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ORIGINAL INVESTIGATION

Dopamine D_4 receptor involvement in the discriminative stimulus effects in rats of LSD, but not the phenethylamine hallucinogen DOI

Danuta Marona-Lewicka · Benjamin R. Chemel · David E. Nichols

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Abstract
Rationale Lysergic acid diethylamide (LSD) differs from other types of hallucinogens in that it possesses direct dopaminergic effects. The exact nature of this component has not been elucidated.
Objective The present study sought to characterize the effects of several dopamine D_4 agonists and antagonists on the discriminative stimulus effect of LSD at two pretreatment times and 2,5-dimethoxy-4-iodoamphetamine (DOI), a selective 5-$HT_{2A/2C}$ agonist.
Materials and methods Male Sprague–Dawley rats were trained in a two-lever, fixed ratio (FR) 50, food-reinforced task with LSD-30 (0.08 mg/kg, i.p., 30-min pretreatment time), LSD-90 (0.16 mg/kg, i.p., 90-min pretreatment time), and DOI (0.4 mg/kg, i.p., 30-min pretreatment time) as discriminative stimuli. Substitution and combination tests with the dopamine D_4 agonists, ABT-724 and WAY 100635, were performed in all groups. Combination tests were run using the dopamine D_4 antagonists A-381393 and L-745,870 and two antipsychotic drugs, clozapine and olanzapine.
Results WAY 100635 produced full substitution in LSD-90 rats, partial substitution in LSD-30 rats, and saline appropriate responding in DOI-trained rats. ABT-724 partially mimicked the LSD-90 and LSD-30 cues, but produced no substitution in DOI-trained rats. In combination tests, both agonists shifted the dose–response curve of LSD leftward, most potently for the LSD-90 cue. The D_4 antagonists significantly attenuated both the LSD-90 and LSD-30 cue, but had no effect on the DOI cue.
Conclusion Dopamine D_4 receptor activation plays a significant modulatory role in the discriminative stimulus effects in LSD-90-trained rats, most markedly for the later temporal phase of LSD, but has no effect on the cue produced by DOI.

Keywords Dopamine D_4 receptor · Lysergic acid diethylamide (LSD) · Drug discrimination · Hallucinogen · Rat · 5-HT_{2A}

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Introduction

Activation of the serotonin$_{2A}$ (5-HT_{2A}) receptor is essential for the effects of hallucinogens (see review by Nichols 2004). These receptors are particularly important in prefrontal cortical function where they are found at high densities in many species (Miner et al. 2003; Xu and Pandey 2000). Cortical 5-HT_{2A} receptors also are hypothesized to be involved in the pathology and treatment of schizophrenia (Meltzer 1999; Aghajanian and Marek 2000). Although the pyramidal neuron is the major cortical cell type expressing 5-HT_{2A} receptors, some cortical GABAergic interneurons also express these receptors (Griffiths and Lovick 2002; de Almeida and Mengod 2007).

In drug discrimination assays in rats, the interoceptive cue generated by hallucinogenic phenethylamine derivatives such as 2,5-dimethoxy-4-iodoamphetamine (DOI) or 4-bromo-2,5-dimethoxyamphetamine is monophasic, lasts for several hours after drug administration, and is mediated by stimulation of 5-HT_{2A} receptors (e.g., Glennon 1986; Klodzinska and Chojnacka-Wojcik 1997; Smith et al.

1999). Phenethylamines such as DOI show high affinity only at 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors without detectable effects at other G-protein-coupled receptors, channels, or transporters (http://kidb.cwru.edu/pdsp.php).

The potent hallucinogen lysergic acid diethylamide (LSD) displays high affinity for 5-HT_2 family receptors, but it also has high affinity at many other monoamine receptors (http://kidb.cwru.edu/pdsp.php). Although it has been known for a long time that LSD has direct dopaminergic properties (see Watts et al. 1995 and references therein), activation of 5-HT_{2A} receptors is thought to mediate its hallucinogenic properties as well as its interoceptive cue in drug discrimination studies. That is, when rats are administered LSD (0.08 or 0.16 mg/kg) 30 min before discrimination training, the cue is mediated primarily by 5-HT_{2A} receptor activation.

We have previously demonstrated, however, that the discriminative stimulus effect of LSD in rats proceeds through two temporal phases (Marona-Lewicka et al. 2005). The first phase is mediated by stimulation of 5-HT_{2A} receptors, reaches a maximum 15–30 min after drug administration, and lasts about 1 h, a finding that has been replicated in numerous laboratories (e.g., Colpaert et al. 1982; Glennon et al. 1984; Winter et al. 1999). The second phase of action for LSD appears to be mediated by activation of dopamine D_2-like receptors (Marona-Lewicka et al. 2005) and reaches a maximum at times from 60 to 90 min after LSD administration. That is, when LSD (0.16 mg/kg) is administered 90 min prior to discrimination training, it produces a robust interoceptive cue that is mediated primarily by dopamine D_2-like receptor activation.

Thus, in drug discrimination studies, LSD has a biphasic pharmacology, with an initial phase mediated by 5-HT_{2A} receptor activation and a delayed temporal phase that is mediated by D_2-like dopamine receptor stimulation (Marona-Lewicka et al. 2005, 2007). Although a larger dose of LSD is necessary to maintain the salience of a cue out to 90 min, when the higher dose of LSD is administered 30 min prior to discrimination training, the cue is still mediated by 5-HT_{2A} receptor activation (Marona-Lewicka et al. 2005). Thus, the length of time between LSD administration and discrimination training is the key factor that determines the nature of the discriminative cue. Our findings in rats parallel the observations of Freedman (1984) that the psychological effects of LSD in humans occur in two time-dependent phases.

Hallucinogens may activate dopaminergic pathways either directly, as with LSD, or indirectly by compounds that lack significant dopamine receptor affinity (Bortolozzi et al. 2005; Ichikawa and Meltzer 1995; Pehek et al. 2006; Vollenweider et al. 1999). For example, it is well documented that activation of the 5-HT_{2A} receptor can modulate dopamine levels or physiological responses mediated by dopaminergic systems (Alex and Pehek 2007; Lucas and Spampinato 2000; Pehek et al. 2001; Vollenweider et al. 1999; Yan 2000). In addition, it has been reported previously that pretreatment with 5-HT_{2A} agonists can potentiate the discriminative stimulus effects of amphetamine (Marona-Lewicka and Nichols 1997), methamphetamine (Munzar et al. 1999, 2002), and cocaine (Munzar et al. 2002) in rats. Although studies of hallucinogen-mediated dopaminergic effects or action at dopamine receptors are sparse, this area appears promising for further study.

Little is known, however, concerning the role of specific dopamine D_2 receptor subtypes in the effects of LSD. In an earlier study, we reported that WAY 100635, a mixed 5-HT_{1A} antagonist/D_4 agonist (Chemel et al. 2006a) produced partial substitution in rats trained to discriminate LSD following a 30 min pretreatment time, and full substitution in rats trained to discriminate LSD after a 90-min pretreatment time (Marona-Lewicka and Nichols 2007).

In the present study, we examine for the first time the involvement of dopamine D_4 receptors (Van Tol et al. 1991) in the discriminative stimulus effects of LSD (30- and 90-min pretreatment times) and the hallucinogenic amphetamine derivative, DOI. In addition, LSD and DOI radioligand competition binding studies were performed using HEK cell lines stably expressing human $D_{4.4}$ dopamine receptors. The potency and intrinsic activity of LSD at the dopamine D_4 receptor was determined by its ability to inhibit forskolin-stimulated cyclic adenosine monophosphate (cAMP) production.

