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Serotonin-2A Receptors in Rat Anterior Cingulate Cortex Mediate the Discriminative Stimulus Properties of Lysergic Acid Diethylamide

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Abbreviations: lysergic acid diethylamide (LSD), anterior cingulate cortex (ACC), serotonin-2A (5-HT_{2A}), medial prefrontal cortex (mPFC), fixed ratio 1 (FR1), time-out (TO), variable interval (VI), 2,3-dimethoxy-4-iodoamphetamine (DOI), artificial cerebral spinal fluid (aCSF)

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ABSTRACT

Lysergic acid diethylamide (LSD), an indoleamine hallucinogen, produces profound alterations in mood, thought and perception in humans. The brain site(s) that mediates the effects of LSD is currently unknown. In this study, we combine the drug discrimination paradigm with intracerebral microinjections to investigate the anatomical localization of the discriminative stimulus of LSD in rats. Based on our previous findings, we targeted the anterior cingulate cortex (ACC) to test its involvement in mediating the discriminative stimulus properties of LSD. Rats were trained to discriminate systemically administered LSD (0.085 mg/kg, sc) from saline. Following acquisition of the discrimination, bilateral cannulae were implanted into the ACC (+1.2 mm AP, ±1.0 mm ML, -2.0 mm DV relative to bregma). Rats were tested for their ability to discriminate varying doses of locally infused LSD (0.1875, 0.375, 0.75 μg/side) or artificial CSF (n=3-7). LSD locally infused into ACC dose-dependently substituted for systemically administered LSD, with 0.75μg/side LSD substituting completely (89% correct). Systemic administration of the selective serotonin-2A (5-HT_{2A}) receptor antagonist, M100907 (0.4 mg/kg), blocked the discriminative cue of LSD (0.375 μg/side) infused into ACC (from 68% to 16% drug lever responding). Furthermore, M100907 (0.5 μg/μl/side) locally infused into ACC completely blocked the stimulus effects of systemic LSD (0.04 mg/kg; from 80% to 12% on the LSD lever). Taken together, these data indicate that 5-HT_{2A} receptors in the ACC are a primary target mediating the discriminative stimulus properties of LSD.

Hallucinogenic drugs, such as lysergic acid diethylamide (LSD), produce profound changes in consciousness and perception in humans; these effects are primarily responsible for their abuse potential. Understanding the neuronal substrates that mediate the effects of a drug of abuse is important for developing strategies for treatment and intervention. It is well established that the hallucinogenic effects of LSD and other hallucinogens are mediated via actions at serotonin-2A (5-HT_{2A}) receptors. Evidence for this comes primarily from studies in animals using the drug discriminative paradigm. 5-HT_{2A} receptor agonists substitute for hallucinogens (Glennon et al., 1984; Callahan and Appel, 1988; Winter and Rabin, 1988), a significant correlation exists between the binding of hallucinogens at cortical 5-HT_2 receptors and their potency in drug discrimination (Glennon et al., 1984), and 5-HT_2 receptor antagonists block the discriminative stimulus effects of hallucinogenic drugs (Colpaert et al., 1985; Glennon and Hauck, 1985; Cunningham and Appel, 1987; Meert et al., 1989). More recent behavioral studies utilizing selective 5-HT_{2A} antagonists support a pivotal role for the 5-HT_{2A} receptor in hallucinogen drug discrimination (Ismaiel et al., 1993; Schreiber et al., 1994; Fiorella et al., 1995b). Furthermore, studies in humans suggest that most of the subjective hallucinogenic properties of psilocybin, an indoleamine hallucinogen, are blocked by the 5-HT_{2A} receptor antagonist, ketanserin (Vollenweider et al., 1997; Vollenweider et al., 1998). Thus, 5-HT_{2A} receptor activation is proposed to mediate the behavioral and subjective effects of LSD and other hallucinogens.

However, the precise neuronal circuitry involved in the hallucinogenic drug action is less certain. Earlier studies using microinjections to identify brain regions that may be involved in the actions of LSD have suggested a role for the nucleus accumbens

and doral raphe nucleus (Minnema et al., 1980; Nielsen and Scheel-Kruger, 1986). However, these studies used a relatively high dose of LSD and none applied antagonists to identify the serotonin receptor that mediated the effect of LSD. Our group, using c-fos as a biomarker for neuronal activation, reported an increase in c-fos protein expression in anterior cingulate cortex (ACC), medial prefrontal cortex (mPFC), and amygdala after an acute dose of LSD (Gresch et al., 2002). In addition, we observed that the behavioral tolerance to repeated LSD administration, as measured by an attenuation of the discriminative stimulus, is associated with decreased 5-HT_{2A} receptor signaling and receptor density in the mPFC and ACC (Gresch et al., 2005). Based on these findings and studies indicating the ACC influences many functions including integration of sensory input to modulate complex behaviors, the current study targeted the ACC to test the involvement of this brain region in mediating the discriminative stimulus properties of LSD. Experiments were designed to evaluate the ability of LSD administered directly into the ACC to produce a discriminative stimulus that substitutes for the systemic LSD discriminative stimulus, and to determine whether the discriminative stimulus produced by systemic LSD is mediated by 5-HT_{2A} receptors within the ACC.

METHODS

Animals: Adult male Sprague-Dawley rats (225-249 g; Harlan Sprague-Dawley, Inc; Indianapolis, IN) were individually housed and food deprived to 85% of their free-feeding weight one week prior to the beginning of the drug discrimination experiments. All animals had continuous access to water except during training, and were given enough food immediately following training and on weekends to maintain their weights at 85% of their expected non-deprived weights. Rats were maintained in a colony room (ambient temperature 22-23°C, 12:12 hr light:dark cycle). All animal use procedures were in strict accordance with the NIH *Guide to the Care and Use of Laboratory Animals* and approved by Vanderbilt University Animal Care Committee.

Materials. M100907 (R(+)-alpha-(2,3-dimethoxyphenyl)-1- [2-(4- fluorophenylethyl)]-4-piperidine-methanol) was a gift from Merrill Dow (Cincinnati, OH); lysergic acid diethylamide (LSD) was obtained from the National Institute on Drug Abuse (Bethesda, MD). WAY100635 (N-[2-[4-(2-methoxyphenyl)-1-piperazinyl] ethyl]-N-(2-pyridinyl)cyclohexanecarboxamide) was purchased from Tocris (Ellisville, MO). SB206553 (5-methyl-1-(3-pyridylcarbamoyl)-1,2,3,5-tetrahydropyrrolo[2 ,3-f]indole), mianserin hydorchloride, and ketanserin tartrate were purchased from Sigma Aldrich (St. Louis, MO).

Apparatus: Six commercially available operant conditioning chambers (BRS/LVE Model RTC-024), each housed in a sound attenuated chamber, were used. The operant chambers were equipped with two response levers, a liquid dipper centered between the

two levers and a house light. The equipment and experimental parameters were programmed using MED Associates software and controlled by MED Associates interface and MS-DOS compatible computers.

