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Serotonergic and dopaminergic distinctions in the behavioral pharmacology of (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) and lysergic acid diethylamide (LSD)☆

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ABSTRACT

Rationale: After decades of social stigma, hallucinogens have reappeared in the clinical literature demonstrating unique benefits in medicine. The precise behavioral pharmacology of these compounds remains unclear, however.
Objectives: Two commonly studied hallucinogens, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) and lysergic acid diethylamide (LSD), were investigated both *in vivo* and *in vitro* to determine the pharmacology of their behavioral effects in an animal model.
Method: Rabbits were administered DOI or LSD and observed for head bob behavior after chronic drug treatment or after pretreatment with antagonist ligands. The receptor binding characteristics of DOI and LSD were studied *in vitro* in frontocortical homogenates from naïve rabbits or *ex vivo* in animals receiving an acute drug injection.
Results: Both DOI- and LSD-elicited head bobs required serotonin$_{2A}$ (5-HT_{2A}) and dopamine$_1$ (D_1) receptor activation. Serotonin$_{2B/2C}$ receptors were not implicated in these behaviors. *In vitro* studies demonstrated that LSD and the 5-$HT_{2A/2C}$ receptor antagonist, ritanserin, bound frontocortical 5-HT_{2A} receptors in a pseudo-irreversible manner. In contrast, DOI and the 5-$HT_{2A/2C}$ receptor antagonist, ketanserin, bound reversibly. These binding properties were reflected in *ex vivo* binding studies. The two hallucinogens also differed in that LSD showed modest D_1 receptor binding affinity whereas DOI had negligible binding affinity at this receptor.
Conclusion: Although DOI and LSD differed in their receptor binding properties, activation of 5-HT_{2A} and D_1 receptors was a common mechanism for eliciting head bob behavior. These findings implicate these two receptors in the mechanism of action of hallucinogens.

1. Introduction

Although the mechanism of action of hallucinogens is incompletely understood, serotonin (5-HT) and the serotonin$_{2A}$ (5-HT_{2A}) receptor are thought to play a significant role in mediating their effects (Nichols, 2004). There are two major chemical classes fitting this profile — the phenethylamines (*e.g.* mescaline) and the tryptamines (*e.g.* lysergic acid diethylamide, psilocybin). Hallucinogens have significant value as pharmacological agents. They have been used to model psychosis and to better understand human cognition and perception (Nichols, 2004). Their role in the discovery of the serotonergic system has also proven invaluable (Nichols, 2004; Passie et al., 2008). These compounds have also demonstrated clinical utility in pain, drug addiction, headache, depression, and anxiety disorders (Griffiths et al., 2006, 2008; Grob et al., 2011; Kast and Collins, 1964; Mangini, 1998; Sewell et al., 2006). While the psychedelic effects of hallucinogens may be essential for some types of therapy, these and other physiologic side effects may preclude widespread clinical use of these drugs. As opposed to other recreational drugs, however, hallucinogens are not habit-forming in humans, nor are they reinforcing in animals (Chilcoat and Schutz, 1996; Passie et al., 2008). Deciphering the mechanisms by which hallucinogens exert their various effects will significantly benefit areas of basic and clinical science (Vollenweider and Kometer, 2010).

Drug-elicited head movement is a widely used behavioral model with which to investigate hallucinogens and there is a strong correlation between the dose of hallucinogens used to elicit mouse head twitch behavior and that used recreationally in humans (Corne and Pickering, 1967). Serotonin$_{2A}$ receptors have been implicated in head movements elicited by the phenethylamine hallucinogen, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI; Darmani et al., 1990; Dave et al., 2002, 2007; Schreiber et al., 1995; Willins and Meltzer, 1997), but a role for this receptor in the action of the indoleamine hallucinogen,

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lysergic acid diethylamine (LSD), which also elicits head movements, is not as clear. Serotonergic antagonists such as cyproheptadine (Vetulani et al., 1980), methysergide (Yamamoto and Ueki, 1981), and bromo-LSD (Sloviter et al., 1980) block LSD-elicited rodent head movements, but these antagonists are relatively non-selective for the 5-HT_{2A} receptor.

The report of the absence of head twitch behavior elicited by LSD in mice lacking 5-HT_{2A} receptors suggests that the 5-HT_{2A} receptor is necessary for LSD mediation of this behavior in this species (González-Maeso et al., 2007). The pharmacology that characterizes the head movement response in mice may be more complex than that for other animals, however. For example, 5-HT_{2C} receptors were not implicated in either DOI-elicited head shakes in rat (Schreiber et al., 1995) or head bobs in rabbit (Dave et al., 2002). In contrast, 5-HT_{2C} receptors contributed significantly to DOI-elicited head twitch behavior in mice (Canal et al., 2010). More precisely, Fantegrossi et al. (2010) demonstrated that 5-HT_{2C} receptor antagonism right shifted the descending limb of the DOI dose response curve in mice. These findings support an inhibitory role of 5-HT_{2C} activation at high doses of DOI (Fantegrossi et al., 2010). Further investigation with non-DOI hallucinogens, such as LSD, may help further explain the contribution of 5-HT_{2C} receptors in hallucinogen-elicited behavior.

Although 5-HT_{2A} receptors are found in many brain regions, direct infusion of the hallucinogen, DOI, into the frontal cortex of rats (Willins and Meltzer, 1997) or rabbits (Dave et al., 2007) has been shown to elicit head shakes and head bobs, respectively. Previous studies have also demonstrated that repeated systemic administration of DOI or LSD robustly down-regulates frontocortical 5-HT_{2A} receptors in both rats and rabbits (Aloyo et al., 2001; Smith et al., 1999). Thus, the frontocortical area is an appropriate brain region in which to investigate the role of 5-HT_{2A} receptors in mediating the effects of hallucinogens.

The dopaminergic system is believed to play a major role in human psychosis, a condition that hallucinogens have been shown to mimic (Nichols, 2004). Hallucinogens differ in their dopaminergic pharmacology, however. For example, LSD binds dopamine receptors, but DOI does not (Burt et al., 1976; Watts et al., 1995). Investigating the role of dopaminergic receptors in the animal head movement model would not only improve our understanding of hallucinogen pharmacology, but might also offer new insight into human psychosis. Presently, dopamine$_1$ (D_1) receptor antagonists are known to block DOI-elicited head shakes in rats (Schreiber et al., 1995), but the role of D_1 receptors in LSD-elicited head movement behavior has not been studied.

