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Salvinorin A fails to substitute for the discriminative stimulus effects of LSD or ketamine in Sprague–Dawley rats

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ABSTRACT

Salvia divinorum is a small perennial shrub that has gained recent popularity among the drug-using subculture as a legal alternative to hallucinogens. Salvinorin A, the main active compound found in the *S. divinorum* plant, is an atypical hallucinogen with pharmacological selectivity at kappa opioid (KOP) receptor sites and is a unique non-nitrogenous neoclerodane diterpene which is structurally distinct from other opioid compounds. The novel structure of salvinorin A and its specific binding affinity to KOP receptors provide a unique opportunity to investigate neurochemical mechanisms of hallucination and hallucinogenic compounds. The current investigation assessed the substitution of salvinorin A in 16 male Sprague–Dawley rats trained to discriminate either the prototypical serotonergic hallucinogen, LSD (0.08 mg/kg, S.C., $n=8$) or the dissociative anesthetic and glutamatergic hallucinogen, ketamine (8.0 mg/kg, I.P., $n=8$) from vehicle under a FR 20 schedule of food-reinforced responding. Results indicated that neither LSD nor ketamine discrimination generalized to salvinorin A. These findings are consistent with the growing body of evidence that salvinorin A is pharmacologically distinct from other traditional hallucinogenic compounds.

1. Introduction

Salvia divinorum is a small perennial shrub native to Mexico that has gained recent popularity in the United States and Europe as a legal hallucinogen. The use of *S. divinorum* is not exclusive to the modern world, but has been used for centuries as a divining agent by the native tribes of Oaxaca, Mexico (Wasson, 1962). The recent increase in *S. divinorum* use is presumably due to the widespread availability of both the plant material and highly potent extracts that can be readily purchased through the internet (Drug Enforcement Administration, 2008; Prisinzano, 2005). Recreational use of this substance is rampant within the internet community of YouTube©, where one can view a multitude of videos documenting recreational use of *S. divinorum* and the resulting intoxication.

When *S. divinorum* leaves are smoked or chewed as a quid, the user often experiences a "loss of awareness," which could result in users hurting themselves or others (Gonzalez et al., 2006). In reaction to the growing abuse issue, several states within the U.S. and some European countries have banned the cultivation, use, and distribution of the plant (Siebert, 2007). Recently, the Drug Enforcement Administration has cited *S. divinorum* as a "drug of concern" (Drug Enforcement Administration, 2008) and U.S. federal regulation of *S. divinorum* is a possibility in the near future.

Although recent media attention has primarily focused on the legality and abuse liability of *S. divinorum*, the unique pharmacological profile of salvinorin A, the main active compound found in *S. divinorum*, has gained considerable interest among the scientific community. Salvinorin A was first isolated from this plant by Ortega et al. in 1982 and it remains the most potent naturally occurring hallucinogen known to mankind. Salvinorin A is a unique furanolactone, belonging to the neoclerodane class of diterpenes (Ortega et al., 1982). It is also now well established that salvinorin A is a highly selective and potent kappa opioid receptor (KOP) agonist (Roth et al., 2002). Salvinorin A is chemically and structurally unique from other hallucinogens, being the first known psychoactive diterpene and the first non-nitrogenous hallucinogen (Vortherms and Roth, 2006). Doses ranging from 200 to 600 μg produce profound hallucinations in humans that are also qualitatively distinct from the psychoactive effects produced by more traditional hallucinogens like lysergic acid diethylamide (LSD), mescaline, or psilocybin (Gonzalez et al., 2006; Siebert, 1994).

Hallucinogenic drugs comprise a distinct class of compounds categorized by their chemical structures and pharmacological actions. Direct investigation of the precise neurochemical mechanisms responsible for their psychoactive effects in humans is technically challenging. However, the study of the discriminative stimulus effects of hallucinogens using drug discrimination procedures in nonhumans is a particularly attractive investigative paradigm (Winter, 2009) because of the specificity of discriminative stimuli correlating with underlying cellular and molecular mechanisms of drug action (Holtzman and Locke, 1988; Colpaert, 1999). Drug discrimination investigations have reliably shown that hallucinogens with similar neuropharmacological actions tend to produce cross generalization, while compounds with distinct neuropharmacological actions fail to do so.