Drug discrimination training: Rats (n=60) were shaped to lever-press for food reinforcement (Borden's Condensed Milk diluted 1:1 with tap water) on an Fixed Ratio 1 (FR1) schedule of reinforcement during daily 20 min sessions given Monday through Friday. After shaping to lever-press, the reinforcement contingency was changed to a Variable Interval (VI) 15 sec schedule of reinforcement with a 15 sec time-out (TO) for incorrect responses. The TO contingency, a 15 sec period following incorrect responses during which no responses were reinforced, served to punish incorrect responses. At this point discrimination training began. The VI schedule was changed to a VI 30 sec schedule of reinforcement at the end of the first week of training where it remained for the duration of the experiment. On alternate days the rats were injected subcutaneously 30 min prior to training with either 0.085 mg/kg LSD or saline. For half the rats, responding on the right lever was LSD correct and responding on the left lever was saline correct, for the remainder of the rats the reverse was true. Discrimination learning was monitored twice weekly by calculating the percent correct lever responses (number of correct responses/total number of responses) during 2.5 min extinction test sessions given at the beginning of the training session. During the remaining 17.5 min of the training session the VI-30 sec schedule was in effect. Training continued until the rats were averaging 85% correct or greater during the 2.5 min extinction sessions for both LSD and saline.

In order for the animal's choice behavior to be included in the results, the rat was required to make a minimum of 5 responses. For this reason, the length of the test session for all the experiments described below was 5 min in contrast to the 2.5 min extinction session used to monitor acquisition of the discrimination. The additional time is particularly important when the treatment conditions produce response suppression.

Dose-response curve: Following acquisition of the LSD-saline discrimination, a dose-response function was determined for LSD during 5 min extinction tests. For this experiment, 60 rats were assigned to one of five groups (n=12). Rats were injected with LSD (0.01, 0.02, 0.043, 0.085 mg/kg, s.c.) or saline 30 min prior to testing. At the end of the 5 min extinction test, rats were returned to their home cages.

Antagonist studies: To determine the role of the 5-HT_{2A}, 5-HT_{2C} and 5-HT_{1A} receptors in mediating the discriminative stimulus effects of LSD, animals were tested for their ability to discriminate LSD following pretreatment with M100907, a selective 5-HT_{2A} receptor antagonist, ketanserin and mianserin, selective 5-$HT_{2A/C}$ antagonists, SB206553, a selective 5-$HT_{2B/C}$ antagonist and WAY100635, a selective 5-HT_{1A} receptor antagonist. For the M100907 experiment, animals were divided into four groups (n=12) and injected with one of three doses of M100907 (0.025, 0.1 and 0.4 mg/kg, s.c) or saline 45 min prior to testing. Thirty minutes prior to testing all rats were injected with 0.06 mg/kg LSD, s.c. The test dose of LSD, 0.06 mg/kg, was a submaximum dose, thus insuring sensitivity to even subtle effects produced by the various antagonists. At the end of the pretreatment

interval, animals were placed in the operant conditioning chamber and given 5 min extinction tests. Following testing, all animals were returned to their home cages.

Following a week of re-training, mianserin was tested for its ability to block the discriminative stimulus effects of LSD. Rats were assigned to one of four groups (n=11) and pretreated with mianserin (0.125, 0.25 and 0.5 mg/kg, s.c.) or saline 45 min prior to testing. Fifteen minutes subsequent to mianserin administration, animals received 0.06 mg/kg LSD, s.c. Thirty min after LSD injections the rats were tested during 5 min extinctions sessions. At the end of the test session, all animals were returned to their home cages.

The same procedure was used to test ketanserin, SB206553 and WAY100635. For each experiment animals were assigned to one of four groups (n=11 or 12) and pretreated with three doses of drug or saline. Ketanserin (0.1, 0.3 and 1.0 mg/kg, s.c.) was administered 60 min prior to testing, SB206553 (1, 2 and 8 mg/kg, s.c.) was administered 60 min prior to testing and WAY100635 (0.1, 0.3 and 1.0 mg/kg, s.c.) was administered 60 min prior to testing. LSD (0.06 mg/kg, s.c.) was always administered 30 min before testing.

Stereotaxic surgery: Animals were anesthetized with ketamine and xylazine and placed in a stereotaxic apparatus. Bilateral 26 gauge guide cannulae (Plastics One, Roanoke, VA) were surgically implanted in order for the injection cannulae to target the ACC (+1.2 mm AP and ± 1.0 mm ML relative to bregma and – 2.0 mm DV relative to dura (Paxinos and Watson, 1986). After 5-7 days of recovery, discrimination training and behavioral testing resumed.

Drug infusions and testing sessions: Following recovery from surgery, the animals were re-trained and tested on the LSD-saline discrimination to confirm that the discrimination was still intact. Once re-training was complete (1-2 weeks), microinjections of LSD, M100907 or aCSF in a volume of 1 µl was administered through a 33 gauge internal cannula extending 1 mm below the tip of the guide cannula using a microsyringe and hand-driven micromanipulator. Drugs were simultaneously injected bilaterally into the ACC over a 1-min period and the cannula left in place for an additional 2 min. Immediately following removal of the cannula the animals were placed in the operant chamber and given 5 min extinction tests. Animals were habituated to the injection procedure prior to the onset of the experiment.

To determine if intra-ACC infusions of LSD would substitute for systemic LSD, LSD was locally infused into the ACC once per week with systemic training being given on the remaining four days. All tests were conducted on a Friday which allowed the animals the weekend to recover prior to resuming training on Monday. Tests were run 2 min following local infusions and consisted of 5 min extinction sessions at the end of which the animals were returned to their home cages. During their weekly training the rats were given 2.5 min extinction tests to confirm that they were still discriminating both cues at 85% correct or greater. The order for local infusion of LSD test doses was aCSF, 0.75, 0.375 and 0.1875 µg/µl/side LSD.

To determine if systemically administered M100907 would block intra-ACC infused LSD discrimination, rats were tested for their ability to discriminate locally infused LSD following s.c. pretreatment with M100907. This experiment was conducted

over four weeks. On week one, rats received saline (1 ml/kg, s.c.) 20 min prior to local infusion of aCSF (1 µl). For the second week, rats received saline (1 ml/kg, s.c.) 20 min prior to local infusion with 0.375 µg/µl/side LSD. The third week, the 5-HT_{2A} receptor antagonist, M100907 (0.4 mg/kg, s.c.) was injected 20 min prior to local infusion with 0.375 µg/µl/side LSD. On the fourth week, rats were re-tested for their ability to discriminate local infusion of 0.375 µg/µl/side LSD. The fourth experiment was to confirm that the reduction in drug lever selection observed in week three was due to direct antagonism of the 5-HT_{2A} receptors and not the result of a loss of tissue or cannula viability. Each week during the experiment, rats received four days of training with testing being given on Friday. All tests were 5 min extinction sessions.

To determine if intra-ACC infusions of M100907 would block discrimination of systemically administered LSD, animals were tested for their ability to discriminate s.c. LSD in the presence and absence of locally infused M100907. These experiments were conducted over a three weeks. During week one, rats received intra-ACC infusion of aCSF 20 min prior to 0.04 mg/kg LSD. For week two, animals were given local infusions of M100907 (0.5 µg/µl/side) 20 min prior to s.c. injections of 0.04 mg/kg LSD. For the third week, the week one experiment was replicated (intra-ACC infusion of aCSF 20 min prior to 0.04 mg/kg LSD s.c.) to evaluate possible changes in tissue and cannula viability. Rats were tested on Friday during 5 min extinction sessions and received training Monday-Thursday.

