The 5-HT_{1A} receptor and Hallucinogens

By

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In Memory of: Michael G. Villani, Ph.D

For my Family and loved ones.

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Figure Lists and Legends

Abstract

Use of hallucinogens, such as lysergic acid diethylamide (LSD), continues to be a societal problem and accordingly is a major interest of the National Institute on Drug Abuse. Hallucinogens are worthy of study not only because they have significant abuse liability but also because a detailed understanding of their mechanisms of action may be informative with respect to a variety of psychiatric disorders including psychosis. The psychotropic actions of indoleamine (e.g., LSD) and phenethylamine (e.g., mescaline) hallucinogens appear to involve activation of 5-HT_{2A} receptors. However, this activation is modulated by other serotonergic and non-serotonergic receptors. The objective of the project was to determine the modulatory influence of 5-HT_{1A} receptors on the stimulus effects of LSD, and to determine the mechanism by which this modulation occurs.

It has been suggested that the 5-HT_{1A} receptor plays a significant modulatory role in the stimulus effects of the hallucinogen lysergic acid diethylamide (LSD). Initial studies sought to characterize the effects of several compounds with known affinity for the 5-HT_{1A} receptor on the discriminative stimulus effects of LSD. Rats were trained in a two-lever, fixed ratio10, food reinforced task with LSD (0.1 mg/kg; IP; 15 min pretreatment) as a discriminative stimulus. Combination and substitution tests with the 5-HT_{1A} agonists, 8-hydroxy-2-(di-*N*-propylamino)tetralin (8-OH-DPAT), buspirone, gepirone, and ipsapirone, with LSD-induced stimulus control were then performed. The effects of these 5-HT_{1A} ligands were also tested in the presence of the selective 5-HT_{1A} receptor antagonist, WAY-100,635 (0.3 mg/kg; SC; 30 min. pretreatment). In combination tests stimulus control by LSD was increased by all 5-HT_{1A} receptor ligands

with agonist properties. Similarly, in tests of antagonism, the increase in drug-appropriate responding caused by stimulation of the 5-HT_{1A} receptor was abolished by administration of WAY-100,635. These data support the hypothesis that the 5-HT_{1A} receptor has a significant modulatory role in the stimulus effects of LSD.

Observations from the preceding study resulted in investigations to test the hypothesis that ligands which were active at 5-HT_{2A} receptors had a modulatory influence on 5-HT_{1A} receptor function. Initial studies sought to characterize the effects of the selective 5-HT_{2A} antagonist M100907 on the discriminative stimulus effects of 8-OH-DPAT. Rats were trained in a two-lever, fixed ratio10, food reinforced task with the prototypical 5-HT_{1A} agonist 8-OH-DPAT (0.2 mg/kg; IP; 15 min pretreatment) as a discriminative stimulus. Stimulus control by 8-OH-DPAT was attenuated by the selective 5-HT_{2A} antagonist M100907 (0.1 mg/kg; 30 min pretreatment time). In addition, 8-OH-DPAT generalized to the 5-HT_{1A} agonist buspirone (1.0 mg/kg; 15 min pretreatment time) and this substitution was also attenuated by M100907. These data suggest that 5-HT_{2A} receptors may have a role in the stimulus properties of 8-OH-DPAT and that the interactions between 5-HT_{1A} and 5-HT_{2A} receptors are bidirectional in drug discrimination studies.

Based on our behavioral studies the mechanisms of action of LSD, and the observed 5-HT_{1A}-5-HT_{2A} receptor interactions were investigated. While previous studies have suggested a prominent role for 5-HT_{1A} and 5-HT_{2A} receptors in the mechanism of action of LSD, glial cells and other cytological sites of action may contribute to the drug's effects. To this end, c-Fos was employed as a marker of cellular activation to determine the specific cell types which are activated by administration of LSD. Adult

male F344 rats were injected with LSD and sacrificed 90 min later. Brains were removed, and the prefrontal cortex was examined for c-Fos expression by immunohistochemistry. Dose-response studies revealed that LSD-induced c-Fos expression is dose related. Double labeled immunohistochemistry revealed that LSD induced c-Fos expression occurs in neurons, and oligodendrocytes. Because c-Fos is an inducible transcription factor, these results suggest that dose related changes in gene expression may result from, and contribute to the behavioral effects of LSD. Our finding that LSD-induced c-Fos expression occurs in oligodendrocytes suggests these cells may be involved in the acute behavioral effects of LSD or stimulated as a consequence of LSD administration.