The present study compares and contrasts the 5-HT_{2A} and D_1 receptor actions of hallucinogens, represented by two chemical classes, the phenethylamines (DOI) and the indoleamines (LSD). The experiments include receptor binding properties and behavioral actions. The goal of these experiments is to identify the essential pharmacological components shared among hallucinogenic agents.

2. Materials and methods

2.1. Animals

Adult male New Zealand White rabbits (Covance; Devon, PA), weighing 1.8–2.2 kg upon arrival, were housed individually under a standard light–dark cycle of 12 h in an AAALAC-approved colony maintained at 22 ± 1 °C. Rabbits were fed 2/3 cup of rabbit chow daily and had unlimited access to water. Rabbits were adapted to the colony room and the experimenter (*via* handling) for several days before the initiation of experiments. Experiments were carried out in accordance with the "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research of the National Research Council" (2003) and were approved by the Institutional Animal Care and Use Committee (IACUC) of Drexel University College of Medicine.

2.2. Drugs and solutions

Ritanserin (FW 477.6), SCH23390-HCl (FW 324.2), prazosin-HCl (FW 419.86), (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI)-HCl (FW 357.6), and lysergic acid diethylamide (LSD base; FW 323.4) were purchased from Sigma-Aldrich (St. Louis, MO). Ketanserin tartrate (FW 554.5), SB206553-HCl (FW 328.8), and RS102221-HCl (FW 649.08) were purchased from Tocris Bioscience (Ellisville, MO). [^{3}H]ketanserin and [^{3}H]SCH23390 were purchased from Perkin–Elmer (Boston, MA). [^{3}H]mesulergine was purchased from GE Healthcare (Arlington Heights, IL) or American Radiolabeled Chemicals (St. Louis, MO). All other reagents and supplies were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma-Aldrich (St. Louis, MO). For *in vivo* injections: DOI was dissolved in physiological saline. LSD and ritanserin were dissolved in 2% tartaric acid, pH adjusted to approximately 6.5 with NaOH, and diluted with deionized water. All other drugs were dissolved in deionized water. All drugs were injected subcutaneously at a volume of 1 mL/kg, except the high dose of ketanserin (10 μmol/kg), which required an injection volume of 3.5 mL/kg. The literature primarily reports intravenous administration of LSD (Harvey and Gormezano, 1981; Welsh et al., 1998), but in order to match mode of administration of all other drugs, LSD was also administered subcutaneously. Preliminary studies demonstrated that LSD-elicited head bob behavior was independent of route of administration (subcutaneous or intravenous; unpublished data). LSD was injected at 30 nmol/kg because this was the maximally effective dose (Romano et al., 2010). DOI was injected at 300 nmol/kg because the response at this dose was less variable and equal to that of the maximally effective dose of 1000 nmol/kg (Dave et al., 2002). All pretreatment drugs (ketanserin, ritanserin, SB206553, SCH23390, or vehicle) were injected subcutaneously 1 h prior to hallucinogen administration. Doses of pretreatment drugs were chosen based on previously used doses from both rabbit (Dave et al., 2002; Simansky et al., 1998; Welsh et al., 1998) and rat (Schreiber et al., 1995) head movement studies. For membrane binding studies, all test compounds were dissolved in ethanol and diluted into assay buffer for a final alcohol concentration of 0.025%.

2.3. Experimental procedure

For all behavioral studies, animals were weighed, injected, and immediately replaced in their home cage, and their head bob behavior recorded for 60 min for later analysis. A head bob is a sequential down-up motion of the head without intervening behaviors (*e.g.* sniffing, chewing, hopping; Dave et al., 2002). Food and water were removed from the cage for behavioral recording. In antagonist pretreatment studies, a reduction in head bobs was considered an inhibition or rightward shift of the agonist response. Both DOI and LSD produced inverted "U" dose response curves in the rabbit (Dave et al., 2002; Romano et al., 2010). Behaviors such as lying down and staring increased with higher doses of hallucinogens (unpublished data). This downward turn of the curves likely reflects increasing systemic effects. Such behaviors were not seen when hallucinogens were preceded by antagonist administration (unpublished data). Furthermore, at the doses used, no antagonist produced head bobs or other behaviors alone (unpublished data). For chronic studies, DOI (3 μmol/kg) was injected once daily for 8 days. Twenty four hours following the last DOI treatment, rabbits were challenged by administering either DOI (300 nmol/kg) or LSD (30 nmol/kg) and their behavioral activity recorded for 1 h. Animals were then sacrificed and frontal cortex tissue harvested for later analysis of receptor density as described below. For acute studies, rabbits were administered antagonist (or vehicle) and replaced in their home cage for 60 min. Subsequently, DOI (300 nmol/kg), LSD (30 nmol/kg), or vehicle was administered, the rabbits were replaced in their home cage, and their behavior recorded for 60 min. Where indicated, animals were then sacrificed 10 min later *via* decapitation and frontal cortex

harvested for later binding analysis. For *in vitro* binding studies, drug-naïve rabbits were sacrificed *via* guillotine decapitation, their frontal cortices harvested and frozen immediately on dry ice for later use in receptor binding studies (see below). All *in vitro* binding experiments were performed in dim lighting in order to protect the structural integrity of the serotonergic ligands.

2.4. Washed membrane preparation

Frontal cortex tissue was used in all binding experiments due to the importance of this brain region in eliciting head movement behavior (Dave et al., 2007; Willins and Meltzer, 1997). Except as noted, the washed membrane fraction was prepared as previously described (Aloyo and Harvey, 2000). Frozen frontal cortex was placed in 10 volumes (by weight) of ice-cold homogenization buffer (50 mM Tris–HCl pH7.4 at 4 °C) and homogenized with a Brinkman Polytron (10 s at half power). The cortical homogenate was centrifuged at 40,000 *g* for 20 min at 4 °C. The resulting pellet was washed by resuspension in 50 volumes of homogenization buffer and centrifuged as described above. Where indicated, this standard tissue preparation was modified by the addition of a third wash step. Furthermore, in some experiments, the tissue preparation was warmed to 37 °C for 60 min between the 2nd and 3rd wash in an attempt to remove residual ligand. The final membrane fraction was then dispersed in room temperature assay buffer (20 or 50 mM Tris–HCl pH7.4 at 20 °C; 5 mM Mg^{2+} present in some assays).