Histological analysis: After completion of the study, rats were sacrificed, brains removed, sectioned, mounted on microscope slides and stained. The placement of the

cannulae were verified with reference to the coordinates from the atlas of (Paxinos and Watson, 1986). A schematic diagram of the extent of bilateral cannulation placement for ACC infusions is shown in Figure 1a.

Statistical analysis: The data was analyzed using a one-way ANOVA. Level of significance for the ANOVA was set at $p < 0.05$. Contribution of individual group means to the overall significant *F*-value was determined by Tukey LSD post-hoc test ($p< 0.05$). Statistical analyses were preformed with SPSS 7.0 software (SPSS Inc, Chicago IL)

RESULTS

Discrimination training and dose-response curve. The initial training dose of LSD was gradually incremented over the first 26 days of training from 0.06 to 0.085 mg/kg LSD. The rats were averaging 85% or greater correct lever pressing during the 2.5 min extinction sessions for both training cues by day 31 of training. Following acquisition of the discrimination, a dose-response curve was determined by testing several doses of LSD (0.085, 0.043, 0.02, 0.01 mg/kg, s.c.) and saline (Figure 2). As can be seen, percent choice of LSD lever was dose-dependent [$F(4,50)=47.4$, $p<0.0001$]. All but the lowest dose of LSD tested were significantly different from saline ($p<0.05$, Tukey). Response rates varied significantly as a function of different doses of LSD [$F(4,50)=4.6$, $p<0.0037$]. Rate of responding was significantly higher following 0.01 and 0.02 mg/kg LSD then at the other doses of LSD tested or saline ($p<0.05$, Tukey).

Systemic pretreatment with 5-HT_{2A} receptor antagonists blocks the LSD mediated discriminative stimulus. To determine the role of various 5-HT receptors in mediating the discriminative stimulus effects of LSD, rats were pretreated with increasing doses of M100907, a selective 5-HT_{2A} receptor antagonist; SB206553, a 5-$HT_{2B/2C}$ receptor antagonist; WAY100635, a $5HT_{1A}$ receptor antagonist and mianserin and ketanserin, mixed 5-HT_2 receptor antagonists followed by 0.06 mg/kg LSD, s.c. M100907 [$F(3,40)=31.7$, $p<0.0001$], ketanserin [$F(3,40)=21.5$, $p<0.0001$] and mianserin [$F(3,40)=12.2$, $p<0.0001$] significantly reduced LSD lever selection compared to saline, while SB206553 and WAY100635 did not significantly alter LSD lever selection, thus confirming a major role for the 5-HT_{2A} receptor (Figure 3).

Intra-ACC injection of LSD substitution for systemically administered LSD is dose-dependent. Intra-ACC infusion of 0.1875 μg/side, 0.375 μg/side, and 0.75 μg/side of LSD produced 41.0 ± 15.5 %, 73.5 ± 6.0 and 88.7 ± 4.6 responding on the LSD lever [$F(3,17) = 36.1$, $p < 0.0001$, Figure 4]. The dose of 0.75 μg/side of LSD injected into the ACC substituted fully for systemically administered LSD, while aCSF injection elicited only 14% responding on the LSD lever. A dose of 0.29 μg per side produces 50% LSD lever selection (based on liner regression analysis). Response rates did not differ significantly over the course of the dose-response curve [$F(3,17) = 0.347$, $p = 0.792$, Figure 4, lower panel]. LSD (0.75 μg/side, n=4) injected into brain sites outside of ACC was ineffective in substituting for systemically administered LSD (Figure 1b) eliciting a mean of 24.3 ± 6.7 % responding on the LSD lever.

Systemic M100907 pretreatment blocks the substitution produced by intra-ACC LSD injection. Administration of the 5-HT_{2A} receptor antagonist, M100907 (0.4 mg/kg, s.c) 20 min prior to LSD infusion into the ACC blocked the ability of LSD (0.375 μg/μl/side) locally infused into the ACC to substitute for systemic LSD [$F(3,26) = 38.7$, $p<0.0001$, Figure 5]. Systemic M100907 reduced LSD lever responding by 52% (from 68% to 16%, $p < 0.001$). A week later, the animals were pretreated with saline and retested with the same dose of LSD (0.375 μg/μl/side). The percent responding on the LSD lever was similar to that observed previously (74% compared to 68%) indicating that the discrimination was still intact and confirming that the effects of M100907 were specific and not due to cannula malfunction or loss of ACC tissue viability. Rate of

responding did not differ significantly between the test conditions [$F(3,26) = 0.772$, $p<0.520$, Figure 5, lower panel].

Intra-ACC injection of M100907 blocks systemic LSD substitution. Local infusion of the 5-HT_{2A} receptor antagonist, M100907 (0.5 μg/μl/side) 20 mins prior to systemic administration of LSD (0.04 mg/kg, s.c) blocked the discriminative stimulus effects of systemically administered LSD [$F(2,11) = 37.3$, $p< 0.0001$, Figure 6]. Local infusion of M100907 reduced LSD lever responding from 80% to 12% ($p <0.001$, Tukey). When the animals were retested with LSD 0.04 mg/kg sc, percent LSD lever selection was similar to that which occurred prior to local infusions of M100907 (85% compared to 80%), confirming tissue and cannula viability. Response rates were not altered significantly by the test conditions [$F(2,11) = 0.996$, $p= 0.40$, Figure 6, lower panel].

DISCUSSION

In the present study we demonstrate that systemic administration of the specific 5-HT_{2A} receptor antagonist, M100907, and the 5-HT_2 receptor antagonists, ketanserin and mianserin, completely blocked the LSD-induced discriminative stimulus whereas the 5-HT_{1A} antagonist, WAY100635, and 5-$HT_{2B/2C}$ receptor antagonist, SB 206553, had no effect on choice behavior. This finding is in agreement with reports demonstrating the blockade of the discriminative cue by DOI or LSD in rats with M100907 pretreatment (Schreiber et al., 1994; Winter et al., 2004), supporting the general conclusion that 5-HT_{2A} receptors are key mediators of the discriminative stimulus effects of hallucinogens in rats (see Nichols, 2004, for review). However, because LSD also has high affinity for the 5-HT_{1A} receptor, many drug discrimination studies have investigated a possible role for the 5-HT_{1A} receptor in the stimulus effects of LSD. The results have been variable, but in general suggest that there is not a large 5-HT_{1A} component to the LSD stimulus in the rat. For example, the 5-HT_{1A} receptor agonist 8-hydroxy-2-(di-*n*-propylamino)-tetralin (8-OH-DPAT) has been found to either partially substitute (Reissig et al., 2005) or not substitute at all for LSD (Cunningham and Appel, 1987) in rats. Interestingly, the involvement of 5-HT_{1A} receptor to the LSD discriminative stimulus may be species dependent; the LSD discriminative stimulus in the monkey, pigeon, and mouse is reported to have a 5-HT_{1A} receptor component (Nielsen, 1985; Walker et al., 1991; Benneyworth et al., 2005). The possible role for the 5-HT_{2C} receptor is less certain due to the lack of specific agonists available for testing (Nichols, 2004), however, (Fiorella et al., 1995a) demonstrated that 5-HT_{2C} receptors mediate an enhanced sensitivity to LSD discriminative stimulus following serotonin depletion. In summary, our results confirm

that 5-HT_{2A} receptors are the primary mediators of the discriminative stimulus of LSD in the rat.