Materials and methods

Animals

Male Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN, USA) weighing 180–200 g at the beginning of the study were used as subjects. Rats were divided into three groups and trained to discriminate LSD (186 nmol/kg, 0.08 mg/kg, i.p.) with a 30-min pretreatment time (LSD-30), LSD (372 nmol/kg, 0.16 mg/kg, i.p.) with a 90-min pretreatment time (LSD-90), and DOI (1.12 μmol/kg, 0.4 mg/kg, i.p., 30 min before training) from saline using a two-lever, food-reinforced operant conditioning task. All experimental conditions and the feeding procedure were described in detail in our previous paper (Marona-Lewicka and Nichols 2007). Animals used in these studies were maintained in accordance with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals as amended August 2002, and the protocol was approved by the Purdue University Animal Care and Use Committee.

Apparatus

Six standard operant conditioning chambers (model E10-10RF, Coulbourn Instruments, Lehigh Valley, PA, USA) consisted of modular test cages enclosed within sound-attenuated cubicles with fans for ventilation and background white noise. A white house light was centered near the top of the front panel of the cage, which also was equipped with two response levers, separated by a food hopper (combination dipper pellet trough, model E14-06, module size 1/2) all positioned 2.5 cm above the floor. Solid state logic in an adjacent room, interfaced through a Med Associates (Lafayette, IN, USA) interface to a personal computer, controlled reinforcement and data acquisition with locally written software.

Discrimination training and testing

A FR 50 schedule of food reinforcement (45 mg dustless pellets, Research Diets, NJ, USA) in a two-lever paradigm was used. The drug discrimination procedure details have been described elsewhere (Marona-Lewicka and Nichols 1994). At least one drug and one saline session separated each test session, and rats were required to maintain the 85% correct responding criterion on the two prior training days in order to be tested. In addition, test data were discarded when the accuracy criterion of 85% was not achieved on either of the two training sessions following a test session. Training sessions lasted 15 min, and test sessions lasted 5 min and were run under conditions of extinction, with rats removed from the operant chamber when 50 presses were emitted on either lever. In a test session, if 50 presses on one lever were not completed within 5 min, the session was ended and scored as a disruption. For substitution tests, drugs were administered i.p. 30 min prior to test sessions. Inhibition tests were carried out by administering different doses of antagonist 30 min before the training dose of training drugs, that is, 60 min before tests in LSD-30 and DOI-trained rats and 120 min in LSD-90-trained animals. For combination tests, a single dose chosen from substitution or inhibition tests for agonists or antagonists, respectively, was administered 30 min before different doses of training drug. For a dose–response effect of the training drugs, all groups of rats were tested when they first passed the required criteria and later at least once during each 8-month period.

Drugs

The training drugs used were LSD [(+)-lysergic acid diethylamide tartrate, NIDA] and DOI [1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride, synthesized in our laboratory]. LSD training doses were 0.08 mg/kg (186 nmol/kg) or 0.16 mg/kg (372 nmol/kg.), and the training dose used for DOI was 0.4 mg/kg (1.12 μmol/kg). WAY 100635 (*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl] ethyl]-*N*-(2-pyridinyl)cyclohexanecarboxamide), ABT-724, A-381393 (2-[4-(3,4-dimethylphenyl)piperazin-1-ylmethyl]-1*H*-benzimidazole), and WAY 100635 were prepared in our synthesis laboratory using previously reported methods (Nakane et al. 2005; Zhuang et al. 1994). Other drugs used for this study include: L-745,870 (3-[[4-(4-chlorophenyl)piperazin-1-yl]methyl]-1*H*-pyrrolo [2,3-*b*]pyridine trihydrochloride) and clozapine (8-chloro-11-(4-methyl-1-piperazinyl)-5*H*-dibenzo[b,e][1,4]diazepine), which were purchased from TOCRIS (Ellisville, MO, USA). Olanzapine hydrochloride was a generous gift from Eli Lilly (Indianapolis, IN, USA). All drug solutions were prepared by dissolving the compounds in sterile saline (0.9% NaCl) at a concentration that allowed the appropriate dose to be given in a volume of 1 ml/kg, identical to the volume of the saline injection. Stock solutions of clozapine (10 mg/ml) and olanzapine (10 mg/ml) were prepared by dissolving the drugs in a minimal volume (one to two drops) of 80% L-lactic acid followed by dilution with distilled water to the desired concentration (final pH 6.2–6.7).

Chemicals and reagents used for in vitro experiments were as follows: [^{3}H]Spiperone (95 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ, USA). [^{3}H]Cyclic AMP (30 Ci/mmol) was purchased from Perkin Elmer life and analytical sciences (Boston, MA, USA). Quinpirole, dopamine, spiperone, butaclamol, isobutylmethylxanthine, forskolin, and most other reagents were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Growth media, antibiotics, and most other cell culture reagents were purchased from Gibco Invitrogen Corporation (Carlsbad, CA, USA).

Cell culture

HEK-h$D_{4.4}$ stable cells were created previously (Watts et al. 1999) and maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal clone serum, 5% bovine calf serum, 0.05 μg/ml penicillin, 50 μg/ml streptomycin, and 2 μg/ml puromycin. Cells were grown on 10-cm tissue culture plates in a humidified incubator with 5% CO_2 at 37°C.

Cyclic AMP accumulation assay

Cells were grown to confluent monolayers in 48-well clear tissue culture plates. Prior to assay, growth media was decanted and the plates placed on ice. Drug dilutions made in Earle's balanced salt solution (EBSS) assay buffer (EBSS containing 2% bovine calf serum, 0.025% ascorbic acid, and 15 mM HEPES, pH 7.4) were added on ice. cAMP accumulation was stimulated by 5 μM forskolin, and each

assay was performed in the presence of 500 μM isobutylmethylxanthine. Incubations were performed for 15 min in a 37°C water bath. To terminate the stimulation, the assay media was decanted and cells were lysed by adding 100 μl of 3% trichloroacetic acid on ice. Plates were stored at 4°C for at least 1 h before quantification of cAMP.

Quantification of cyclic AMP

Cyclic AMP accumulation was assessed using a previously described competition binding assay (Watts and Neve 1996). Briefly, cell lysate (12 μl) was added in duplicate to assay tubes with cAMP binding buffer [100 mM Tris–HCl, pH 7.4, 100 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA)] containing [^{3}H]cAMP (1 nM final concentration) and cAMP binding protein (100–150 μg in 500 μl buffer). The reaction tubes were incubated on ice at 4°C for 2–3 h before harvesting by filtration (GF/C filterplates; Whatman, Maidstone, UK) using a 96-well Packard Filtermate cell harvester. Filter plates were dried, and 30 μl of Packard Microscint O scintillation fluid was added to each well. Radioactivity per well was determined using a Packard TopCount scintillation counter. The concentration of cAMP in each sample was estimated from a standard curve ranging from 0.01 to 300 pmol of cyclic AMP.

Radioligand competition assay

Cells were grown to confluence on 15-cm plates. Growth media was decanted and replaced with 10 ml ice-cold lysis buffer (1 mM HEPES, pH 7.4, and 2 mM EDTA). After 10 min, cells were scraped from the plate and centrifuged at 30,000×*g* and 4°C for 20 min. The resulting pellet was resuspended in 4 ml receptor binding buffer (50 mM Tris–HCl, pH 7.4, and 4 mM MgCl) using a Kinematica homogenizer at a setting of 6 for 5 s before 1.0 ml aliquots were centrifuged again at 13,000×*g* for 10 min. The pellets were stored at −80°C until use.