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At the present time, only five published studies have examined salvinorin A using drug discrimination procedures. Only one study trained animals to discriminate another hallucinogen (Li et al., 2008) and only two studies trained animals to discriminate salvinorin A (Baker et al., 2009; Butelman et al., 2010). Butelman et al. (2004) published the first study to demonstrate that salvinorin A substituted in rhesus monkeys (one male and two females) trained to discriminate the synthetic KOP agonist, U69,593 (0.0056 or 0.013 mg/kg; S.C.). The opioid antagonist, quadazocine blocked this substitution in all three animals, whereas the selective kappa antagonist, 5′-guanidinonaltrindole (GNTI) blocked these effects in only two of three animals. Willmore-Fordham et al. (2007) reported similar results in male Sprague–Dawley rats trained to discriminate U69,593 (0.56 mg/kg, I.P.). Baker et al. (2009) replicated these findings in male Sprague–Dawley rats trained to discriminate a lower dose of U69,593 (0.13 mg/kg, S.C.) or another KOP agonist, U-50,488H (3.0 mg/kg, I.P.). That study also showed full substitution with U69,593 and U-50,488H in rats trained to discriminate salvinorin A (2.0 mg/kg, I.P.).

In the only published study to examine salvinorin A in animals trained to discriminate a traditional hallucinogen, Li et al. (2008) trained four rhesus monkeys (two males and two females) to discriminate the serotonergic hallucinogen 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (DOM; 0.32 mg/kg, S.C.) under a FR 5 schedule of stimulus shock termination. In these monkeys, DOM discrimination generalized to other 5-HT agonists, but failed to generalize to salvinorin A, ketamine, or PCP. Consistent with these findings, Butelman et al. (2010) recently reported that monkeys (three males) trained to discriminate salvinorin A (0.015 mg/kg S.C.) generalized to other kappa agonists, but did not generalize to psilocybin or ketamine. Moreover, salvinorin A discrimination was blocked by quadazocine but not the 5-HT_2 antagonist, ketanserin.

To our knowledge, there are currently no published studies on the effects of KOP agonists in animals trained to discriminate any of the dissociative hallucinogens. However, a few studies have tested the noncompetitive NMDA-receptor antagonists, phencyclidine (PCP), ketamine, and MK-801 for substitution in animals trained to discriminate synthetic KOP agonists. The results of these studies are somewhat equivocal. Shearman and Herz (1982) trained male Sprague–Dawley rats to discriminate between ethylketocyclazocine (0.32 mg/kg) or bremazocine (0.04 mg/kg) and saline in a food-reinforced discrimination procedure. They reported that PCP and ketamine substituted in some rats trained to discriminate bremazocine, but not in any of the rats trained to discriminate ethylketocyclazocine. In other studies, PCP failed to substitute in male Sprague–Dawley rats trained to discriminate the KOP agonist, spiradoline (U62,066; 3.0 mg/kg S.C.) in a discrete-trial shock-avoidance/escape procedure (Holtzman et al., 1991) or male Long Evans hooded rats trained to discriminate U-50,488H under a fixed ratio 20 schedule of food reinforcement. In contrast to these earlier findings, a recent study by Mori et al. (2006) reported that PCP, ketamine, and MK-801 all produced full substitution for U-50,488H in male Fischer 344 rats. These findings along with the recent discovery of salvinorin A's selective KOP receptor affinity warrant further investigations comparing the discriminative stimulus effects of salvinorin A with dissociative hallucinogens as well as other traditional hallucinogens. The primary aim of the current study was to assess the effects of salvinorin A in rats trained to discriminate either a prototypical serotonergic hallucinogen, LSD (Experiment 1) or the noncompetitive NMDA antagonist and dissociative hallucinogen, ketamine (Experiment 2).