In addition, we showed that direct bilateral injections of LSD into the ACC of rats substituted completely for systemically administered LSD in drug discrimination. This substitution was dose-dependent with a dose of approximately 0.29 μg of LSD per side producing 50% LSD lever selection. The discriminative stimulus produced by LSD infused directly into the ACC was completely blocked by systemic administration of the selective 5-HT_{2A} receptor antagonist, M100907. Injections of LSD into the orbital or frontal cortex did not substitute for systemic LSD suggesting that not all cortical sites that express 5-HT_{2A} receptors (Cornea-Hebert et al., 1999) are involved in producing the discriminative cue LSD. The major finding of the present study was that direct bilateral injections of M100907 into the ACC completely blocked the discriminative stimulus produced by systemic LSD. Taken together, these results suggest that activation of 5-HT_{2A} receptors located within the ACC is necessary to produce the discriminative stimulus of LSD.

The hypothesis that the hallucinogenic drug, LSD, is acting centrally on the 5-HT system was proposed almost immediately after the discovery of 5-HT as a CNS neurotransmitter. Based primarily on the similarities in chemical structure between LSD and 5-HT, Gaddum and Hameed, (1954) proposed that the effects of LSD might result from actions within the CNS acting on the 5-HT system. Furthermore, Freedman, (1961) demonstrated that administration of LSD elevated brain 5-HT levels. More recently, it was demonstrated that direct injections of 7.5 μg or 25 μg LSD into the lateral ventricle of rats trained to discriminate systemically administered 0.1 mg/kg LSD from saline

produced a full generalization with a similar time course as systemic LSD (Doat et al., 2003). Indeed, we replicated this finding in rats trained to discriminate the hallucinogen, 2,3-dimethoxy-4-iodoamphetamine (DOI), a mixed 5-HT_{2A} and 5-HT_{2C} agonist. We observed that LSD injected into the lateral ventricle in rats trained to discriminate 0.75 mg/kg of DOI substitutes completely for systemic DOI with a dose of 0.45 μg producing 50% DOI lever selection, confirming that LSD acts centrally (unpublished data).

The first evidence that a discrete brain site might be mediating the effects of LSD was from the early studies of Aghajanian and colleagues. They reported that LSD administered either systemically or directly by iontophoresis to the serotonergic raphe cell bodies suppressed the firing of raphe neurons (Aghajanian et al., 1968; Aghajanian et al., 1970; Aghajanian et al., 1972). This lead to the hypothesis that hallucinogens exert their profound effects by altering raphe function thus having extensive influence on many brain regions because of the widespread projections of raphe neurons. However, not all hallucinogenic drugs used by humans have this property, for example the phenethylamines such as mescaline do not suppress raphe neuron firing (Aghajanian et al., 1970; Haigler and Aghajanian, 1973), therefore other brain areas were hypothesized to be involved in the actions of hallucinogenic drugs.

The strategy of intracranial drug injection has been successfully used to identify brain areas in which drugs of abuse initiate their actions. For instance, one of the most intensely studied brain systems is the dopamine system that is involved in the actions of cocaine. Through various methodologies including direct injections, it has been demonstrated that the nucleus accumbens and frontal cortex are involved in the rewarding and reinforcing properties of cocaine's actions (see Wise and Hoffman, 1992). This

method of direct injection has also been used to explore the brain regions that mediate the actions of LSD. Mokler and Rech, (1984) demonstrated that LSD administered intracerebrally disrupted responding on an FR-40 operant task with an ED_{50} of 15 μg. Other investigators have used the drug discrimination paradigm to determine the involvement of brain regions in mediating LSD's discriminative stimulus. The drug discrimination paradigm is a powerful and sensitive method that has been used in both animals and humans to evaluate the stimulus properties of drugs. Results from most (Schuster and Johanson, 1988; Kamien et al., 1993; Johanson et al., 2006), but not all (Brauer et al., 1997) drug discrimination studies in humans, show that laboratory animals and humans place drugs into similar drug classifications . A dose of LSD (1μg) injected bilaterally into nucleus accumbens substituted completely for systemic LSD in rats trained to discriminate 0.16 mg/kg LSD in a FR32 schedule (Nielsen and Scheel-Kruger, 1986). The raphe nucleus was targeted in the study by Minnema et al., (1980) using a two lever VI 15 operant procedure with rats trained to discriminate 96 μg/kg LSD. Direct injection of LSD into the dorsal raphe produced a stimulus that generalized to systemically administered LSD with a similar time course.

The doses of LSD used in most of these studies were relatively high, clouding inteerpretation. For example, the study of Nielsen and Scheel-Kruger (1986) used a training dose of 160 μg/kg compared to our training dose of 60 μg/kg and Minnema et al., (1980) injected 60 μg/kg into the raphe compared to our approximate doses of 1.0 – 5.0 μg/kg injected into the ACC. At high doses, LSD interacts with multiple receptors including 5-HT_{1A} and dopamine D_2 receptors (Nichols, 2004). Furthermore, none of these studies used selective serotonin receptor antagonists to identify which receptors were

mediating the actions of the centrally administered LSD. Thus, the results presented here are the first to demonstrate that 5-HT_{2A} receptors within the ACC are essential for the stimulus produced by LSD. The current results do not rule out a role for 5-HT_{2A} receptors in other brain sites.

Major methodological issues inherent in direct brain microinjections include tissue damage due to cannula implantation and region of influence of a lipid soluble,diffusable drug. Although tissue damage to the cortex could alter behavior in the drug discrimination paradigm, we observed a similar ~80% correct lever pressing with 0.043 mg/kg LSD in non-cannula-implanted animals (figure 2) and with 0.04 mg/kg LSD in bilateral-cannulated animals (figure 6) indicating that surgery and subsequent tissue damage did not alter the ability of systemic LSD to produce discrimination. This conclusion was reinforced in our experimental design in which the stimulus properties of LSD were compared the week before and week after microinjections and found to be identical. We injected dye in a subset of rats to help localize the injection site; qualitative analysis indicated that diffusion of the dye was limited to the ACC. However, the diffusion coefficient of the dye differs compared to the drugs used in this study and post mortem tissue manipulation confounds the use of dye diffusion to conclude the precise anatomical localization of the drugs infused. Placement of the injection cannula extended into border regions of the ACC and frontal cortex (figure 1), thus we can not exclude the possible involvement of this region of the frontal cortex in the LSD discriminative stimulus. However, direct injection of LSD bilaterally into the frontal cortex 0.5 to 1 mm anteriorly was ineffective, suggesting that the diffusion of LSD is relatively anatomically discrete.