2.5. Saturation binding analysis

Serotonin$_{2A}$ receptor density was quantified using the antagonist radioligand, [^{3}H]ketanserin (SA = 67 Ci/mmol), in the presence of RS102221 and prazosin (30 nM each) to block binding to 5-HT$_{2C}$ and α_1 adrenergic receptors, respectively. Saturation analysis was performed using eight concentrations of [^{3}H]ketanserin (0.02–4.0 nM). The assay was initiated by the addition of washed membranes derived from 4 mg of tissue prepared as described above for a final assay volume of 1 mL. The mixture was incubated for 120 min at 25 °C before being terminated by rapid filtration through Whatman GF/B filters (presoaked in 0.5% polyethylenimine) followed by four washes each consisting of 5 mL of wash buffer (20 mM Tris–HCl, pH 7.4 at 4 °C). The amount of radioactivity retained on the filter was then determined by liquid scintillation counting.

Serotonin$_{2C}$ receptor density was quantified using eight concentrations of the antagonist radioligand, [^{3}H]mesulergine (SA = 77 Ci/mmol; 0.02–4.0 nM), in the presence of spiperone (30 nM) to block binding to 5-HT$_{2A}$ and dopamine D$_2$ receptors. [^{3}H]mesulergine was dispensed using siliconized pipette tips and assays were carried out in glass tubes in order to prevent loss of the radioligand. The remainder of the assay was performed with the same procedure as described for 5-HT$_{2A}$ receptors.

Dopamine$_1$ receptor density was quantified using eight concentrations of the antagonist radioligand, [^{3}H]SCH23390 (SA = 85 Ci/mmol; 0.0035–0.45 nM), in the presence of ketanserin (30 nM) to block binding to 5-HT$_{2A}$ receptors. A 50 mM Tris–HCl assay buffer containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$ was used for D$_1$ receptor studies (Davidoff and Benes, 1998). The assay was initiated by the addition of washed membranes derived from 4 mg tissue in 50 mM Tris–HCl buffer (pH7.4 at 20 °C), and the mixture was then incubated for 60 min at 25 °C. The remainder of the assay was performed with the same procedure as described for 5-HT$_{2A}$ receptors except that the wash buffer was 50 mM Tris–HCl, pH 7.4 at 4 °C.

Analysis of saturation binding data was performed using the nonlinear curve-fitting program Ligand, which calculates the Kd (binding affinity, nM) and Bmax (receptor density, Fmol/mg tissue) values (Munson and Rodbard, 1980).

2.6. Competition experiments

The affinity of DOI and LSD for 5-HT$_{2A}$, 5-HT$_{2C}$, and D$_1$ receptors was determined by competition assays using [^{3}H]ketanserin, [^{3}H]mesulergine and [^{3}H]SCH23390, respectively. Each assay tube contained DOI or LSD (0.001 nM–1 mM) and the labeled ligand at its approximate Kd concentration. Nonspecific binding was defined by the addition of spiperone (30 nM), RS102221 (30 nM), or SCH23390 (30 nM), respectively. The remainder of the assays was performed as described in the Saturation Binding Analysis section. Analysis of the competition assays was performed using the curve-fitting program GraphPad Prism 4 (GraphPad Software, Inc., La Jolla, CA).

2.7. Binding reversibility

Separate aliquots of washed frontocortical membranes were mixed with drug plus 5 mM $MgSO_4$ and incubated at 25 °C for 60 min. Drug concentrations were chosen at roughly 10 times their respective Kd values — 4 nM for LSD, ketanserin, and ritanserin, 100 nM for DOI (Aloyo and Harvey, 2000; Leysen et al., 1985). The mixture was then centrifuged at 40,000 *g* for 20 min at 4 °C to remove unbound drug. The final membrane fraction was then dispersed in room temperature assay buffer (20 mM Tris–HCl and 5 mM Mg^{2+} pH7.4 at 20 °C). Each assay contained washed membranes derived from 4 mg of tissue, [^{3}H]ketanserin at 80% 5-HT$_{2A}$ receptor saturation (0.8 nM), and RS10222 (30 nM) and prazosin (30 nM) to block binding to 5-HT$_{2C}$ and α_1 adrenergic receptors, respectively. Separate tubes also contained spiperone (30 nM) in order to define non-specific binding. Assay samples were incubated for 2 h at 25 °C followed by rapid filtration through Whatman GF/B filters (presoaked in 0.5% polyethylenimine) and then four washes each consisting of 5 mL of wash buffer (20 mM Tris–HCl, pH7.4 at 4 °C). The amount of radioactivity retained on the filter was then determined by liquid scintillation counting.

2.8. Statistical analysis

Data were reported as group mean ± SEM. Some results are presented as a fraction of the control group value. The data were analyzed *via* *t*-test, analysis of variance (ANOVA), or 2-way ANOVA. Post hoc Dunnett or Bonferroni tests were carried out when applicable. Significance for all statistical comparisons was set at $p<0.05$ using a two-tailed test.

3. Results

3.1. Effects of systemic drug injection

3.1.1. Manipulation through chronic in vivo treatment

Eight daily injections of DOI (3 μmol/kg) selectively reduced frontocortical 5-HT$_{2A}$ receptor density from 12.36 ± 0.8 (control, saline treatment) to 6.88 ± 0.5 Fmol/mg tissue (DOI treatment; $p<0.005$, *t*-test). Frontocortical 5-HT$_{2C}$ receptor density was unchanged: control value, 5.23 ± 0.2; DOI treatment, 4.68 ± 0.4 Fmol/mg tissue. Similarly, frontocortical D$_1$ receptor density was unchanged: control value, 4.84 ± 0.5; DOI treatment, 4.30 ± 0.01 Fmol/mg tissue. In addition, both DOI- and LSD-elicited head bobs were also significantly decreased from 41.8 ± 3 to 16.3 ± 4 ($p<0.001$, *t*-test) and from 32.3 ± 7 to 8.8 ± 3 ($p<0.01$, *t*-test), respectively. Data are represented graphically as a fraction of the control value (Fig. 1).