2. Materials and methods

2.1. Subjects

Sixteen drug-naive male Sprague–Dawley® rats (Charles River Laboratories, Portage, MI) approximately 180 to 210 days of age at the start of the experiment were used. All rats were individually housed in polycarbonate cages within a climate-controlled animal facility and maintained on a 12-hour light/dark cycle with free access to water. Access to food was limited so that animals were maintained at approximately 85% of their free-feeding weights.

2.2. Apparatus

Behavioral training and test sessions were conducted using eight standard operant chambers (Med-Associates Inc., Georgia, VT) equipped with three retractable levers (left, center, and right) on the front panel, a food delivery mechanism above the center lever, and a 28-V house light located at the top of the rear panel. Experimental events and data collection were computer-controlled using MED-PC (version 4.0 for Windows) instrumentation and software. Lever pressing was reinforced with dustless precision food pellets (45 mg, product # F0021, Bioserv®, Frenchtown, NJ).

2.3. Drugs

Lysergic acid diethylamide tartrate (National Institute on Drug Abuse, Bethesda, MD) was dissolved in sterile 0.9% saline and administered via subcutaneous (S.C.) injection. Ketamine hydrochloride (Sigma Chemical Company, St. Louis, MO) was dissolved in sterile water and administered via intraperitoneal (I.P.) injection. Salvinorin A was generously provided by Mailman Research Center, McLean Hospital (Belmont, MA). Due to limited solubility, salvinorin A was initially dissolved in dimethylsulfoxide (DMSO) and then diluted with sterile water to 75% DMSO and administered via I.P. injection. All drugs were administered at a volume of 1 mg/ml and doses were determined based on the weight of the solid compounds.

2.4. Preliminary training

Prior to the lever-press training, subjects in both groups underwent two 60-minute sessions in which food pellets were delivered under a fixed time 60 s (FT 60″) schedule of pellet delivery in order to familiarize the animals to the stimuli within the operant chamber and the location of the food pellets. All levers remained retracted during these sessions. Animals were then trained to press the center lever for food pellets during a 20-minute session using a fixed ratio 1 (FR 1) schedule of reinforcement. Once lever pressing was acquired, a series of 20-minute errorless training sessions were conducted during which either the left or right lever was extended. Fifteen minutes prior to training sessions, each animal received an injection of either the training compound (Experiment 1: LSD, Experiment 2: ketamine) or its respective vehicle. For half the animals in each experiment, drug injections preceded sessions in which left lever responses were reinforced and vehicle injections preceded sessions in which right lever responses were reinforced. These conditions were reversed for the remaining animals in each group. An equal number of errorless training sessions were conducted for each animal under each training condition. Once animals were reliably responding under both the drug and vehicle conditions, discrimination training sessions commenced.

2.5. Discrimination training

During the discrimination training sessions, both the left and right levers were always present. Twenty-minute sessions were conducted once a day at approximately the same time of day 5 to 7 days per week. Drug and vehicle training sessions were alternated to include two or three of each stimulus condition per week, with the limitation that neither stimulus condition occurred for more than two consecutive days in a row. The programmed fixed ratio (FR) schedule of reinforcement was such that only a fixed number of consecutive responses would result in reinforcer delivery and an incorrect

response would reset the response counter. Responding was initially reinforced under a FR 1 schedule which was gradually incremented to a FR 20 schedule over several training sessions until responding was reliably maintained under the FR 20 schedule. Levers were wiped with isopropyl alcohol in between training sessions to reduce the influence of olfactory cues on discrimination performance (Extance and Goudie, 1981). Discrimination accuracy was determined by calculating the percentage of responses on the correct lever prior to the first food pellet delivery of each session. When discrimination accuracy for any individual animal was 80% or greater for at least eight of ten consecutive sessions, substitution tests commenced.