General Introduction

Hallucinogens have been known by man for thousands of years. The use of psychoactive plants and mushrooms, predates written history where they were used in a variety of sociocultural and religious contexts (Nichols, 2004). Archaeological evidence discovered in Texas indicates that peyote was used ceremonially more than 3, 000 years ago. A Texas resident is believed to have initiated the modern study of hallucinogens by delivering samples of peyote to Park Davis and Co in the early 1880's. In 1897 Arthur Hefter became the first to obtain a relatively pure psychedelic compound from a naturally occurring substance when he isolated mescaline from peyote. Many consider however, the modern era of hallucinogens to have begun when the psychotropic effects of LSD were discovered by Albert Hofmann in 1943 (Hofmann, 1959). In the nearly 60 years since, LSD's mechanisms of action are still not fully elucidated. Greater understanding of the functions of hallucinogens will not only help ameliorate a serious public health concern, but may also add insight into cognitive function and various forms of human psychosis.

A role for serotonin in the effects of LSD was proposed based on results of experiments involving isolated smooth muscle (Wooley, 1954.). Through human (Balistrieri and Fontanari, 1959.) and animal studies (Aghajanian et al., 1970; Winter, 1971; Huang and Ho, 1972.; Cheng et al., 1973) the hypothesis emerged that indoleamine and phenethylamine hallucinogens may act through a common mechanism. With the emergence of drug-induced stimulus control as a tool for the study of psychoactive drugs, it became possible to rigorously test in animals the hypothesis that indoleamine and phenethylamine hallucinogens act via a common serotonergic mechanism. Following the

demonstration that LSD and mescaline could function as discriminative stimuli in the rat (Hirschhorn and Winter, 1971), it was observed that serotonergic antagonists block the stimulus effects of mescaline (Browne and Ho, 1975b; Browne and Ho, 1975a; Winter, 1975). Subsequently, serotonergic antagonists were shown to block the stimulus effects of other hallucinogens, including LSD, 2,5-dimethoxy-4-methylamphetamine (DOM) and dimethyltryptamine (DMT) (Kuhn et al., 1978; Winter, 1978; Glennon et al., 1983).

The original classification of serotonin receptors by Gaddum and Picarelli (1957) has been refined and expanded such that presently there are 14 serotonin receptors categorized into 7 families (5-HT_{1-7}). The 5-HT_2 receptor family, especially the 5-HT_{2A} receptor, appears to be essential for the stimulus effects of indoleamine and phenethylamine hallucinogens. Glennon and colleagues implicated the 5-HT_2 receptor in hallucinogenesis based upon a high degree of correlation between affinities for the 5-HT_2 receptor and both potency in substituting for DOM-induced stimulus control as well as hallucinogenic potency in man (Glennon et al., 1983; Glennon et al., 1984). However, the subsequent discovery of a 5-HT_{2C} receptor (Pazos et al., 1985; Pazos et al., 1998) with a high level of structural and functional similarity to the 5-HT_{2A} receptor and the demonstration that indoleamine and phenethylamine hallucinogens act as partial agonists at the 5-HT_{2C} receptor (Sanders-Bush et al., 1988; Sanders-Bush and Breeding, 1991) demanded consideration of this serotonin receptor subtype. To address this question, experiments in our laboratory used antagonist correlation analysis. Results of these experiments led to the conclusion (Fiorella et al., 1995c) that [i] the *in vivo* potency of the antagonists to block the LSD stimulus and the generalization of the LSD stimulus to [-]-DOM is directly proportional to the *in vitro* affinity of those same antagonists for 5-HT_{2A}

receptors and [ii] interactions with 5-HT_{2C} receptors cannot account for the remaining variance in the potencies of the antagonists to block the LSD stimulus or the generalization of the LSD stimulus to [-]-DOM. These conclusions were further buttressed by the observation that stimulus control by DOM and its iodo analog are antagonized by drugs which are relatively selective for the 5-HT_{2A} receptor (Ismaiel et al., 1993; Schreiber et al., 1994).