3.1.2. Antagonist modulation of drug-elicited head bob behavior

Pretreatment of rabbits with the 5-HT$_{2A/2C}$ receptor antagonist, ritanserin (0.67 μmol/kg, $p<0.0001$, *post hoc* Dunnett test), or the D$_1$ receptor antagonist, SCH23390 (0.05 μmol/kg, $p<0.0001$, *post hoc* Dunnett test), significantly reduced DOI elicited head bobs ($F = 36.9$, $p<0.0001$, ANOVA, Fig. 2). Similarly, ritanserin ($p<0.0001$, *post hoc*

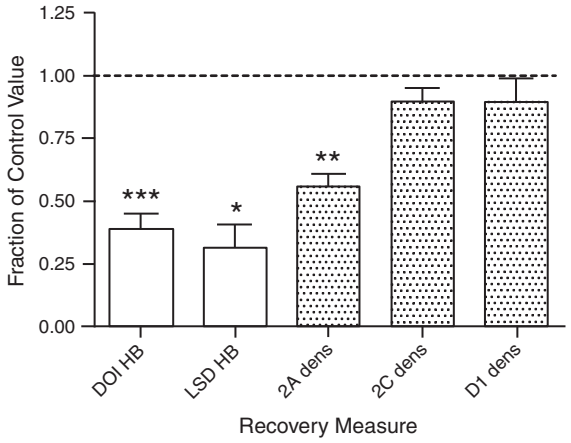

Fig. 1. Effects of chronic *in vivo* DOI administration on head bob and receptor density measures. Twenty-four hours after 8 daily injections of DOI (3 μmol/kg) DOI- and LSD-elicited head bobs as well as frontocortical 5-HT_{2A}, 5-HT_{2C}, and D_1 receptor densities were measured. Data are represented as the mean fraction of control ± SEM. For each measure investigated, the head bob response or receptor density of the animal that received chronic DOI treatment is divided by the head bob response or receptor density of the paired animal that received chronic vehicle treatment (control). These calculations are made for each animal pair. *$p<0.01$, **$p<0.005$, ***$p<0.001$, *t*-test, significantly different from vehicle treated group, n = 3 to 4.

Dunnett test) or SCH23390 ($p<0.0001$, *post hoc* Dunnett test) significantly reduced LSD-elicited head bobs ($F=9.22$, $p<0.0001$, ANOVA, Fig. 3A). Pretreatment with the 5-$HT_{2A/2C}$ antagonist, ketanserin (3 μmol/kg), did not affect LSD-elicited head bobs ($p>0.5$, *post hoc* Dunnett test; Fig. 3A). A higher dose of ketanserin (10 μmol/kg) was required to significantly inhibit the LSD-elicited response ($p<0.001$, *post hoc* Dunnett test, Fig. 3A). Pretreatment with the 5-$HT_{2B/2C}$ antagonist, SB206553 (1 μmol/kg), failed to alter LSD-elicited head bobs (Fig. 3A).

Time course analysis of the effect of ketanserin pretreatment (3 μmol/kg) on LSD (30 nmol/kg)-elicited head bobs revealed an effect of time ($F=2.43$, $p<0.039$, 2-way ANOVA), but not of treatment ($F=1.88$, $p>0.184$, 2-way ANOVA; Fig. 3B). There was a clear tendency for ketanserin to suppress LSD-elicited head bobs early in the time course, however. The LSD response then returned in the second half of the time course (Fig. 3B).

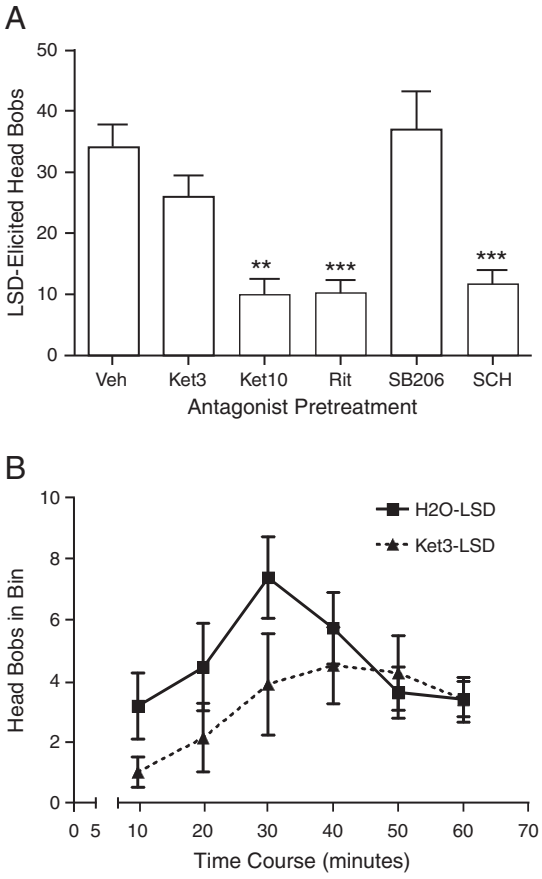

Fig. 3. Effect of antagonist pretreatment on LSD-elicited head bobs. *A* Rabbits were pretreated with ketanserin (3 or 10 μmol/kg), ritanserin (0.67 μmol/kg), SB206553 (1 μmol/kg), or SCH23390 (0.05 μmol/kg) before administration of LSD (30 nmol/kg). LSD-elicited head bobs are shown as the mean ± SEM. *B* Analysis of the behavioral time course of LSD-elicited head bobs following pretreatment with vehicle (H_2O) or ketanserin (3 μmol/kg). LSD-elicited head bobs are shown in 10 minute bins. The first 5 min is not included in the analysis due to the large stress response immediately after injection. **$p<0.001$, ***$p<0.0001$, *post-hoc* Dunnett test, significantly different from vehicle pretreated group, n = 4 to 18.

3.2. Ligand-receptor interactions

3.2.1. Frontocortical receptor binding affinity

Both DOI and LSD bound frontocortical 5-HT_{2A} receptors as measured by competition with [^{3}H]ketanserin; affinity values (Ki) were 12.5 ± 0.74 nM (n = 3) and 0.46 ± 0.045 nM (n = 4), respectively. The affinity for frontocortical 5-HT_{2C} receptors as measured by [^{3}H] mesulergine competition was 73.5 ± 14 nM (n = 4) and 7.5 ± 1.5 nM (n = 4) for DOI and LSD, respectively. Dopamine$_1$ receptor binding affinity in frontal cortex as measured by [^{3}H]SCH23390 competition was 16980 ± 3610 nM (n = 4) and 161.2 ± 32.8 nM (n = 3) for DOI and LSD, respectively.