2.6. Substitution tests

Once an animal met the above stated criteria for stimulus discrimination, substitution tests were conducted with different doses of the training drug and dose response curves were determined for each training drug. Subsequently, substitution tests were conducted with salvinorin A in both training groups. In Experiment 1, LSD-trained rats were tested for substitution with salvinorin A at doses of 0, 0.125, 0.25, 0.5, 1.0, and 2.0 mg/kg. In Experiment 2, ketamine-trained rats were tested for substitution with salvinorin A at doses of 0, 0.25, 0.5, 1.0, 2.0, and 3.0 mg/kg. Individual doses were tested in a randomized order among the animals in each group. For each test dose, approximately half of the animals were tested following a discrimination training session in which drug was administered, and the remaining half were tested following a discrimination training session in which vehicle was administered. Test sessions were conducted according to each animal's performance during the training sessions. All animals received at least one drug training session and one vehicle training session between test sessions and were required to exhibit 80% or greater discrimination accuracy during these sessions in order to proceed with subsequent test sessions. Test sessions were conducted similar to training sessions, with the exception that reinforcers were not delivered and sessions ended when the first 20 consecutive responses on either lever was completed or after 20 min, which ever occurred first. Animals were removed from the chambers immediately following completion of a test session.

2.7. Data analysis

For both experiments, the mean (±S.E.M.) number of sessions required to meet the discrimination criterion was calculated. Substitution test data were analyzed for the percentage of drug-lever responses and overall response rate. The percentage of drug-lever responses was expressed as the number of responses emitted on the drug-lever divided by the total number of responses emitted during a test session and multiplied by 100. Response rate was expressed as the number of responses per second during a test session. For each test dose, group means were calculated and plotted in dose response curves depicting the percentage of responses on the drug-lever and response rate over a range of test doses. Results from the range of doses for each test compound were tested for normality using the Kolmogorov–Smirnov test. Those data sets that were not normally distributed were analyzed with a nonparametric statistical test, Kruskal–Wallis. For those data sets that did pass the test for normality, a one-way repeated measures analysis of variance (ANOVA) was conducted. If a specific dose of a particular compound produced 80% or greater drug-lever responses, it was considered to show full substitution for the training drug. Drug-appropriate responding below 80% but significantly different from vehicle-appropriate responding was considered evidence for partial substitution.

3. Results

3.1. Experiment 1

Animals trained to discriminate 0.08 mg/kg LSD met the discrimination criterion within an average of 17.25 (±3.25 S.E.M.) training sessions (range: 10–32). Dose response curves depicting the results of substitution tests in these animals are shown in Fig. 1. The mean percentage of LSD-lever responses increased in a dose-dependent manner. The lowest dose of LSD (0.01 mg/kg) engendered vehicle-appropriate responding, whereas 0.04 and 0.08 mg/kg produced LSD-appropriate responding. These data were analyzed using the Kruskal–Wallis test and were found to be significant ($H = 22.7$, 5 d.f., $p < 0.0001$). Response rates following LSD administration were distributed normally and a repeated measures ANOVA indicated that they were not significantly different from vehicle response rates ($F_{5,47} = 0.51$, $p = 0.76$).

None of the salvinorin A doses tested in the LSD-trained animals produced drug-appropriate responding greater than 40%. These data were also analyzed using Krusal–Wallis test, which indicated a significant dose effect ($H = 11.71$, 5 d.f., $p < 0.05$). A modest dose-dependent decrease in response rate was observed following salvinorin A administration in LSD-trained animals. Response rates