The region of the rat ACC targeted in our experiments corresponds to areas 24a and 24b of the rat cingulate cortex (Vogt and Peters, 1981; Vogt et al., 2003). Areas 24a and 24b contain most neuronal types found in neocortical areas; in addition to pyramidal cells, there are a variety of multipolar, bitufted, and bipolar cells (Vogt and Peters, 1981; Devinsky et al., 1995; Gabbott et al., 1997; Vogt et al., 2003). In the ACC, 5-HT_{2A} receptors are located throughout the cortical layers, with the densest expression on layer V pyramidal cells (Cornea-Hebert et al., 1999). This region of the ACC receives projections from the anteriomedial and laterodorsal thalamus and amygdala and projects to motor and visual cortexes, periaqueductal grey, striatum, nucleus accumbens, raphe nucleus, nucleus of the solitary tract, and dorsal nucleus of the vagus (Vogt and Peters, 1981; Vogt and Miller, 1983 ; Devinsky et al., 1995; Vogt et al., 2003). The ACC is unique in that its role is to integrate inputs from multiple thalamic nuclei for the initiation of goal-directed behaviors. The unifying theory of ACC function is to integrate the individual's affect into their cognitive and motor behaviors (Devinsky et al., 1995). Since the ACC influences many functions including modulating autonomic activity, endocrine function, nociceptive processing, visuomotor integration, and decision making behaviors (Devinsky et al., 1995; Vogt et al., 2003), our finding that LSD produced a discriminative cue by acting on 5-HT_{2A} receptors in the ACC may reflect modulation of these systems to produce its unique introceptive stimulus.

Psilocybin, an indoleamine hallucinogen that shares similar psychological effects in humans as LSD, in normal human subjects increases cerebral glucose utilization in cortical regions including the ACC as measured by PET (Vollenweider et al., 1997). Vollenweider hypothesized that the association between the subject's drug-induced

derealization and ego pathologies and the increase in glucose metabolism in the ACC may be a result of disruption of ACC network circuits leading to a mismatch of internal and external reality, change of sense of time and space, and impairment of ego functioning. Interestingly, neuroimaging studies have reported that both auditory and visual hallucinations in schizophrenics are associated with activation of the ACC (Silbersweig et al., 1995; Weiss and Heckers, 1999; Shergill et al., 2000). Moreover, the ACC has been implicated in the neuropathology of schizophrenia because of structural and morphological differences observed from postmortem analyses of schizophrenic brains, including a reduction of gray matter volume, reduced laminar thickness, and reduction in number and size of pyramidal neurons (Benes et al., 2001; Bouras et al., 2001; Chana et al., 2003). These clinical observations point to a role of a dysfunctional ACC in schizophrenia.

In conclusion, we confirmed previous studies demonstrating that 5-HT_{2A} receptors mediate the discriminative stimulus of systemic LSD in rats, whereas 5-HT_{2C} receptors and 5-HT_{1A} receptors do not appear to be involved in the LSD discriminative stimulus in the rat. In addition, we observed that direct bilateral injections of LSD into the ACC of rats substituted completely for systemically administered LSD. Furthermore, we showed that local antagonism of 5-HT_{2A} receptors in the ACC completely blocked the effects of systemic LSD, suggesting that activation of 5-HT_{2A} receptor in the ACC is necessary for the production of the unique effects of LSD.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Illustration of histological verification of the injection sites for the intracerebral administration of drugs. A. Cannula placements indicated by black dot are the sites of drug injection as determined from cannula tracks that were within the ACC. B. Cannula tip placements indicated by open circles in the orbital and frontal cortex are the sites that LSD infusion (0.75 μg/side) did not substitute for systemic LSD. Illustrations are adapted from the atlas of Paxinos and Watson (1986). Measurements in millimeters refer to distance from bregma.

Figure 2. LSD dose-response curve. The data represent mean percent responding ± SEM (n=12) on the LSD lever (*upper panel*) and mean number of responses ± SEM made during 5-min test session (*lower panel*) as a function of various doses of LSD. * in upper panel indicates LSD treated groups whose choice behavior was significantly different from vehicle controls ($p < 0.05$, Tukey). * in lower panel rate of responding was significantly different from other doses of LSD tested or saline ($p<0.05$, Tukey).

Figure 3. Pharmacological properties of LSD discrimination cue. The ability of increasing doses of M100907 (M), a selective 5-HT_{2A} receptor antagonist; SB206553 (SB), a $5HT_{2B/2C}$ receptor antagonist; WAY100635 (WAY), a $5HT_{1A}$ receptor antagonist and mianserin (Mian) and ketanserin (Ket), mixed 5-HT_2 receptor antagonists to block 0.06 mg/kg s.c. LSD is shown in the upper panel (mean percent responding ± SEM on LSD lever; n=11-12). Mean response rates ± SEM made during the test session is shown in the lower panel. Test doses are plotted as equal log units.

Figure 4. LSD infusion into the ACC . Direct microinjection of LSD into ACC substituted for systemic LSD in a dose dependent manner (*upper panel)*. A dose of 0.75 µg/side of LSD injected bilaterally into the ACC substituted completely for systemically administered LSD. ** = significantly different from aCSF ($p < 0.001$, Tukey); † = significantly different from 0.1875 mg LSD ($p < 0.05$, Tukey); (n = 3-7). *Lower panel* depicts the mean ± SEM total responses the rats made during the 5 min test period.

Figure 5. Systemic M100907 blocks intra-ACC injected LSD. Pretreatment of animals with the specific 5-HT_{2A} antagonist, M100907 (M) (0.4 mg/kg s.c.) administered 20 mins prior to LSD blocked the ability of bilateral, intra-ACC injection of LSD (0.375 µg/µl/side) to substitute for systemic LSD (upper *panel)*. ** = $p < 0.001$ compared to aCSF; † † = $p < 0.001$ compared to intracerebral (ic) LSD; §§ = $p < 0.001$ compared to retest ic LSD; (n = 6-8). *Lower panel* indicates the mean ± SEM total responses the rats made during the 5 min test period.

Figure 6. Intra-ACC injection of M100907 blocks systemic LSD. The 5-HT_{2A} receptor antagonist, M100907 (M)(0.5 µg/µl/side) was injected bilaterally into the ACC 20 mins prior to systemically administered LSD (0.04 mg.kg s.c.). As illustrated in the *upper panel*, intra-ACC injection ofM100907 significantly reduced the ability of rats to discriminate LSD, given systemically . ** = $p < 0.001$ compared to intracerebral (ic) aCSF- sc LSD; † † = $p < 0.001$ compared to retest ic aCSF- sc LSD; (n = 4-5). The mean

± SEM total responses the rats made during the 5 min test period are shown in the *lower panel*.