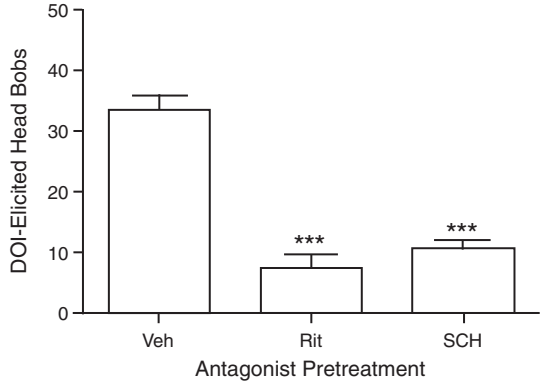

Fig. 2. Effect of antagonist pretreatment on DOI-elicited head bobs. Rabbits were pretreated with ritanserin (0.67 μmol/kg) or SCH23390 (0.05 μmol/kg) before administration of DOI (300 nmol/kg). DOI-elicited head bobs are shown as the mean ± SEM. ***$p<0.0001$, *post-hoc* Dunnett test, significantly different from vehicle pretreated group, n = 5 to 14.

3.2.2. In vitro binding reversibility at frontocortical 5-HT_{2A} receptors

Drug pre-incubation significantly affected the ability of [^{3}H]ketanserin to bind rabbit frontocortical membranes ($F=590.5$, $p<0.0001$, ANOVA; Fig. 4). Pre-incubation of membranes with LSD ($p<0.0001$, *post-hoc* Dunnett test) and ritanserin ($p<0.0001$, *post hoc* Dunnett test) inhibited [^{3}H]ketanserin binding (Fig. 4). In contrast, pre-incubation with DOI or ketanserin did not inhibit [^{3}H]ketanserin binding in frontocortical rabbit membranes (Fig. 4). Interestingly, ketanserin pre-incubation led to a 43% increase in [^{3}H]ketanserin binding (1238 ± 36 to 1766 ± 51disintegrations per minute; Fig. 4; $p<0.0001$, *post hoc* Dunnett test).

3.2.3. Ex vivo binding at frontocortical 5-HT_{2A} receptors

Acute administration of DOI (300 nmol/kg) did not change the maximum binding (Bmax) of [^{3}H]ketanserin, [^{3}H]mesulergine, or [^{3}H]SCH23390 to the frontocortical membranes, indicating no change

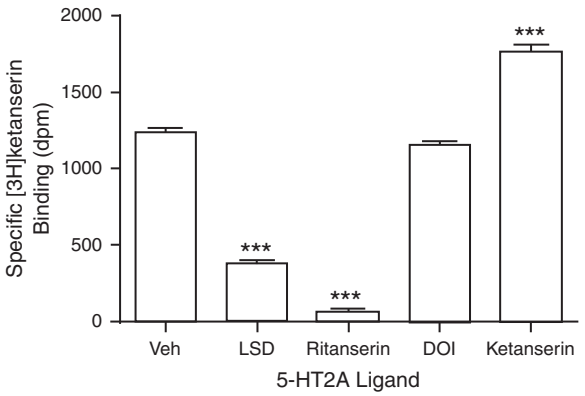

Fig. 4. Binding reversibility of 5-HT_{2A} receptor ligands in rabbit frontal cortex. Frontocortical membranes were pre-incubated with LSD (4 nM), ritanserin (4 nM), DOI (100 nM), or ketanserin (4 nM). Following the pre-incubation, the membranes were washed and [^{3}H]ketanserin binding was measured (see Methods 2.7). Specific binding is shown as mean disintegrations per minute (dpm) ± SEM. ***p<0.0001, *post-hoc* Dunnett test, significantly different from vehicle pre-incubated membranes, n = 4.

in frontocortical 5-HT_{2A}, 5-HT_{2C}, or D_1 receptor binding, respectively (data not shown). As with DOI, acute administration of LSD (30 nmol/kg SC) had no effect on frontocortical [^{3}H]mesulergine (3.84 ± 0.5 to 3.53 ± 0.9 Fmol/mg tissue) or [^{3}H]SCH23390 (3.47 ± 0.7 to 3.66 ± 0.2 Fmol/mg tissue) binding. In contrast to DOI, however, acute LSD administration (30 nmol/kg SC) significantly reduced the maximum binding of [^{3}H]ketanserin in frontocortical membranes from 11.29 ± 0.9 to 5.21 ± 0.6 Fmol/mg tissue ($p<0.001$, *t*-test). Data are represented graphically as a fraction of the control value (Fig. 5A). This reduction in [^{3}H]ketanserin binding after acute *in vivo* LSD administration was dose-dependent ($F=27.9$, $p<0.0001$, 2-way ANOVA; Fig. 5B). The doses of 30 and 300 nmol/kg LSD significantly reduced maximum binding of [^{3}H]ketanserin in frontocortical membranes ($p<0.001$ and $p<0.0001$, *post-hoc* Bonferroni tests), but the dose of 3 nmol/kg did not (Fig. 5B). As residual drug would artificially decrease the Bmax for [^{3}H]ketanserin binding, membranes were warmed prior to [^{3}H]ketanserin incubation in order to remove any potentially residual drug. The LSD-elicited reduction in frontocortical membrane [^{3}H]ketanserin binding did not differ between cold and warm membrane preparations ($F=2.0$, $p>0.18$, 2-way ANOVA, Fig. 5B). Neither acute DOI nor acute LSD injection altered receptor binding affinity (Kd) for any of the radioligands tested (data not shown).

Pre-treatment of rabbits with ketanserin (3 μmol/kg) did not affect the LSD-induced decrease in frontocortical [^{3}H]ketanserin binding. Interestingly, acute injection of ketanserin (3 μmol/kg) led to an increase in the maximum binding (Bmax) of [^{3}H]ketanserin to rabbit frontocortical membranes ($F=13.1$, $p<0.004$, 2-way ANOVA). In animals that received the LSD vehicle, the control value, 10.83 ± 0.8 Fmol/mg tissue, increased to 13.46 ± 0.2 Fmol/mg tissue with *in vivo* ketanserin pretreatment ($p<0.001$, *post hoc* Bonferroni test). In animals that received LSD (30 nmol/kg), the control value, 6.05 ± 0.2 Fmol/mg tissue, increased to 8.04 ± 1 Fmol/mg tissue with *in vivo* ketanserin pretreatment ($p<0.001$, *post hoc* Bonferroni test). ([^{3}H]Ketanserin binding affinity (Kd) remained unchanged in all treatment groups. A representative Scatchard plot of these effects is shown in Fig. 6A.