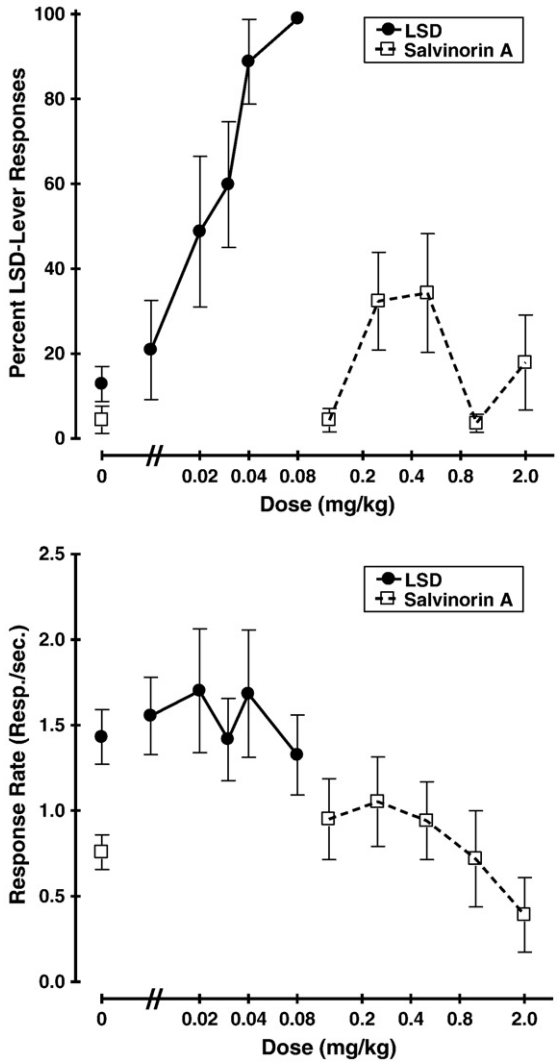

Fig. 1. Dose response curves for LSD and salvinorin A in rats trained to discriminate 0.08 mg/kg LSD ($n = 8$). Percent LSD-lever responses for both compounds are depicted in the top graph and the response rate for both compounds is depicted in the lower graph. Data points represent group means (±S.E.M.) at each dose tested.

were normally distributed and a repeated measures ANOVA indicated they were not significantly different ($F_{5,47}=2.05$, $p=0.10$). Unfortunately, higher doses of salvinorin A were not examined in these because they were determined to markedly reduce responding in a previous study (Baker et al., 2009).

3.2. Experiment 2

The initial training dose of ketamine (5.0 mg/kg, I.P., 15 min) established stimulus control in 6 of 8 rats within an average of 46.7 (±6.7 S.E.M.) training sessions. After 80 training sessions, the training dose was increased to 6.0 mg/kg and then to 8.0 mg/kg after another 19 training sessions. After the dose was increased to 8.0 mg/kg, all rats met the criterion for discrimination within an average of 21 (±4.8 S.E.M.) additional training sessions (range: 10–45).

Results obtained from dose response tests with ketamine and salvinorin A are displayed in Fig. 2. Vehicle and 1.0 mg/kg ketamine engendered responding primarily on the vehicle-associated lever. A dose-dependent increase in responding on the ketamine-associated lever was observed, but only the training dose (8.0 mg/kg) produced full substitution. The Kruskal–Wallis test indicated a significant dose effect on percent ketamine-lever responding ($H=13.36$, 4 d.f., $p<0.01$). Response rates were distributed normally and generally similar across all doses of ketamine, with no statistically significant difference by dose ($F_{4,39}=0.88$, $p=0.49$).