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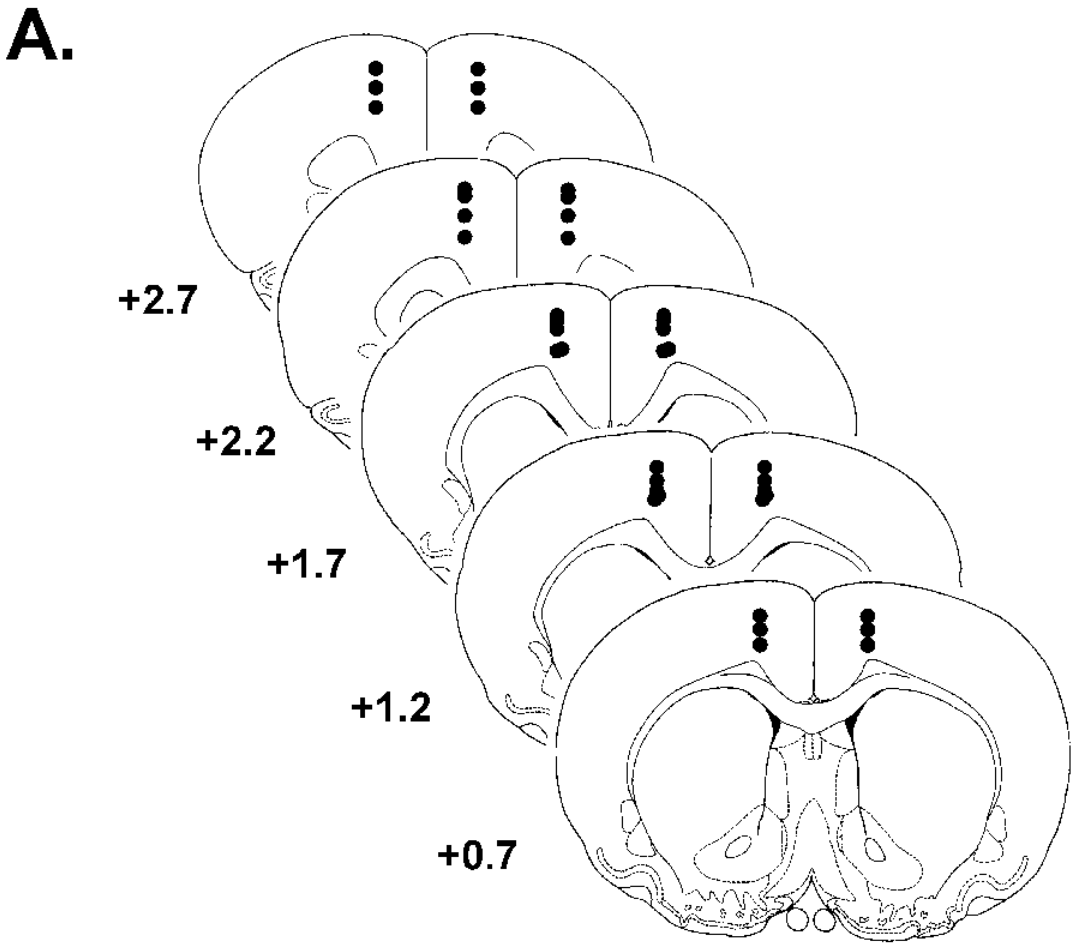

Figure 1

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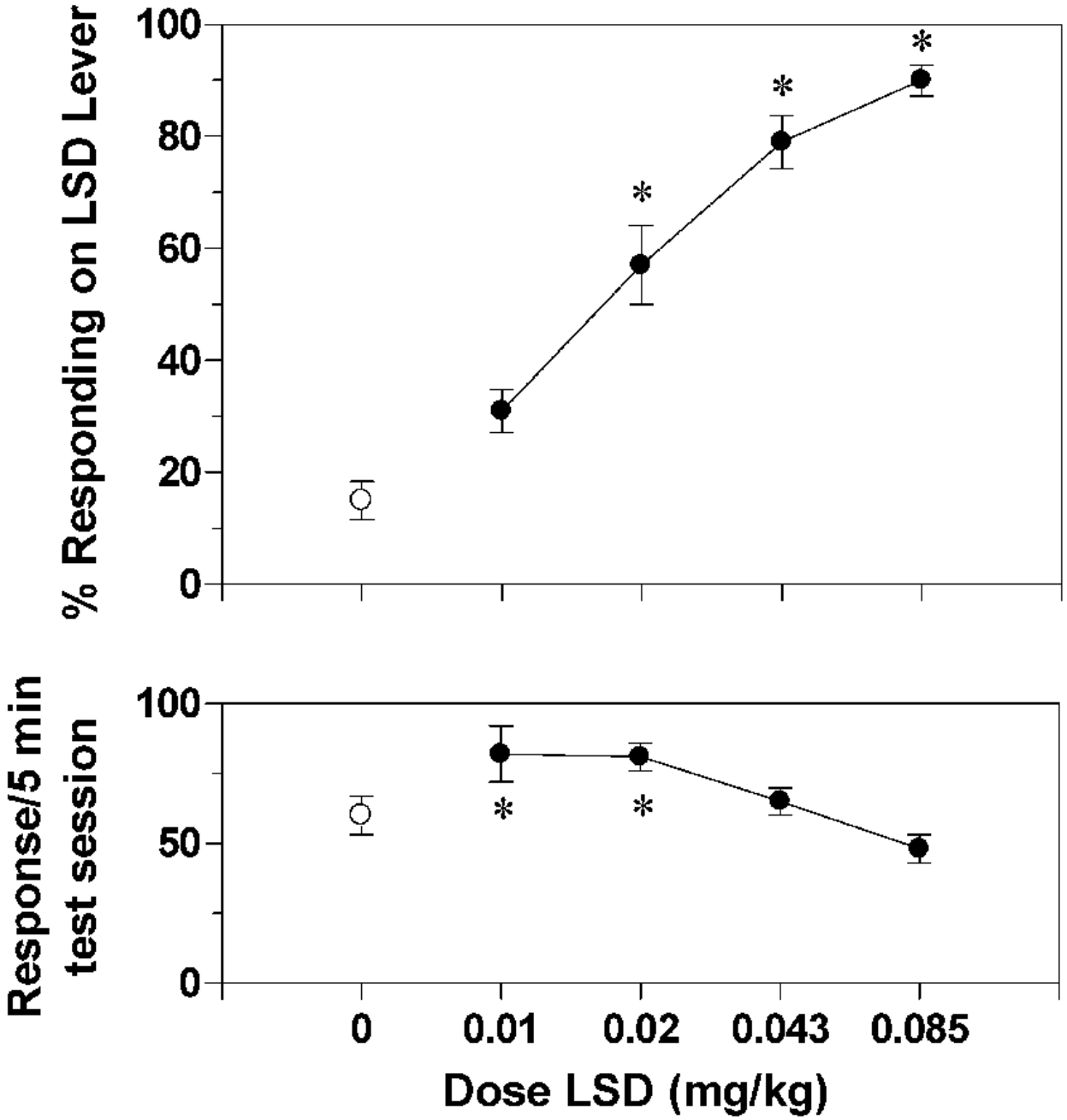