Pellets were then resuspended for use by trituration in receptor binding buffer (50 μg protein/100 μl) and added in duplicate to assay tubes containing 0.1–0.2 nM [^{3}H] spiperone and appropriate drugs. Non-specific binding was determined using 5 μM (+)-butaclamol. Assay tubes were incubated at 37°C for 30 min before filtration, as described for cAMP binding assays. Filter plates were dried, 30 μl of Packard Microscint O scintillation fluid was added to each well, and radioactivity per well was determined using a Packard TopCount scintillation counter.

Data analysis

Data from the drug discrimination study were scored in a quantal fashion with the lever on which the rat first emitted 50 presses in a test session scored as the "selected" lever. The percentage of rats selecting the drug lever (%SDL) for each dose of test compound was determined. Full, partial, and no substitution were statistically determined using a binomial test (Zar 1999). This test is very conservative, and additional details for the use of the binomial test to analyze quantal drug discrimination data are provided in our earlier publication (Marona-Lewicka et al. 2005). If the drug was one that completely substituted for the training drug, the method of Litchfield and Wilcoxon (1949) was used to determine the ED_{50} and 95% confidence interval (95% CI).

GraphPad Prism software was used to generate dose–response and competition binding curves (GraphPad Software, San Diego, CA, USA). Data from cAMP inhibition assays were normalized to percent maximum forskolin-stimulated cAMP accumulation. IC_{50} values were generated by GraphPad Prism using the sigmoidal dose–response (variable slope) equation. $K_{0.5}$ values for agonists were calculated from IC_{50} values by GraphPad Prism using the Cheng–Prusoff equation (Cheng and Prusoff 1973).

Results

Generalization tests with D_4 agonists

Figure 1 shows results from generalization tests for the highly selective D_4 dopamine agonists ABT-724 (Cowart et al. 2004) and for the mixed 5-HT_{1A} antagonist/D_4 agonist WAY100635 (Chemel et al. 2006a) tested in DOI (Fig. 1a), LSD-30 (Fig. 1b), and LSD-90 (Fig. 1c) rats. Neither compound mimicked DOI at any dose tested, although WAY 100635 produced a significantly higher percentage of disruption than ABT-724 in DOI-trained rats. Data from generalization tests for both D_4 receptor agonists were presented earlier (Marona-Lewicka and Nichols 2007), but we included them for comparison with results from substitution tests in DOI-trained rats. ABT-724 produced partial substitution in both LSD-30- and LSD-90-trained rats, with a bell-shaped dose–response curve in LSD-30 rats (62.5% maximum %SDL). The doses of WAY 100635 required for effects in the generalization tests were about ten times larger than those of ABT-724. To test for significant differences in the maximum degree of WAY 100635 substitution in each colony of rats, the Pearson χ^2 statistic (8.325, 2 *df*) gave $p=0.016$, indicating that the proportion of responding rats in these three conditions is not plausibly the same. Further, maximal drug-appropriate responding in the LSD-30 rats is intermediate between the DOI and LSD-90 rats, tested using a χ^2 test for linear trend (χ^2, 1 $df=8.27$, $p=0.004$).

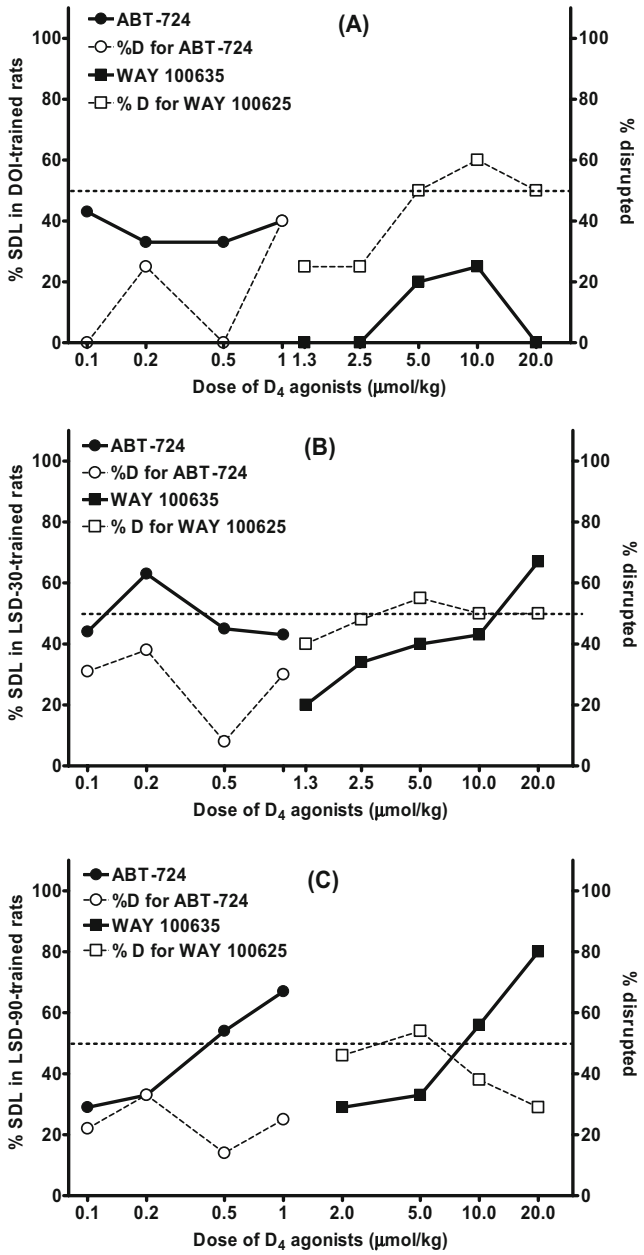

Fig. 1 Results from substitution tests with the dopamine D_4 agonist ABT 724 and 5-HT_{1A} antagonist/D_4 agonist WAY 100635 performed in rats trained to discriminate DOI (**a**, *upper panel*), LSD-30 (**b**, *middle panel*), and LSD-90 (**c**, *bottom panel*) rats. All drugs were injected 30 min before tests. %SDL is the percentage of rats that selected the drug-appropriate level. *N*=6–14 rats per data point. Right *Y*-axis and *open symbols* show the percentage of rats disrupted. Data from LSD-trained rats were presented in Marona-Lewicka and Nichols (2007)

Results from combination tests of different doses of dopamine D_4 antagonists on discrimination of the training drugs

Pretreatment with WAY 100635 had no effect on the discriminative stimulus of DOI, although the highest dose of WAY 100635 inhibited the response rate, producing as much as 75% disruption (Fig. 2a). The lowest doses of WAY 100635 had an inhibitory effect LSD-30, but this effect was lost as the dose was increased, with no effect at the two highest doses of WAY 100635 (10 and 20 µmol/kg; Fig. 2b). The low dose of WAY 100635 (0.74 µmol/kg) produced the greatest inhibitory effect against the LSD-30 cue, whereas the high dose (10 µmol/kg) had no effect on the stimulus effects of the training dose of LSD in LSD-30 rats. This divergent effect of WAY 100635 on the LSD-30 cue prompted us to use two different doses of WAY 100635 in subsequent combination tests with different doses of LSD.

In contrast to the marked inhibition by low doses of WAY 100635 on the LSD-30 cue, it inhibited the LSD-90 cue by only 20% at the lowest dose (Fig. 2c). This inhibition was not statistically significant because 80% of the rats were still able to emit drug-appropriate responses, a condition scored as full substitution. Moreover, WAY 100635 did not produce significant behavior disruption in LSD-90-trained rats (Fig. 2c).