Seventy minutes after acute administration of ritanserin (0.67 μmol/kg), there was a significant reduction in the Bmax for [^{3}H]ketanserin binding in rabbit frontocortical membranes (9.40 ± 1.2 to 1.91 ± 0.5 Fmol/mg tissue; $p<0.0005$, *t*-test). A representative Scatchard plot of the effect is shown in Fig. 6B. Acute ritanserin injection significantly decreased frontocortical [^{3}H]ketanserin binding affinity (0.21 ± 0.01 to 0.5 ± 0.1 nM; $p<0.01$, *t*-test), but this apparent Kd shift is probably due to calculation errors resulting from the very low Bmax values (1.9 ± 0.5 Fmol/mg tissue).

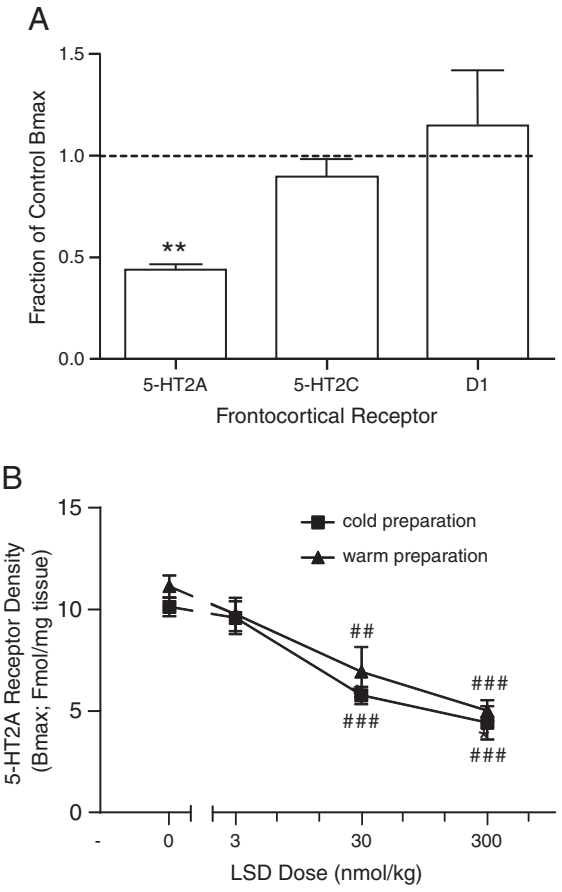

Fig. 5. Effect of acute in vivo LSD administration on frontocortical [^{3}H]ketanserin binding. *A* Seventy minutes after in vivo LSD (30 nmol/kg SC) or vehicle (control) injection, the frontocortical density (Bmax) of 5-HT_{2A}, 5-HT_{2C}, and D_1 receptors were measured using [^{3}H]ketanserin, [^{3}H]mesulergine, and [^{3}H]SCH23390, respectively. Data are represented as the mean fraction of control ± SEM. For each receptor, the receptor density of the animal that received acute LSD treatment is divided by the receptor density of the paired animal that received acute vehicle treatment. These calculations are made for each animal pair. *B* Seventy minutes after in vivo LSD injection (0, 3, 30, or 300 nmol/kg IV), the density of frontocortical 5-HT_{2A} receptors was measured using [^{3}H]ketanserin. Receptor density is reported as Fmol/mg tissue and shown as mean ± SEM. Frontocortical membranes were prepared with either a cold (square) or warm (triangle) pre-incubation. **p<0.001, *t*-test, significantly different from vehicle treated group, n = 4; ##p<0.001, ###p<0.0001, post-hoc Bonferroni test, significantly different from vehicle treated group, n = 4.

4. Discussion

4.1. Serotonin$_{2A}$-mediated effects of DOI and LSD

The present work demonstrates that the indoleamine class of hallucinogens, exemplified by LSD, elicited head bobs in rabbits through 5-HT_{2A} receptor activation. This conclusion is consistent with previous reports establishing that the phenethylamine hallucinogen, DOI, elicits head movements in rodents *via* 5-HT_{2A} receptors (Fantegrossi et al., 2010; González-Maeso et al., 2007; Schreiber et al., 1995; Willins and Meltzer, 1997). Likewise, we have previously demonstrated that DOI elicits head movements in the rabbit by activation of this same receptor (Dave et al., 2002, 2007). The current study provides two lines of evidence to support the conclusion that DOI and LSD elicit rabbit head bobs by activation of 5-HT_{2A} receptors. Firstly, downregulation of frontocortical 5-HT_{2A} receptors resulted in decreased head movement behavior elicited by both hallucinogens. Secondly, ritanserin inhibited head bobs elicited by both DOI and LSD. Although ritanserin is also an antagonist at 5-HT_{2C} receptors, the inability of SB206553, which is 750 times more selective for rabbit frontocortical 5-HT_{2C} receptors over 5-HT_{2A} receptors, to affect either