Figure 2

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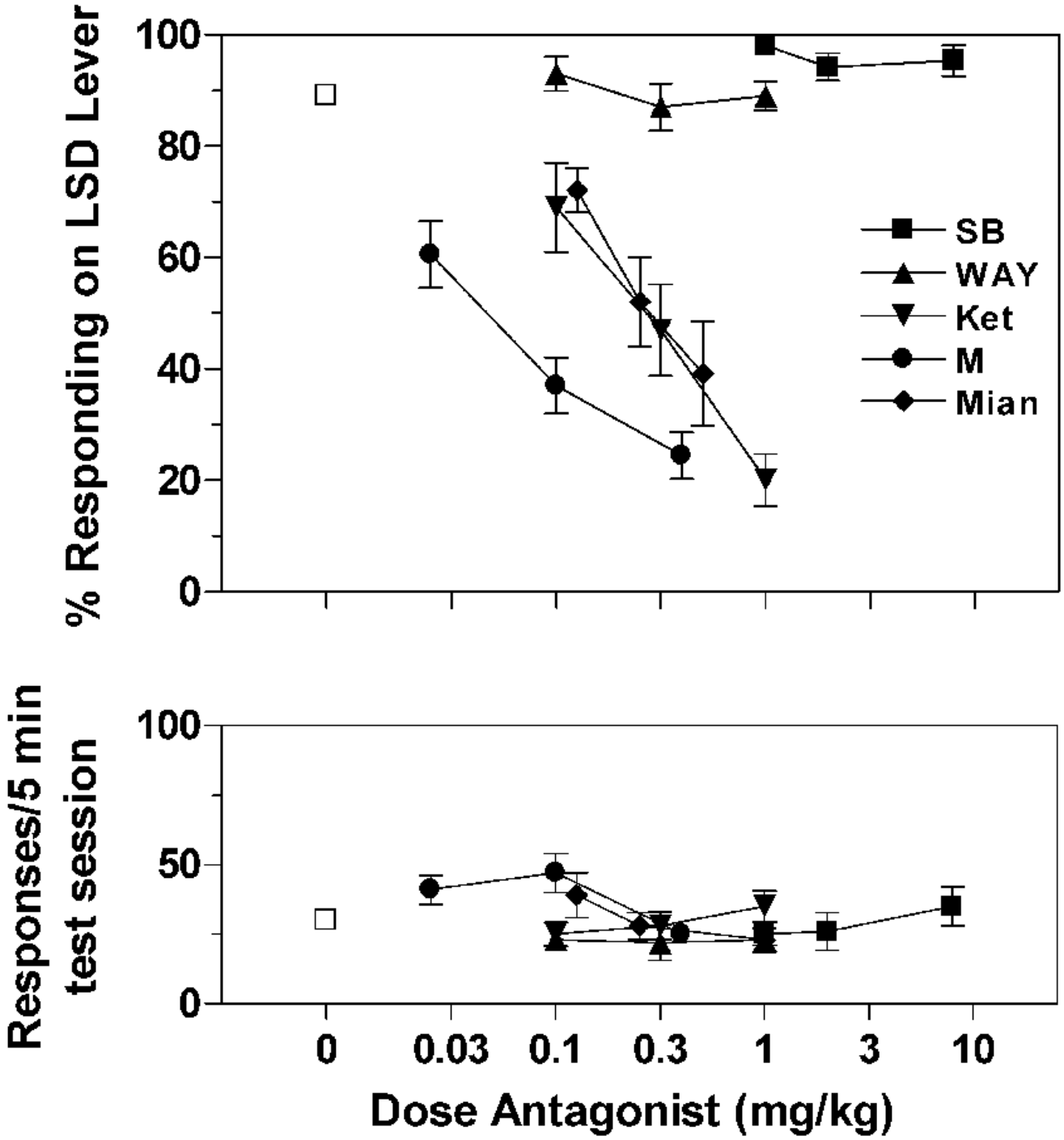

Figure 3

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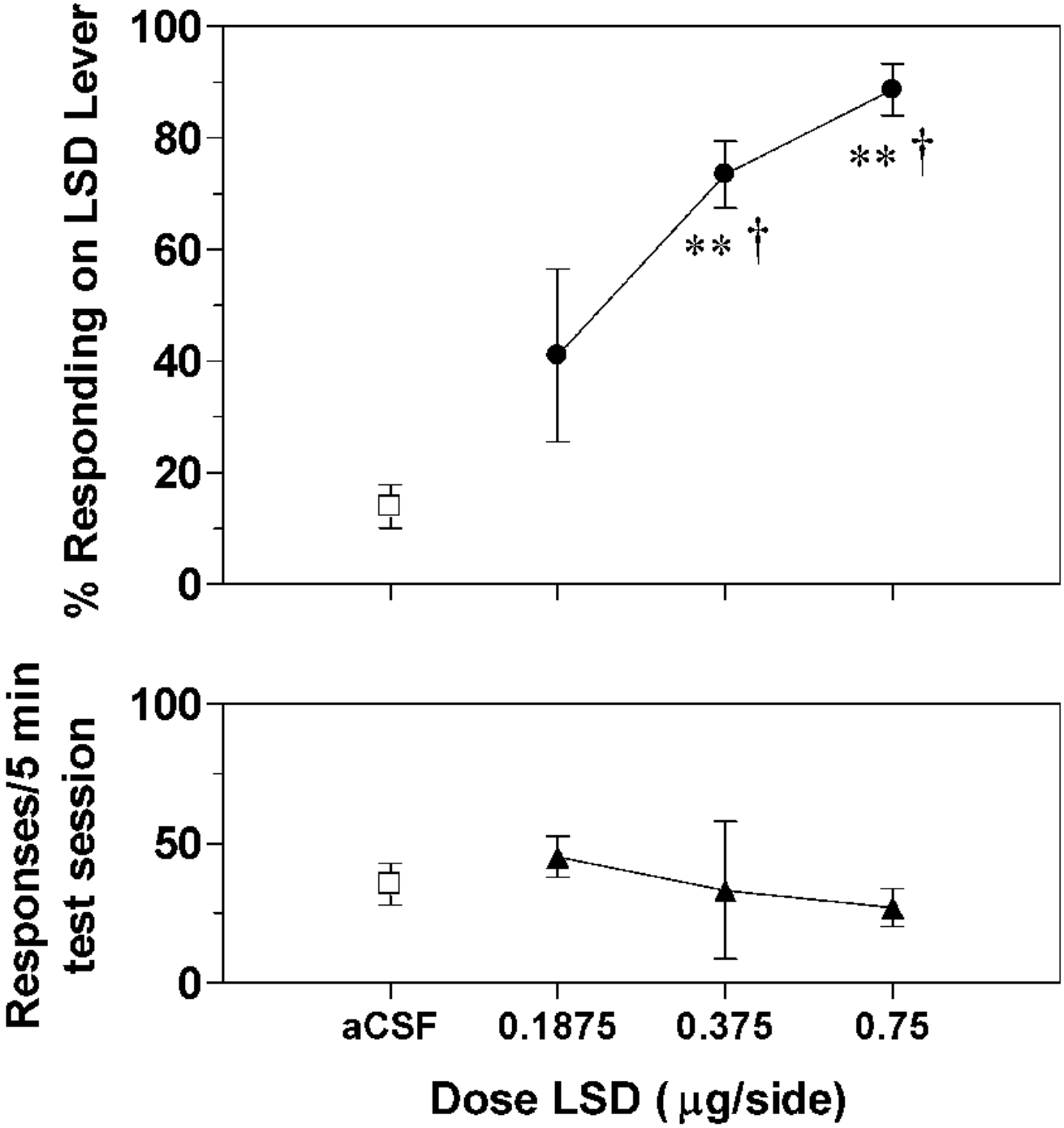