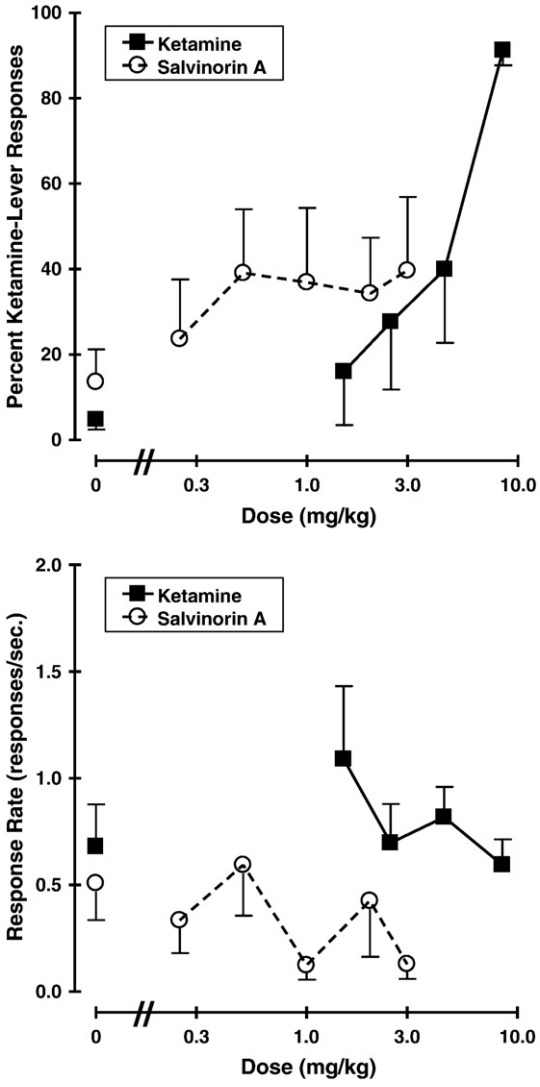

Fig. 2. Dose response curves for ketamine and salvinorin A in rats trained to discriminate 8.0 mg/kg ketamine ($n=8$). Percent ketamine-lever responses for both compounds are depicted in the top graph and the response rate for both compounds is depicted in the lower graph. Data points represent group means (±S.E.M.) at each dose tested.

Salvinorin A did not substitute for ketamine up to doses that significantly suppressed responding. Following administration of salvinorin A, responding was suppressed in 1 of 8 rats at 1.0 and 2.0 mg/kg and in 3 of 8 rats at 3.0 mg/kg. Neither the percent drug-lever responses nor the response rate data were distributed normally, so the Kruskal–Wallis test was used to analyze both these measures. Salvinorin A's effects on the percentage of ketamine-lever responses were not statistically significant ($H=2.11$, 5 d.f., $p=0.834$), although its effects on response rate in these animals was statistically significant ($H=13.56$, 5 d.f., $p<0.05$).

4. Discussion

The current findings demonstrate that the highly selective KOP agonist, salvinorin A exerts stimulus effects in rats that are distinct from those of the 5-HT hallucinogen, LSD and the noncompetitive NMDA antagonist and dissociative hallucinogen, ketamine. Only two previously published studies have compared the discriminative stimulus effects salvinorin A to other hallucinogenic drugs and both studies were conducted in nonhuman primates. Li et al. (2008) reported that salvinorin A failed to substitute for the 5-HT hallucinogen, DOM in rhesus monkeys. Butelman et al. (2010) reported that rhesus monkeys trained to discriminate salvinorin A did not generalize to either psilocybin or ketamine. The results reported herein are consistent with these reports and expand these findings to another species.

The current results are also consistent with some earlier findings that PCP failed to substitute in rats trained to discriminate synthetic KOP agonists (Holtzman et al., 1991; Picker et al. 1990). However, Mori et al. (2006) recently offered fairly convincing evidence suggesting that noncompetitive NMDA-receptor antagonists share similar discriminative stimulus effects with the synthetic KOP receptor agonist, U-50,488H in rats. They tested several NMDA-receptor antagonists in male Fisher 344 rats trained to discriminate either U-50,488H or TRK-820. Animals trained to discriminate U-50,488H generalized to PCP, ketamine, and MK-801, whereas only partial substitution was observed with the competitive NMDA-receptor antagonist (CPP), and the NR1 /NR2B NMDA-receptor antagonist, ifenprodil. However, PCP and MK-801 failed to produce significant drug-appropriate responding in rats trained to discriminate another KOP receptor agonist,TRK-820.

It is noteworthy that the synthetic KOP agonists have been reported to produce hallucinations in humans (Siebert, 1994). Although initial investigations of synthetic KOP agonists demonstrated that activation of KOP receptors can produce aversive and dissociative effects in humans, KOP agonists were not considered true hallucinogens until recent scientific investigations of salvinorin A. Human recreational use of salvinorin A as a "legal alternative" to restricted hallucinogens is at least suggestive that salvinorin A may approximate the effects of other hallucinogenic substances. Laboratory investigations have yet to demonstrate any similarities in the cellular mechanisms underlying the hallucinogenic effects of salvinorin A and other more traditional hallucinogens.