The selective D_4 antagonist L-745,870 (Patel et al. 1997) had no effect on DOI discrimination (Fig. 2a), but produced significant disruption of behavior. L-745,870 partially inhibited LSD-30 (Fig. 2b), but more strongly antagonized the discriminative stimulus effects in LSD-90 rats (Fig. 2c). Similar to the results with WAY 100635, this combination did not produce significant behavioral disruption. Unfortunately, the limited amount of L 745,870 available precluded testing additional doses. Another selective D_4 antagonist A381393 produced effects similar to L-745,870 (Fig. 2a–c) in all groups of rats. The 10-µmol/kg dose of A381393 produced significant inhibitory effects in LSD-90 rats, induced a relatively low percentage of disruption, and therefore was chosen for more extensive combination tests.

The atypical antipsychotic drugs clozapine and olanzapine, which have both 5-HT_{2A} and dopamine D_4 antagonist activity (Roth et al. 1995; Meltzer 1999; Seeman et al. 1997; Richelson and Souder 2000), showed a significant inhibitory effect when tested in combination with training drugs in all three groups of rats (Fig. 2). In combination with DOI (Fig. 2a) or LSD-30 (Fig. 2b), however, they also produced a high percentage of disruption. In contrast, clozapine and olanzapine blocked drug-appropriate lever selection in more than 80% of LSD-90-trained rats without affecting their response rate (Fig. 2c). The inhibitory effects of these atypical antipsychotics, based on the data presented in Fig. 2, were strongest against the LSD-90 cue, were less pronounced against the LSD-30 cue, and only partially inhibited the DOI cue.

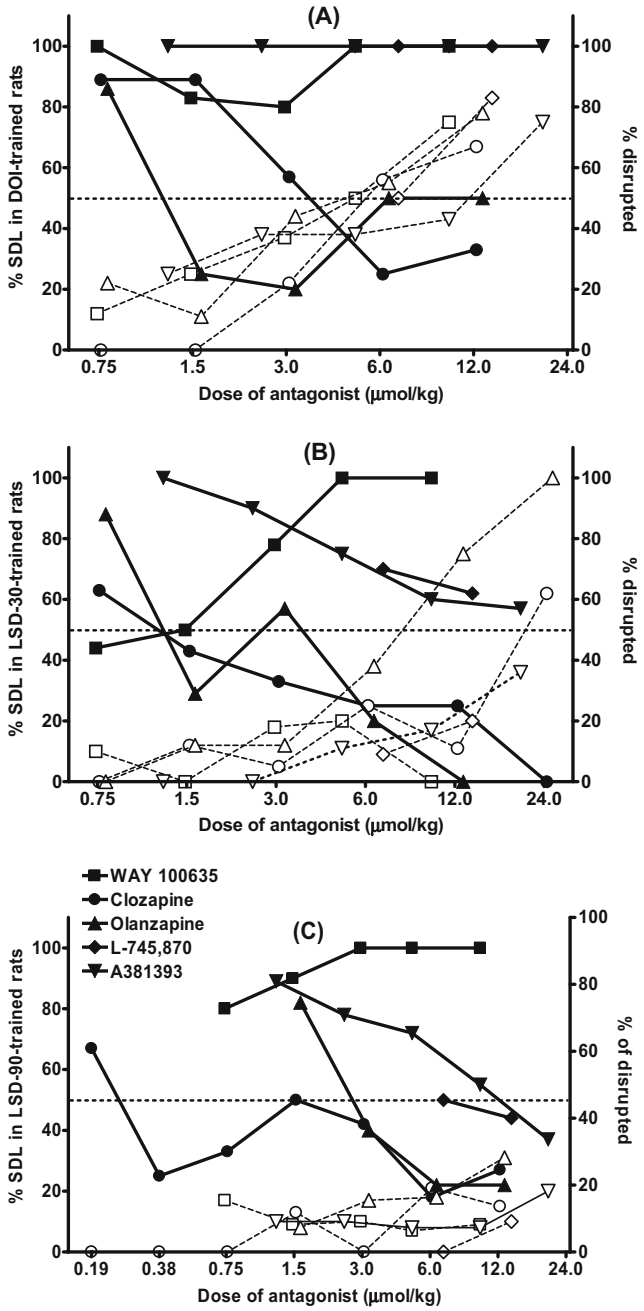

Fig. 2 Results from combination tests of different doses of the dopamine D_4 antagonists: L-745,870 (*diamonds*) and A381393 (*inverted triangles*), the 5-HT_{1A} antagonist/D_4 agonist WAY 100635 (*squares*), and the atypical antipsychotic drugs: clozapine (*circles*), and olanzapine (*triangles*) with 1.12 μmol/kg of DOI in DOI-trained rats (**a**, *upper panel*), 186 nmol/kg of LSD in LSD-30-trained rats (**b**, *middle panel*), and 372 nmol/kg of LSD in LSD-90-trained rats (**c**, *bottom panel*). All drugs were injected 30 min prior to the training drug. %SDL is the percentage of rats that selected the drug-appropriate lever. Right *Y*-axis and *open symbols* show the percentage of rats disrupted. *N*=6–20 rats per data point

Results of combining the dopamine D_4 agonist ABT-724 with different doses of training drugs

Co-administration of ABT-724 with DOI (Fig 3a) significantly decreased drug-appropriate lever selection for the 0.28 and 0.56 μmol/kg doses of DOI, but was without

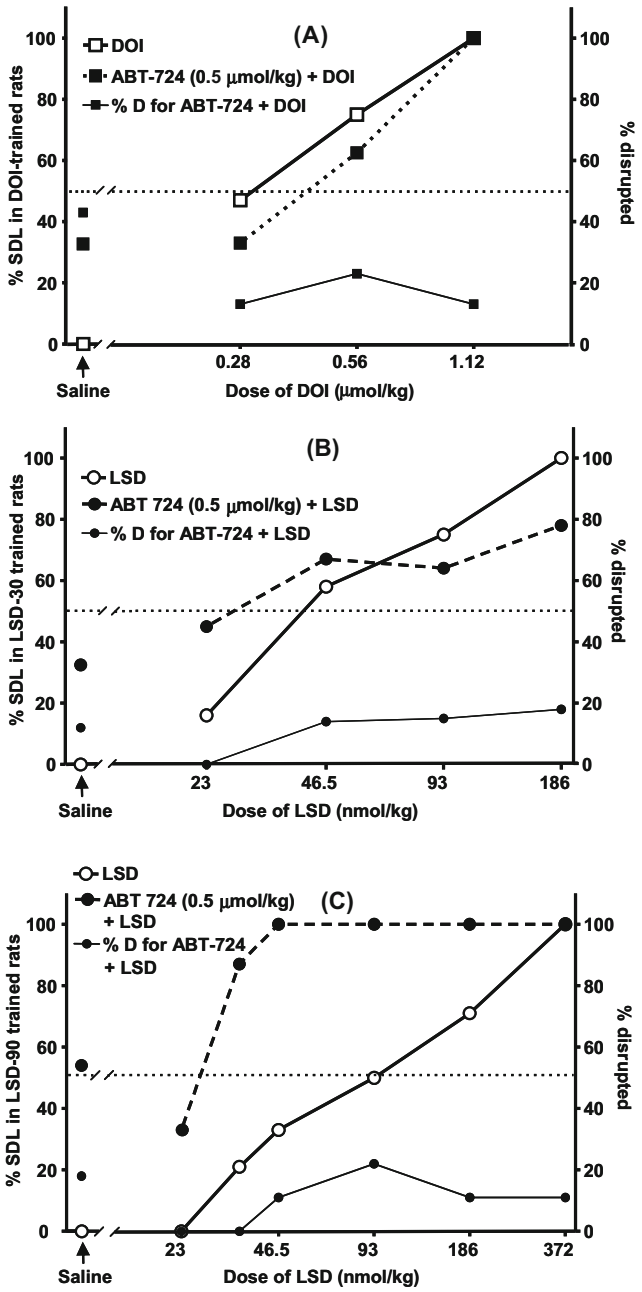

Fig. 3 Results from combination tests of the dopamine D_4 agonist ABT 724 (0.5 μmol/kg, 0.2 mg/kg) with different doses of DOI in DOI-trained rats (**a**, *upper panel*), with different doses of LSD in LSD-30-trained rats (**b**, *middle panel*), and in LSD-90-trained rats (**c**, *bottom panel*). ABT 724 was injected 30 min before the training drug. %SDL is the percentage of rats that selected the drug-appropriate lever. *N*=8–16 rats per data point. There was 0% disruption for DOI, LSD-30 and LSD-90 alone for all doses tested

effect when combined with the 1.12 μmol/kg training dose of DOI. The ED_{50} for this combination was not significantly different than for DOI alone (Table 1).