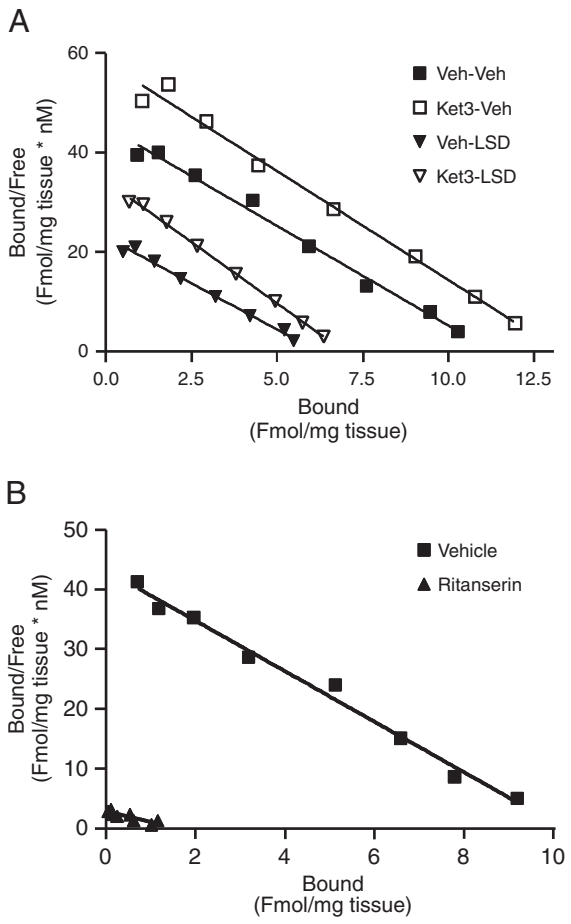

Fig. 6. Effects of acute *in vivo* ketanserin or ritanserin on frontocortical [^{3}H]ketanserin binding. *A* Rabbits were injected with ketanserin (3 μmol/kg) or vehicle (H_2O) followed 60 min later by LSD (30 nmol/kg) or vehicle (control). Seventy minutes later, rabbits were sacrificed and frontal cortex tissue harvested for analysis of [^{3}H]ketanserin binding. A Scatchard plot shows binding in representative animals from Veh–Veh (filled square), Ket–Veh (open square), Veh–LSD (filled triangle), and Ket–LSD (open triangle) groups. *B* Other rabbits were acutely injected with ritanserin (0.67 μmol/kg), sacrificed 70 min later, and frontal cortex tissues harvested for analysis of [^{3}H]ketanserin binding. A Scatchard plot shows binding in a representative vehicle (square) and ritanserin (triangle) animal. All membranes were warmed for 60 min at 37 °C (see Methods 2.4). n = 3 to 5.

DOI- or LSD-elicited head bobs highlights the 5-HT$_{2A}$ receptor in the effects of ritanserin (Dave et al., 2002).

The present study also explored the receptor binding characteristics of DOI and LSD at the rabbit frontocortical 5-HT$_{2A}$ receptor. Preincubation of rabbit frontocortical membranes with LSD, but not DOI, resulted in a significant reduction in [^{3}H]ketanserin binding. To test the hypothesis that residual, unbound LSD in the membrane preparation accounted for the observed change in [^{3}H]ketanserin binding, the tissue was warmed to facilitate drug removal. Warming the tissue during preparation, however, failed to alter this binding parameter. Acute *in vivo* administration of LSD, but not DOI, also significantly reduced [^{3}H]ketanserin binding *ex vivo*. Radioligand binding to 5-HT$_{2C}$ and dopamine D$_1$ receptors was unaffected by this *in vivo* manipulation. The selective and dose-dependent effects of LSD suggest that the drug remains pseudo-irreversibly bound to frontocortical 5-HT$_{2A}$ receptors for at least 60 min after *in vitro* incubation or 70 min after *in vivo* administration. In contrast, DOI binds the rabbit frontocortical 5-HT$_{2A}$ receptor in a reversible manner.

An alternative hypothesis is that the acute administration of LSD to rabbits results in the down-regulation of 5-HT$_{2A}$ receptors. Indeed, Ferry et al. (1993) demonstrated rapid reduction of 5-HT$_{2A}$ receptor density in cultured P11 cells after LSD incubation. This group showed the same effect and time course for DOI-induced 5-HT$_{2A}$ receptor reduction in P11 cells (Ferry et al., 1993). These findings are in contrast to the current study, which showed no change in [^{3}H]ketanserin binding after either *in vitro* incubation or *in vivo* injection with DOI. Similar to the findings in cells, DOI significantly decreased 5-HT$_2$ receptor binding in rat aortic smooth muscle, but required at least 4 h to do so (Rinaldi-Carmona et al., 1994). Thus, it is unlikely that the 1 h following drug administration used in the current study would be sufficient to down-regulate 5-HT$_{2A}$ receptor density. Instead, the reduction in [^{3}H]ketanserin binding observed in the current study likely represents the presence of pseudo-irreversibly bound LSD. This conclusion is supported by previous studies that used similar methods to show that LSD binds the rat and blowfly 5-HT$_{2A}$ receptor in an irreversible or pseudo-irreversible manner (Berridge and Prince, 1974; Burris and Sanders-Bush, 1992).

Consistent with our previous results demonstrating rapid dissociation of [^{3}H]ketanserin from rabbit 5-HT$_{2A}$ receptors (Aloyo et al., 2001), the present studies found that ketanserin bound reversibly to rabbit cortical membranes. Interestingly, frontocortical [^{3}H]ketanserin binding was increased after *in vitro* incubation or acute *in vivo* administration of ketanserin. This effect suggests that ketanserin may possess unique interactions with the 5-HT$_{2A}$ receptor or its associated proteins. Further research will be required to test this hypothesis.

In behavioral studies, a relatively low dose of ketanserin (1 μmol/kg) maximally inhibited DOI-elicited head bobs in rabbits (Dave et al., 2002). In contrast, the current study showed that a ten-fold higher dose of ketanserin (10 μmol/kg) was required to inhibit the LSD-elicited behavior. On a molar basis, the high dose of ketanserin was more than 300 times the dose of LSD. The present work shows that pretreatment with a lower dose of ketanserin (3 μmol/kg) was also able to reduce LSD-elicited head bobs, but only in the early period of agonist exposure (0–30 min). The behavioral effect of LSD then returned in the latter portion of the time course (30–60 min). Thus, when averaged over the entire 60 minute observation period, the data show that ketanserin (3 μmol/kg) did not alter LSD elicited head bobs. Based on our demonstration that LSD binds in a pseudo-irreversible manner whereas ketanserin binds reversibly, a likely explanation is that LSD displaced ketanserin from the 5-HT$_{2A}$ receptor and thereby elicited sufficient head bob behavior to compensate for the initial ketanserin-induced inhibition.

In keeping with previous work in rodents (Leysen et al., 1985), the current studies demonstrated that ritanserin differed from ketanserin in that it bound to frontocortical 5-HT$_{2A}$ receptors in a pseudo-irreversible manner, a property demonstrated both *in vitro* and *ex vivo*. In contrast to ketanserin, ritanserin blocked head bobs elicited by either DOI or LSD at a relatively low dose (0.67 μmol/kg). The pseudo-irreversible binding of ritanserin likely prevented its displacement by LSD, allowing it to inhibit LSD-elicited effects at low doses. This property of ritanserin supports its preferred use over ketanserin in studying the 5-HT$_{2A}$-mediated effects of the pseudo-irreversibly binding LSD. Ritanserin is also preferable to more selective 5-HT$_{2A}$ antagonists such as M-100,907 or sarpogrelate, which have both been described as reversible antagonists (Ito et al., 1998; Kanamori et al., 1994). If LSD displaced ketanserin in the current experiments, it would likely displace these reversibly binding antagonists as well.