Ergolines, phenethylamines, and tryptamines make up three structural classes of hallucinogens that exert hallucinogen-induced stimulus control through interactions with serotonergic receptor sites. The 5-HT_{2A} receptor site has been shown to be primarily responsible for the discriminative stimulus effects of LSD and psilocybin (Fiorella et al., 1995; for review see Fantegrossi et al., 2008). For example, the selective 5-HT_{2A} antagonist MDL100, 907 completely blocks the discriminative stimulus effects of LSD in mice (Sorensen et al., 1993). A more recent report indicated that mice lacking the serotonin transporter gene do not readily discriminate LSD from vehicle (Krall et al., 2008).

Probably the most convincing evidence for serotonin-mediated hallucinogenesis is the extremely high correlation between a

compound's affinity for the 5-HT_{2A} receptor and hallucinogenic activity in humans (Sadzot et al., 1989). However, a myriad of data show that interactions between multiple receptor systems, and not just serotonin systems, are characteristic of hallucinogenic effects. Gonzalez-Maeso et al. (2007) demonstrated that the neurobehavioral effects of the tryptamine type hallucinogens are dependent on secondary signaling pathways, and are not limited to 5-HT_{2A} receptor activation. Glutamatergic and serotonergic interactions have been demonstrated in co-localized cortical pyramidal neurons and 5-HT_{2A} receptor stimulation is correlated with an increase in glutamate-mediated synaptic activity (Lambe et al., 2000; Lambe and Aghajanian, 2001). Furthermore, dopaminergic D_1/D_5 receptors have been shown to attenuate glutamatergic activity and to oppose the effects of both phenethylamines and tryptamines (Lambe and Aghajanian, 2007). There has yet to be determined a plausible causal mechanistic link between these receptor systems and the hallucinogenic effects of drugs, despite their apparent interactions.

The noncompetitive NMDA antagonists, PCP and ketamine are also known to produce dissociative hallucinations in humans (Krystal et al., 1994). Although the role of NMDA receptors or other glutamate receptors in hallucinogenesis is not well understood, evidence from drug discrimination studies in rats indicate that noncompetitive NMDA antagonists (PCP, dizocilpine, and ketamine) potentiate the discriminative stimulus effects of the serotonergic hallucinogens DOM (Winter et al., 2000) and LSD (Winter et al., 2004). It is also of interest that the administration of the 5-$HT_{2A/2C}$ hallucinogen, 1-(2,5-dimethoxy-4-iodophenyl-2-aminopropane (DOI) has been reported to increase glutamatergic activity in the prefrontal cortex (Lambe et al., 2007). Based on electrophysiological studies, it has been proposed that stimulation of 5-HT_{2A} receptors and NMDA-receptor antagonism both result in glutamate release. The above-mentioned findings by Winter et al. (2000; 2004) provide support for the hypothesis that glutamate release may be a common mechanism responsible for hallucinogenesis produced by 5-HT_2 agonists and NMDA-receptor antagonists.

Endogenous hallucinogenic neurochemicals, in particular N,N-dimethyltryptamine (DMT), have recently been shown to activate a small subgroup of receptor sites called the trace amine associated receptors (Wallach, 2009). Recent evidence suggests DMT may be an endogenous agonist for the sigma-1 receptor (Fontanilla et al., 2009). Salvinorin A has not yet been compared to DMT or other sigma-1 receptor agonists. Interactions between KOP agonists and the trace amine receptors may warrant further investigation.

The failure of salvinorin A to substitute for either LSD or ketamine in the present study suggest that the mechanisms underlying the psychoactive effects of salvinorin A are uniquely different from either serotonergic or glutamatergic hallucinogens. Thorough receptor profiling has determined that salvinorin A has little to no affinity for the 5-HT_{2A} receptor subtype and virtually no affinity for the mu or delta opioid receptors (Roth et al., 2002; O'Connor and Roth, 2005). Moreover, as noted above, there is now considerable evidence that the discriminative stimulus effects of salvinorin A are dependent on kappa opioid receptors (Butelman et al., 2004; Willmore-Fordham et al., 2007; Baker et al., 2009). In consideration of the apparent dissimilarities between the psychoactive effects of salvinorin A and other hallucinogens in an animal model of subjective drug effects, further investigations into the subjective effects and abuse liability of salvinorin A in humans may be warranted.

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