Co-administration of the D_4 dopamine agonist ABT-724 with LSD in LSD-30 rats produced a bimodal effect (Fig. 3b). We observed potentiation of the discriminative effect at low doses of LSD, but attenuation of drug-appropriate responding at higher doses of LSD.

The effect of ABT-724 on the LSD-90 cue was, however, quite remarkable, with significant potentiation of the LSD effect (Fig. 3c). The ED_{50} of LSD for this combination is more than twofold lower than for LSD-90 alone (Table 1). Yet, the shift in ED_{50} fails to convey the very steep dose–response curve evident in Fig. 3c.

The results of combining a low dose of the 5-HT_{1A} antagonist/dopamine D_4 agonist WAY 100635 with different doses of training drugs

Administration of 0.74 μmol/kg of WAY 100635 prior to DOI decreased drug-appropriate responding only for the 0.56 μmol/kg dose of DOI (Fig. 4a), whereas it was without effect either at the lower or higher dose of DOI.

Figure 4b shows the effect of combining different doses of LSD with 0.74 μmol/kg of WAY 100635 administered 30 min before LSD (60 min before testing). At this dose, WAY 100635 slightly increased the %SDL when combined with the lower 23 and 46.5 nmol/kg doses of LSD, but significantly decreased drug-appropriate responding in LSD-30 rats when combined with higher LSD doses, although the difference between the ED_{50} for the combination and for LSD alone was not significant (Table 1). Moreover, this combination did not produce a simple rightward shift of the dose-dependent response. The cue produced by the combination of WAY 100635 plus LSD is not parallel to the cue generated by LSD alone, suggesting that a mechanism different from simple antagonism is involved. Similar results were observed in LSD-90-trained rats (Fig. 4c), but a combination of 0.74 μmol/kg of WAY 100635 with 93 nmol/kg of LSD in LSD-90 rats was without effect on drug-appropriate responding, whereas in LSD-30 rats, the same combination significantly decreased drug-appropriate lever selection.

The results of combination tests of a high dose of the 5-HT_{1A} antagonist/dopamine D_4 agonist WAY 100635 with different doses of training drugs

Combinations of 10 μmol/kg of WAY 100635 with DOI (a), LSD-30 (b), and LSD-90 (c) gave the results shown in Fig. 5. Pretreatment with the high dose of WAY 100635 produced a leftward but non-significant shift of the dose–response curve for both DOI and LSD-30. By contrast, the 10-μmol/kg dose of WAY 100635 significantly enhanced drug-appropriate lever selection when combined with different doses of LSD in LSD-90 rats (Fig. 5c), with a significant difference between the ED50 for this combination versus LSD-90 alone (Table 1).

The results of combination tests of the dopamine D_4 antagonist A-381393 with different doses of training drugs

Results from combination tests with the selective dopamine D_4 receptor antagonist A-381393 are presented in Fig. 6. A-381393 had no effect on the DOI cue (Fig. 6a), but pretreatment with 10 μmol/kg before different doses of LSD in LSD-30 (Fig. 6b) or in LSD-90 (Fig. 6c) rats inhibited drug-appropriate lever selection, especially at the higher doses of LSD. In LSD-90-trained rats, the combination of A-381393 with LSD produced a more than sixfold shift to the right compared with LSD alone (Table 1).

Table 1 The ED_{50} values and 95% CI (shown in parenthesis) determined using the method of Litchfield and Wilcoxon (1949) for LSD and DOI alone and in combination with dopamine D_4 receptor agonists and antagonists tested in DOI-, LSD-30-, and LSD-90-trained rats

Training drug tested in combination with	Tested in rats trained with		
	DOI (μmol/kg)	LSD-30 (nmol/kg)	LSD-90 (nmol/kg)
DOI	0.32 (0.23–0.44)		
LSD		44.6 (25–77)	57.3 (37–87)
+ ABT-724 (0.5 μmol/kg)	0.39 (0.25–0.62)	52.2 (22–116)	26.5 * (22–32)
+WAY 100635 (0.74 μmol/kg)	0.35 (0.22–0.64)	61.2 (10–345)	56.7 (26–159)
+WAY 100635 (10 μmol/kg)	Not calculated[a]	23.2 (14–37)	17.2 * (7–31)
+A-381393 (10 μmol/kg)	0.46 (0.22–0.84)	93.4 (21–412)	367 (68–988)

The "+" symbol before the drug name indicates that this drug was combined with different doses of DOI or LSD and tested in DOI-, LSD-30, or LSD-90-trained animals. N=8–16 rats per dose of drugs

*P<0.05 compared to LSD alone

[a] Could not be calculated with only two data points

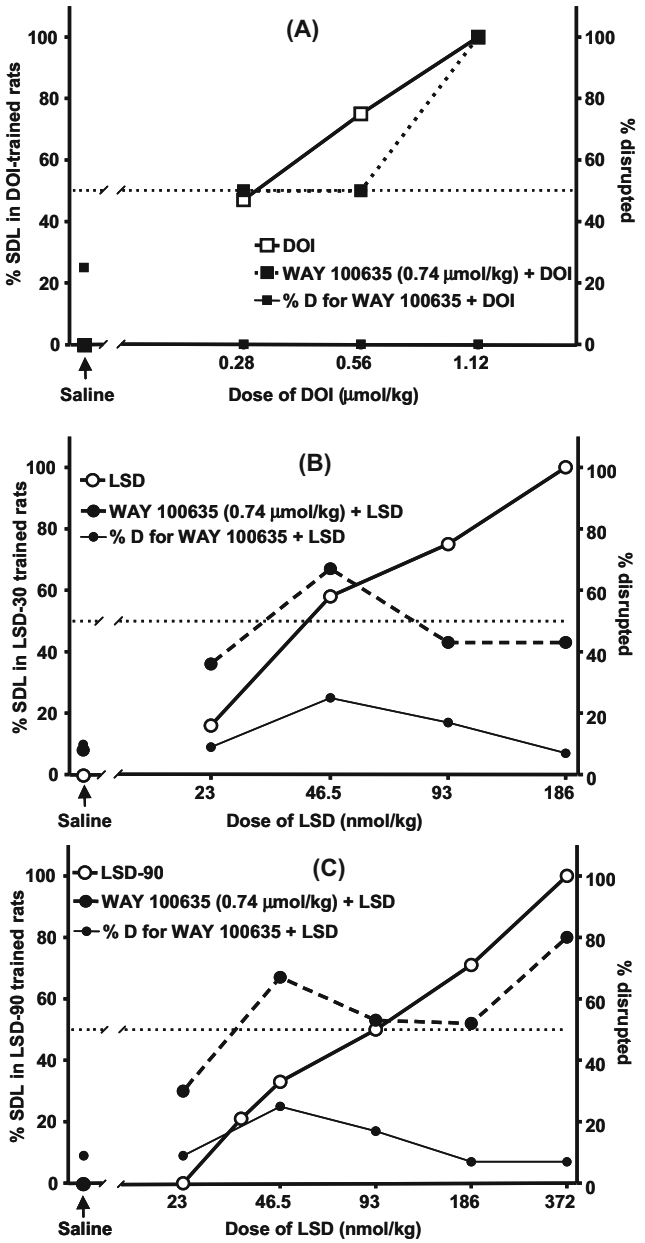

Fig. 4 Results from combination tests with the low dose (0.74 µmol/kg; 0.4 mg/kg) of WAY 100635 and different doses of DOI in DOI-trained rats (**a**, *upper panel*), with different doses of LSD in LSD-30-trained rats (**b**, *middle panel*), and in LSD-90-trained rats (**c**, *bottom panel*). WAY 100635 was administered 30 min before the training drug. %SDL is the percentage of rats that selected the drug-appropriate lever. N=8–16 rats per data point. There was 0% disruption for DOI, LSD-30, and LSD-90 alone for all doses tested