Figure 4

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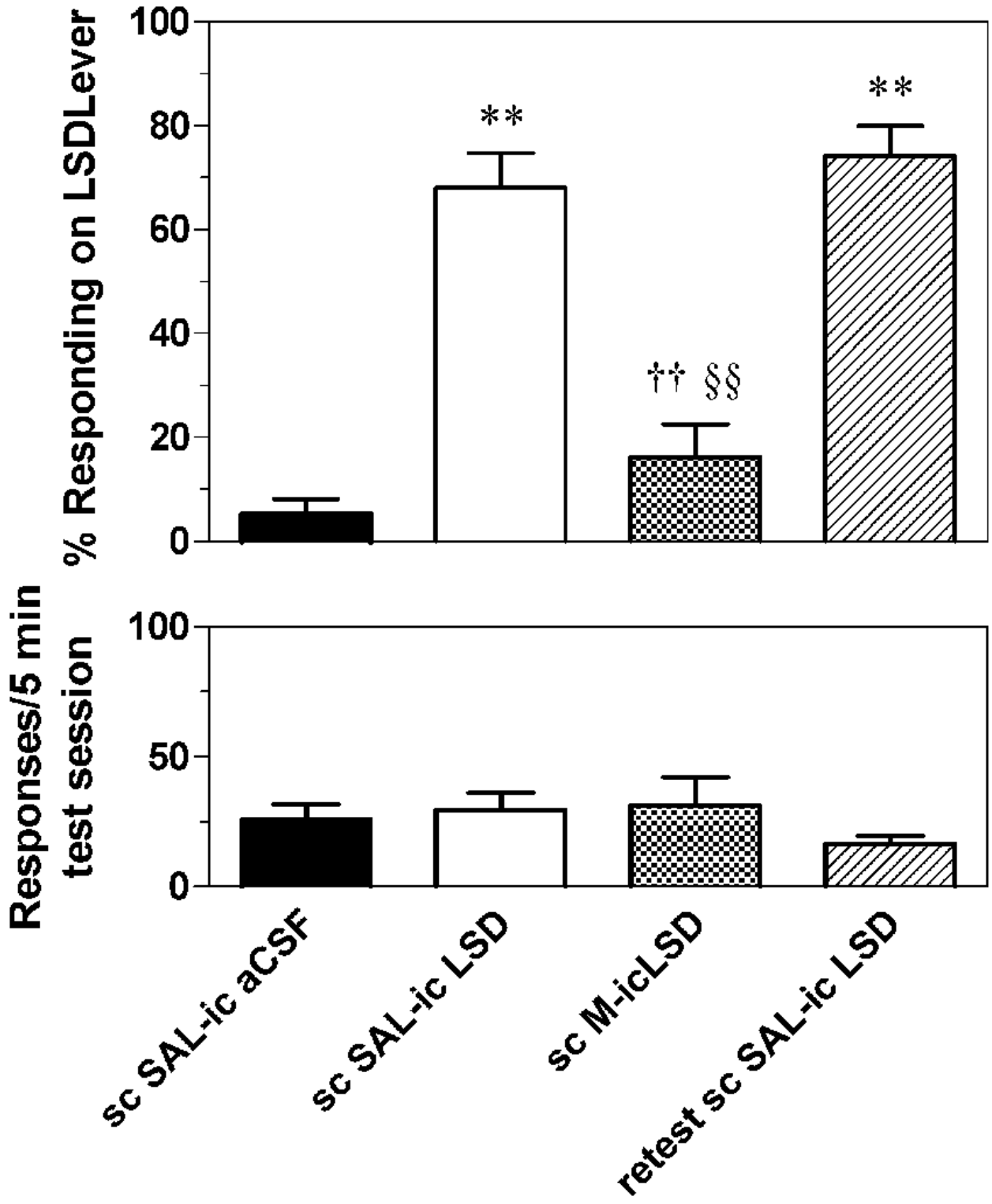

Figure 5

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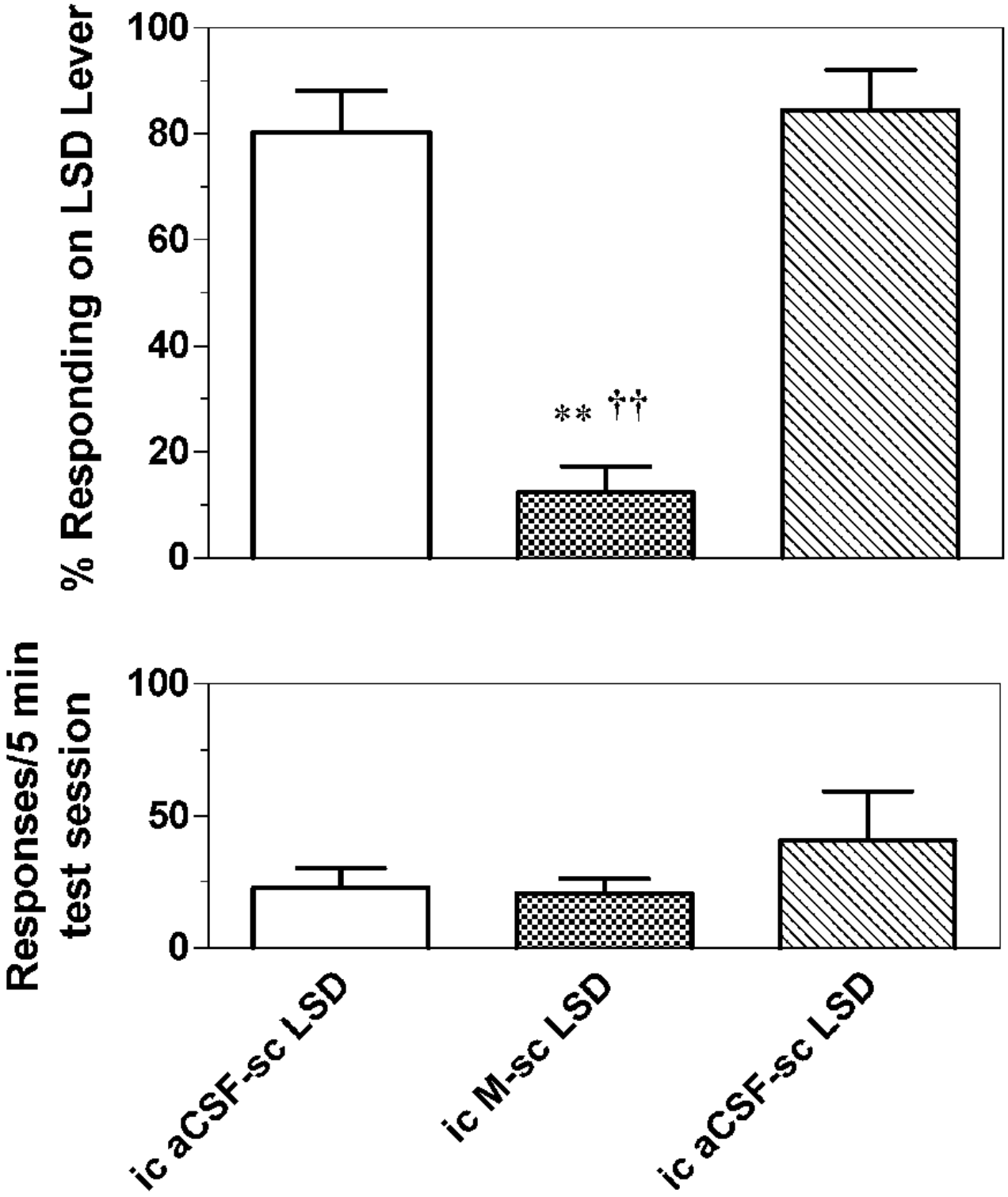

Figure 6