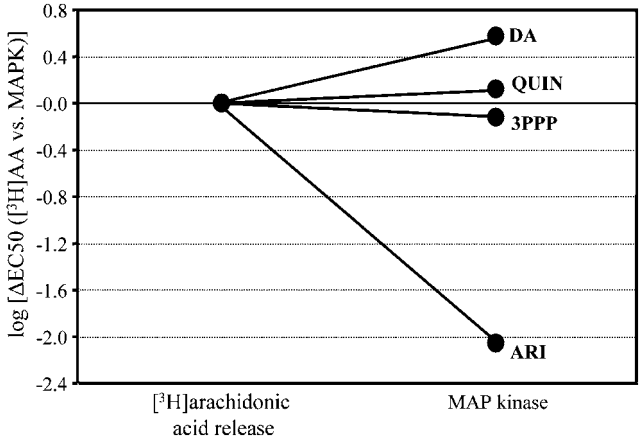

Figure 9 Relative potency of D_{2L} ligands for the potentiation of AA release and phosphorylation of MAPK. Ligands with similar potency for both AA release and MAPK phosphorylation have a log (fold change) of close to zero (quinpirole (QUIN) and (−)3PPP), whereas compounds having decreased potency for MAPK phosphorylation relative to AA release display a negative change (aripiprazole (ARI)), and ligands with an increased potency for MAPK phosphorylation compared to AA release display a slight positive change (dopamine (DA)). Data illustrated for each ligand are expressed as the log of EC_{50} (nM) for MAPK phosphorylation divided by their respective log of EC_{50} value for AA release.

The model commonly used to illustrate GPCR internalization describes an agonist–receptor interaction that promotes receptor phosphorylation via a GRK or other kinase, with subsequent arrestin binding and receptor sequestration via clathrin-coated pits (von Zastrow, 2003). Not all GPCRs follow the same mechanism of internalization, and the intracellular loops of a receptor, as well as the local cellular milieu, play an important role in the mechanism of endocytosis (Ferguson, 2001). D_2 receptors have been shown to internalize in a manner dependent upon GRKs, clathrin, and dynamin (Kim *et al*, 2001), although dynamin-independent internalization can be observed in cells expressing GRKs at lower levels (Vickery and von Zastrow, 1999). Studies in an HEK293-fD_{2L} system showed that the D_{2L} receptor displays both basal (constitutive) and dopamine-induced internalization, and that haloperidol can block dopamine-induced, but not constitutive, internalization (Vickery and von Zastrow, 1999). Our study indicates that neither aripiprazole nor the typical partial agonist (−)3PPP induce a significant degree of internalization relative to basal levels, indicating that either D_2 ligands with low intrinsic activity fail to produce D_2 receptor internalization or that these two compounds are unique in their promotion of receptor conformations that cannot be phosphorylated by the appropriate kinases. Although (−)3PPP can desensitize the D_2 receptor (Lahti *et al*, 1998), it is clear that this does not necessarily predict internalization (Lewis *et al*, 1998; Ryman-Rasmussen *et al*, 2005). Further experiments are needed to determine whether D_2 receptor internalization is a product unique to ligands that are full agonists, or if aripiprazole and (−)3PPP are unique in their inability to internalize the D_2 receptor.

Some of the most efficacious atypical APDs (clozapine, amisulpride, olanzapine), as well as a compound considered to have good atypical APD potential (melperone), were also studied. Vanhauwe *et al* (2000) had reported that these compounds should be considered as D_2 receptor antagonists based on their inhibition of AC, although melperone was not studied by them. We hypothesized that a study of their regulation of D_{2L} function might reveal functional characteristics that explained some of their behavioral atypicality. The current study demonstrates that none of these four atypicals have any D_{2L} intrinsic activity for either MAPK phosphorylation or AA release, and all four blocked the activity of quinpirole for both endpoints. Thus, these data suggest that neither the efficacy nor low EPS of these compounds involves functional selectivity.

In conclusion, the promise of even more effective D_2 partial agonists has been widely proposed based on the success of aripiprazole (Burris *et al*, 2002; Lieberman, 2004; Bolonna and Kerwin, 2005). Conversely, we have suggested that functional selectivity at D_2 receptors, probably combined with actions at nondopamine receptors (Lawler *et al*, 1999; Shapiro *et al*, 2003), is the more likely mechanism responsible for the atypicality of this drug. It seems clear to us that the available evidence (including the current work) supports only the latter hypothesis. No other D_2 partial agonist has shown similar therapeutic promise to aripiprazole. More importantly, even those who have advocated for simple partial agonism (Burris *et al*, 2002) now have shown cell-dependent differences in the intrinsic activity of aripiprazole (Tadori *et al*, 2005). The most parsimonious way to reconcile the available data is accept the hypothesis that the pattern of D_2 functional selectivity, and/or combined with actions at other receptors systems (eg 5-HT_{1A}, 5-HT_{2C}, etc), mediate the novel actions of aripiprazole, rather than simple partial agonism. More detailed understanding of these mechanisms may help find a drug with an improved clinical profile.

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NOTE ADDED IN PROOF

After acceptance of this paper, Bruins Slot *et al* (2006) reported that aripiprazole also mediated Erk phosphorylation via D_{2S} receptors (CHO cells) with similar low potency as we report for D_{2L}.

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