WAY 100635, dopamine D_4 receptor antagonists, and atypical antipsychotic drugs have significant affinity at the dopamine D_4 receptor

Table 2 presents data from radioligand competition binding experiments and from inhibition of forskolin-stimulated cAMP accumulation in HEK cells stably expressing the human dopamine $D_{4.4}$ receptor. Competition binding experiments were performed with [^{3}H]spiperone to label the h$D_{4.4}$ receptor; this radioligand had a K_d of 0.11±0.01 nM in these cells. LSD, like quinpirole, acts as full agonist (Fig. 7), dose-dependently inhibiting forskolin-stimulated cAMP accumulation. LSD is more potent than ABT-724,

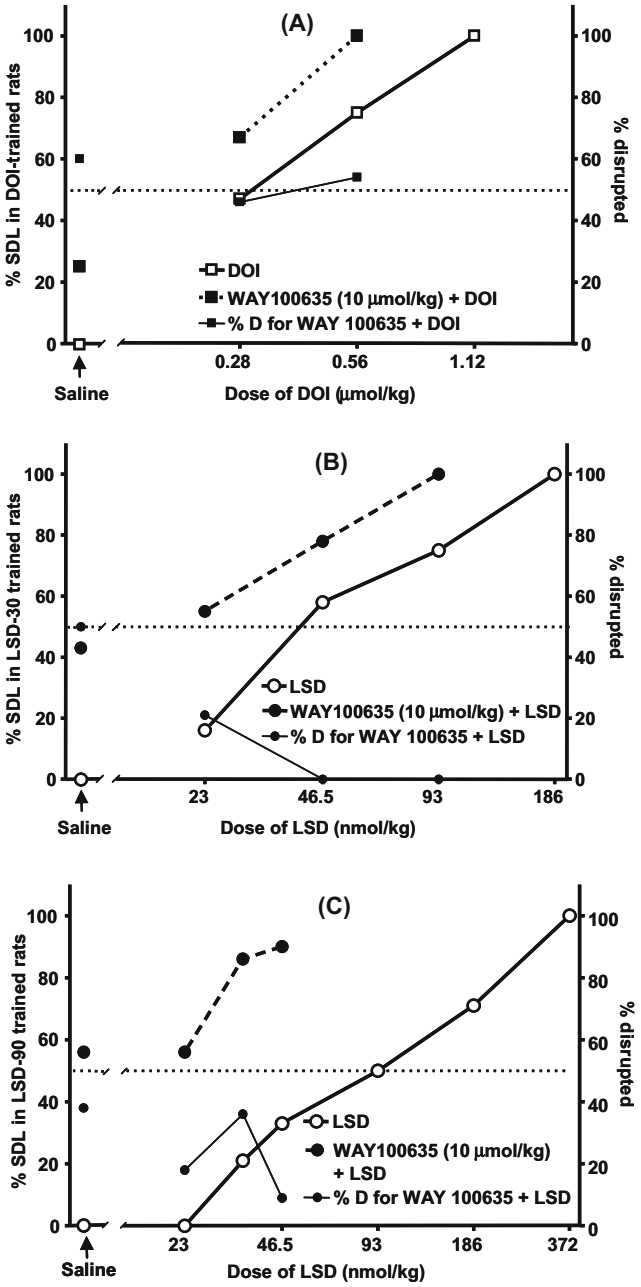

Fig. 5 Results from combination tests with the higher dose of WAY 100635 (10 µmol/kg; 5.4 mg/kg) and different doses of DOI in DOI-trained rats (**a**, *upper panel*), with different doses of LSD in LSD-30-trained rats (**b**, *middle panel*), and in LSD-90-trained rats (**c**, *bottom panel*). WAY 100635 was administered 30 min before the training drug. %SDL is the percentage of rats that selected the drug-appropriate lever. N=8–16 rats per data point. There was 0% disruption for DOI, LSD-30, and LSD-90 alone for all doses tested

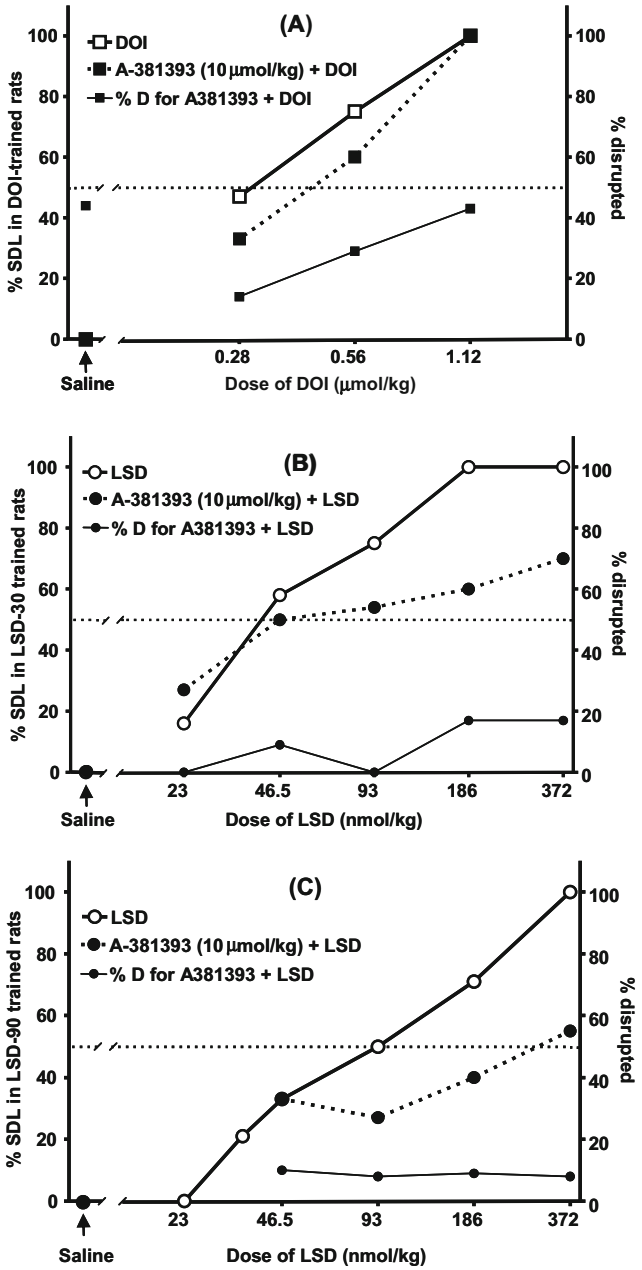

Fig. 6 Results from combination tests of the dopamine D_4 antagonist A381393 (10 µmol/kg, 4.37 mg/kg) with different doses of DOI in DOI-trained rats (**a**, *upper panel*), with different doses of LSD in LSD-30-trained rats (**b**, *middle panel*), and in LSD-90-trained rats (**c**, *bottom panel*). A381393 was administered 30 min before the training drug. %SDL is the percentage of rats that selected the drug-appropriate lever. N=8–16 rats per data point. There was 0% disruption for DOI, LSD-30, and LSD-90 alone for all doses tested

which shows only partial agonist activity at the D_4 receptor. We reported earlier (Chemel et al. 2006a) that WAY 100635 behaves as a full agonist at human $D_{4.4}$ receptors stably expressed in HEK cells, with efficacy and affinity comparable to quinpirole. In contrast to LSD, the hallucinogenic amphetamine derivative DOI had no detectable affinity at the $D_{4.4}$ receptor. For the D_4 receptor antagonists used in the drug discrimination experiments, the most potent is L-745,870, showing more than an order of magnitude higher affinity for the $D_{4.4}$ receptor than the atypical antipsychotic drug clozapine.