4.2. The role of 5-HT$_{2B/2C}$ receptors in hallucinogen-elicited behavior

The present work demonstrates that LSD did not elicit head bobs through 5-HT$_{2B/2C}$ receptor activation. The inability of SB206553 to alter LSD-elicited head bobs is, to our knowledge, the first reported investigation into the role of 5-HT$_{2B/2C}$ receptors in LSD-elicited head movement behavior. This finding also extends the work by Dave et al. (2002) showing that 5-HT$_{2B/2C}$ receptors were not involved with DOI-elicited head bobs in rabbits. Other reports have

also shown that 5-HT_{2C} receptors are not involved in either DOI-elicited rat head twitches (Schreiber et al., 1995) or the DOI stimulus cue in rats (Smith et al., 1999). The lack of a role for 5-HT_{2C} receptors in hallucinogen-mediated head movements in both rabbits and rats contrasts with the findings in mice, which identify a role for 5-HT_{2C} receptors in both DOI-elicited head twitches (Canal et al., 2010) and the DOI stimulus cue (Smith et al., 2003). Additional studies should examine the possibility that a species factor accounts for the differential roles of 5-HT_{2C} receptors in rat, rabbit, and mouse.

Dave et al. (2002) showed that 5-$HT_{2B/2C}$ receptors were in fact involved in another rabbit behavior, the body shake. DOI-elicited body shakes were blocked by SB206553 (1 μmol/kg), but not by ketanserin (1 and 3 μmol/kg; Dave et al., 2002). The authors concluded that head bobs and body shakes were mediated by 5-HT_{2A} and 5-HT_{2C} receptors, respectively (Dave et al., 2002). Given the inability of this same behaviorally active dose of SB206553 (1 μmol/kg) to modify LSD-elicited head bobs (either inhibit or potentiate) we concluded that 5-$HT_{2B/2C}$ receptors were not involved in this behavior. As Fantegrossi et al. (2010) clearly demonstrated, the role of 5-HT_{2C} receptors in mouse head twitches is conditional on the dose of DOI administered. Further studies using doses of DOI and LSD on the descending arm of the dose response curve are warranted to test the hypothesis that 5-HT_{2C} antagonism may potentiate rabbit head bobs under these conditions.

4.3. The role of D_1 receptors in hallucinogen-elicited behavior

The dopaminergic system is not a major focus of hallucinogen study, although dopamine is strongly tied to human psychotic disease, a condition mimicked by hallucinogens (Nichols, 2004). Dopamine$_1$ receptors have been reported to mediate DOI-elicited rat head shakes (Schreiber et al., 1995), but the role of this or any dopaminergic receptor in LSD-elicited head movement behavior was not previously investigated. The current studies found that both DOI and LSD required the activation of D_1 receptors to elicit head bobs, strongly supporting the hypothesis that D_1 receptors may play a part in the hallucinogenic mechanism of action. The antagonist used to reach this conclusion was SCH23390, a ligand used previously to demonstrate the role of D_1 receptors in DOI-elicited head movement behavior (Schreiber et al., 1995). SCH23390 is known to bind 5-HT_{2A} receptors as well as D_1 receptors, suggesting that SCH23390 may be inhibiting head bobs *via* antagonist action at 5-HT_{2A} receptors. Although the relative affinity for these two receptors varies among animal species, SCH23390 exhibits approximately 100 fold selectivity for D_1 receptors as compared to 5-HT_{2A} receptors (Ekelund et al., 2007; Neumeyer et al., 2003; Schreiber et al., 1995). Thus, it is unlikely that SCH23390 is inhibiting head movement behavior *via* 5-HT_{2A} receptors.

Previously it was shown that LSD binds dopamine receptors whereas DOI and other hallucinogens do not (Burt et al., 1976; Watts et al., 1995). The present work showed that LSD had modest binding affinity for D_1 receptors in rabbit frontal cortex, whereas DOI had negligible affinity. Thus, LSD may act directly on D_1 receptors to elicit head bobs in rabbits, whereas the dopaminergic component of the DOI-elicited behavior is likely an indirect effect. One possibility for an indirect mechanism is the release of dopamine in frontal cortex mediated *via* 5-HT_{2A} receptor activation (Gobert and Millan, 1999; Ichikawa et al., 2001). Importantly, both ritanserin and SCH23390 independently and equally diminished head bob behavior elicited by the hallucinogens studied. Thus, both 5-HT_{2A} and D_1 receptors appear to play key roles in producing head bobs in rabbits. To confirm the role of D_1 receptors in DOI- and LSD-elicited head bobs, other more selective D_1 receptor antagonist should be tested (see Schreiber et al., 1995).

5. Conclusion

In keeping with the original goals of this study, the current report delineates some of the pharmacology behind hallucinogen-mediated behavior. Importantly, these studies showed that both 5-HT_{2A} and D_1 receptors were crucial for DOI- and LSD-elicited head bobs in rabbits. This conclusion suggests a central role for these receptors in the effects of hallucinogens in humans. The pseudo-irreversible binding of LSD at frontocortical 5-HT_{2A} receptors along with its affinity for D_1 receptors may also explain some of the unique characteristics of this particular hallucinogen. The various findings discussed in this report are supported by the known effects of hallucinogens in humans, which are also similar, yet distinct (Shulgin and Shulgin, 1991; Shulgin and Shulgin, 1997). Ultimately, the ability of these compounds to have such profound and lasting effects on the human psyche, even after limited exposure (Griffiths et al., 2006, 2008; Grob et al., 2011), implicates complex interactions, such as genetic effects or perhaps even changes in neurocircuitry (Passie et al., 2008; Vollenweider and Kometer, 2010). These and other post-receptor effects are driving the latest studies (Nichols and Sanders-Bush, 2004; Schmid and Bohn, 2010). The benefits of continued investigation of hallucinogenic compounds are clear. The contributions of this distinctive group of compounds to science and medicine have only begun to be realized.

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