Discussion

Our main finding is that stimulation or inhibition of the dopamine D_4 receptor has a significant modulatory effect on the discriminative stimulus properties of LSD, but not the discriminative stimulus effect produced by the hallucinogenic phenethylamine DOI. The role of the D_4 receptor is most pronounced in the later temporal effects of LSD (in LSD-90 rats). Our earlier results had provided evidence that the delayed temporal phase in the behavioral pharmacology of LSD is mediated by D_2-like dopamine receptor activation (Marona-Lewicka et al. 2005, Marona-Lewicka and Nichols 2007), and results from this study identify the D_4 receptor as the relevant D_2-like isoform.

Thus, we evaluated the effect of stimulation or inhibition of dopamine D_4 receptors on $5HT_{2A}$ (DOI and LSD-30) or D_2-like (LSD-90)-mediated discriminative stimulus effects. We demonstrate that the interoceptive cue of LSD, when given 90 min before training, is mediated through activa-

Table 2 Effects at cloned human dopamine $D_{4.4}$ receptors for compounds used in the drug discrimination assay

Compound	Intrinsic activity (%)	EC_{50} (nM)	$K_{0.5}$ (nM)
Dopamine	100±2.3[a]	3.0±0.5[a]	
Quinpirole	100±3[a]	9.6±2.4[a]	16.2±2
LSD	101±7	11.1±2.7	10.3±0.9
WAY 100635	98±2.3[a]	9.7±2.2[a]	6.7±1.1
ABT-724	76±5	122±2.3	61±3.7[b]
DOI	N.D.	N.D.	>10,000
L-745,870	N.D.	N.D.	0.44±0.05[c]
A381393	N.D.	N.D.	1.5±0.3[c]
Clozapine	N.D.	N.D.	5.2±0.2
			33±3.0[d]
Olanzapine	N.D.	N.D.	26±3.2[d]

Affinities ($K_{0.5}$) at the human dopamine $D_{4.4}$ receptor stably expressed in HEK cells were determined from competition binding experiments with [^{3}H]spiperone. Agonist potencies (EC_{50}) and intrinsic activities were determined by inhibition of forskolin-stimulated cyclic AMP accumulation. Intrinsic activity is expressed relative to that of dopamine (100%). Results are expressed as mean±SEM of at least three independent experiments.

N.D. not determined

[a] All of the data for these compounds were obtained in parallel experiments, done at the same time, but these results for dopamine, quinpirole, and WAY 100635 were published in Chemel et al. (2006a).

[b] Data from Brioni et al. 2004

[c] Data from Nakane et al. 2005

[d] Data from Newman-Tancredi et al. 1997 (used $D_{4.4}$ receptors stably expressed in CHO cells)

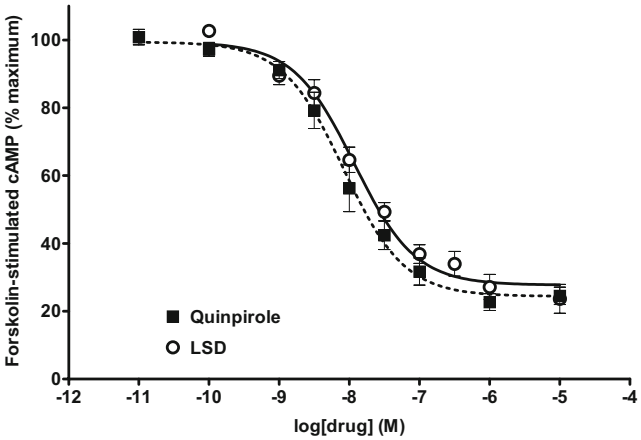

Fig. 7 Dose–response curves for $D_{4.4}$ receptor-mediated inhibition of forskolin-stimulated cyclic AMP accumulation. HEK-h$D_{4.4}$ cells were incubated with 5 μM forskolin (*FSK*) in the presence of increasing concentrations of the indicated agonists for 15 min at 37°C. Experiments were performed in duplicate. Data from each assay were normalized to percent of maximum cAMP accumulation stimulated by forskolin, as estimated from the upper limits of dose–response curves generated by GraphPad Prism. Data points represent mean±SEM of combined data from at least four experiments

tion of dopamine D_4 receptors. Consistent with that conclusion, the selective dopamine D_4 receptor antagonists L-745,870 and A-381393 significantly attenuated the LSD-90 cue to a somewhat greater extent than LSD-30 responding and had no effect on the DOI cue. The D_4 dopamine full agonist WAY 100635 mimics the LSD-90 cue, produces only partial substitution in LSD-30 rats, and only saline-appropriate responding in DOI-trained rats. Finally, the partial D_4 agonist ABT-724 produced no substitution in DOI-trained rats, a bell-shaped dose–response curve in LSD-30 rats, and partial substitution in LSD-90 trained rats.

WAY 100635 has highest affinity for the 5-HT_{1A} receptor (Fletcher et al. 1996; Forster et al. 1995) and for more than a decade has been widely used as the selective antagonist of choice for this receptor. Although WAY 100635 has tenfold higher affinity for the 5-HT_{1A} than for the D_4 receptor (Chemel et al. 2006a), at higher doses, it induces behavioral effects mediated by D_4 receptor activation (Chemel et al. 2006b). Although Martel et al. (2007) have recently suggested that WAY 100635 is only a very weak partial D_4 agonist with greater than 200-fold selectivity for the 5-HT_{1A} receptor, our behavioral results are not consistent with that characterization and are more in line with our earlier report that the selectivity at the 5-HT_{1A} receptor versus the D_4 dopamine receptor is only about ten-fold (Chemel et al. 2006a). Low doses of WAY 100635 did not produce a discriminative stimulus effect in rats, as rats were unable to discriminate between WAY 100635 and saline. By contrast, at higher doses, WAY 100635 generates a discriminative stimulus that is not mimicked by other 5-HT_{1A} antagonists, is blocked by D_4 antagonists, and is not reversed by co-administration of 5-HT_{1A} agonists (unpublished). It also has been proposed that 5-HT_{1A} receptor agonists are able to modulate the discriminative stimulus effects of LSD given at short times before testing (Reissig et al. 2005). Consistent with all these findings, in the present study, low doses of WAY 100635 were able to attenuate partially the LSD-30 cue, but not the DOI cue.

In the combination tests, we have clearly demonstrated that co-administration of LSD with either a low or high dose of WAY 100635 has divergent effects. Pretreatment with 0.4 mg/kg of WAY 100635 flattened the LSD dose–response curve in the LSD-30 group by partially attenuating drug-appropriate responding, most notably at higher doses of LSD. A similar but less marked effect was observed with the LSD-90 cue, whereas the low dose of WAY 100635 had no effect on the DOI cue. It therefore seems reasonable to speculate that the effect of the low dose of WAY on the LSD cue is mediated by its 5-HT_{1A} receptor antagonism.

Although conflicting results have emerged from a variety of behavioral paradigms employed to study interactions between 5-HT_{1A} and 5-HT_{2A} receptors, Reissig et al. (2005) have reported that in their drug discrimination studies, 5-HT_{1A} receptor agonists enhanced the discriminative stimulus effects of LSD and 5-HT_{1A} antagonists attenuated it. Recently, Reissig et al. (2008) have reported that antagonism of the 5-HT_{2A} receptor attenuated a 5-HT_{1A}-mediated drug discrimination cue, and the authors suggested that in drug discrimination studies, the interaction between 5-HT_{1A} and 5-HT_{2A} receptors is bidirectional. In our laboratory, we were never able to potentiate the LSD cue by co-administration of a 5-HT_{1A} agonist (unpublished results) because this combination induces a serotonin syndrome that interferes with lever responding, leading to behavioral disruption in our FR50 paradigm.

In combination tests with the D_4 partial agonist ABT 724, we observed enhancement of the discriminative stimulus effect of LSD-30 only at low doses, but robust potentiation of the LSD cue in LSD-90 rats. Combination of 10 μmol/kg of WAY 100635 with LSD or DOI resulted in leftward shifts of the dose–response curves quite markedly in LSD-90 rats and less so in LSD-30- and DOI-trained rats. We believe that the high degree of potentiation observed in LSD-90-trained rats occurs because the primary mechanism responsible for the discriminative stimulus effects in these rats occurs through stimulation of D_2-like receptors (Marona-Lewicka et al. 2005), the dopamine D_4 receptor being a member of the D_2 receptor family.

A different mechanism is likely responsible for enhancement of the 5-HT_{2A}-mediated cue. It is possible that stimulation of D_4 dopamine receptors potentiates the

function of 5-HT_{2A} receptors. The dopamine D_4 receptor mediates changes in neuronal excitability and synaptic plasticity in the brain (Rubinstein et al. 2001; Wang et al. 2002, 2003) and is thought to play a major role in the control of integrative functions underlying the organization of complex behavior (Fuster 2001). Dopamine D_4 receptors are localized on cortical pyramidal neurons, with particularly high expression in the anterior cingulate as well as on GABAergic interneurons throughout the frontal cortex (Mrzljak et al. 1996; Wedzony et al. 2000); some cortical GABAergic interneurons also express 5-HT_{2A} receptors (Griffiths and Lovick 2002; de Almeida and Mengod 2007). D_4 receptor activation attenuates both GABA-mediated inhibition of medial prefrontal cortex pyramidal neurons and *N*-methyl-D-aspartic-acid-mediated synaptic responses (Seamans et al. 2001; Wang et al. 2002, 2003). Another mechanism for 5-HT_{2A} interactions with dopaminergic systems that has been proposed includes the possibility that 5-HT_{2A} receptors might act as presynaptic heteroreceptors on dopamine axon terminals (Pehek 1996; Pehek et al. 2001), a hypothesis that finds support in the study by Miner et al. (2003), showing that in the prefrontal complex (PFC), some 5-HT_{2A} receptors are localized on dopaminergic terminals. Thus, dopamine D_4 receptors are co-localized in some of the same cortical layers where 5-HT_{2A} receptors are highly expressed and might directly or indirectly modulate 5-HT_{2A} receptor function.

Dopamine D_4 receptor stimulation can activate multiple intracellular pathways, including inhibition of cAMP synthesis (Seamans and Yang 2004) and modulation of G-protein-regulated ion channels (Pillai et al. 1998; Wedemeyer et al. 2007). Moreover, one of the important targets of dopamine D_4 receptors is calmodulin-dependent protein kinase II (CaMKII), which distinguishes D_4 receptor signaling from dopamine D_2 signaling (Wang et al. 2003; Gu and Yan 2004; Gu et al 2006). In PFC neurons, D_4 receptor stimulation increases CaMKII activity through phospholipase C (PLC)/inositol-1,4,5-triphosphate (IP_3)-receptor-dependent pathways, resulting in elevation of intracellular Ca^{2+}. This effect is similar to Ca^{2+} release from intracellular stores through activation of G_q-coupled receptors, the family to which the 5-HT_{2A} receptor belongs. Thus, D_4 receptor activation might enhance 5-HT_{2A}-mediated effects through the PLC/IP_3 pathway.

It is well known that atypical antipsychotics, such as clozapine and olanzapine, have high affinities for the 5-HT_{2A} and dopamine D_2 and D_4 receptors (Roth et al. 1995; Meltzer 1999; Seeman et al. 1997; Richelson and Souder 2000). We examined the ability of these drugs to affect the DOI, LSD-30, and LSD-90 cues, assuming that the antagonistic promiscuity of these antipsychotics might be important to explain differences between mechanisms responsible for blockade of discriminative stimulus effects. Both clozapine and olanzapine partially inhibited the DOI cue, and we hypothesize that the antagonist properties of clozapine and olanzapine at the 5-HT_{2A} receptor are responsible for inhibition of the DOI cue, whereas their D_2 and D_4 antagonism is responsible for the marked behavior disruption observed during these combination tests. In LSD-30- and LSD-90-trained rats, clozapine and olanzapine produced dose-dependent full inhibition with limited behavioral disruption in LSD-30 rats and no disruption in LSD-90 rats. We call attention to the very high potency of clozapine in blocking the LSD-90 cue and note that clozapine is considered to be one of the most efficacious atypical antipsychotics.

In our previous paper (Marona-Lewicka et al. 2005), we showed that MDL 100907, a selective 5-HT_{2A} antagonist, produced some degree of LSD-90 cue inhibition, and haloperidol, a nonselective dopamine receptor antagonist, also slightly attenuated drug-appropriate lever selection in LSD-30 rats. Thus, we conclude that although activation of 5-HT_{2A} receptors plays a central role in the discriminative stimulus effects in LSD-30 rats, a minor dopaminergic pharmacology component is also probably involved. By contrast, D_2-like receptor activation, presumably the D_4 receptor, is the central element in the LSD-90 discriminative stimulus, but the 5-HT_{2A} receptor also must play some role. As was noted earlier, pretreatment with 5-HT_{2A} agonists can dramatically potentiate the discriminative stimulus effects of amphetamine (Marona-Lewicka and Nichols 1997) and methamphetamine (Munzar et al. 1999, 2002) and slightly enhances the cocaine cue (Munzar et al. 2002) in rats. If activation of 5-HT_{2A} receptors serves to sensitize the dopamine system, it seems possible that the 5-HT_{2A} agonist effect of LSD is a necessary but not sufficient condition for the strength of the LSD-90 cue.

In summary, the results presented here suggest that the dopamine D_4 receptor plays a modulatory role in the discriminative stimulus effects produced by LSD, a drug with complex pharmacological properties. We also show that manipulation of D_4 dopamine receptors has a much more marked effect on the discriminative cue generated during the later temporal phase of LSD action than for discriminative stimulus effects occurring 15–30 min after LSD administration. No similar role of the D_4 receptor was evident for the hallucinogenic amphetamine DOI, which possesses affinity only at the 5-HT_2 family receptors. The involvement of both dopamine and serotonin pathways in the pharmacology of LSD, but not DOI, is very intriguing given the vast body of research demonstrating that LSD is a more potent hallucinogen than phenethylamines, even though their affinities at the 5-HT_{2A} receptor are not significantly different, with the intrinsic activity of LSD actually being lower than DOI at these receptors (Nichols 2004).

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