5-HT_{1A} Receptor

The 5-HT_{1A} receptor is found throughout the brain, with high concentrations in dorsal raphe nucleus (DRN), medial raphe nucleus (MRN), hippocampus, lateral septum, entorhinal cortex, and central amygadala. The raphe nuclei are the major source of serotonergic cell bodies in the brain and send projections to cortical and limbic areas (Aghajanian et al., 1968). In general, projections from DRN and MRN overlap one another with the former sending projections to the frontal cortex (Molliver, 1987) an area containing a high density of 5-HT_{2A} receptors (Pazos et al., 1985; Pazos and Palacios, 1985) and thought to play a significant role in hallucinogenesis and psychosis (Arvanov et al., 1999; Gewirtz and Marek, 2000). Previous studies support a significant role of the medial raphe nucleus in hallucinogenesis, as DOM infused directly into this area is able to produce full generalization to the discriminative effects of DOM (Doat et al., 2003).

The 5-HT_{1A} receptor is a member of the superfamily of heptahelical receptors typically linked to G protein activation. Agonist binding of G protein coupled receptors (GPCRs) results in the exchange of GTP for GDP on the α subunit of the heterotrimeric G protein. This binding of GTP causes a dissociative activation of the G protein into an

α-GTP complex and βγ subunits, which can then interact with the same or different types of effectors. In the case of the 5-HT_{1A} receptor, the activity of numerous downstream targets (e.g. adenylyl cyclase, K^+ channels, phospholipase C and protein kinase C) can be affected (Raymond et al., 2001). Interestingly, the inhibition of adenylate cyclase activity observed by 5-HT_{1A} stimulation in various tissues is not seen in the DRN (Clarke et al., 1996; Johnson et al., 1997). Hydrolysis of GTP by an intrinsic GTPase on the α subunit allows reformation of the subunits into a αβγ heterotrimer and return of the system to the basal state. Of the 4 families of G proteins, 5-HT_{1A} receptor activation has been coupled to activation of the $G_{i/0}$ family which includes: G_{i1}, Gi_2, Gi_3, Gz, G_0.

5-HT_{2A} receptor modulation

Although the 5-HT_{2A} receptor seems to be central to the effects of hallucinogens, modulation by other serotonergic receptors and non-serotonergic receptors can have a significant impact on the system as well. Previous studies have demonstrated that the stimulus effects of LSD are potentiated by selective serotonin reuptake inhibitors (SSRI's) (Fiorella et al., 1996) and modulated by 5-HT_{2C} receptors (Fiorella et al., 1995c). Various behavioral effects of 5-HT_{2A} receptor activation are modulated by 5-HT_{1A} receptor stimulation. The head-twitch response, a behavior typically associated with 5-HT_{2A} receptor stimulation, was shown to be attenuated by the prototypical 5-HT_{1A} receptor agonist 8-hydroxy-2-(di-N-propylamino)tetralin (DPAT) (Darmani et al., 1990a). Further supporting a modulatory role of the 5-HT_{1A} receptor is an isobolographic analysis suggesting that 5-HT_{1A} receptors are involved in an inhibition of 5-HT_{2A} receptor-stimulated locomotor activity (Krebs-Thomson and Geyer, 1998).

Based on electrophysiological studies, Pennington and Fox (1994) suggested that inhibition of 5-HT release resulting from 5-HT_{1A} receptor activation may play a role in the hallucinogenic actions of LSD. Behavioral evidence in support of such an interaction is provided by the observation that the stimulus effects of DOM are potentiated by 8-OH-DPAT (Glennon, 1994). The mechanism of this potentiation is unknown. Although 8-OH-DPAT is considered a relatively specific 5-HT_{1A} agonist, this compound also has appreciable affinity for the 5-HT_7 receptor subtype (Wood et al., 2000). These data raise a number of questions. For example: can the potentiation of the discriminative effects of DOM caused by direct 5-HT_{1A} receptor stimulation be extended to include LSD? Which area of the brain is responsible? How does the 5-HT_{1A} receptor cause this potentiation? The objective of my investigations was to answer these questions in a systematic fashion.

METHODS

A. Behavioral Experiments

Subjects

All behavioral studies were conducted using adult male Fischer 344 rats obtained from Harlan Sprague-Dawley Inc. (Indianapolis, Ind., USA). Subjects were housed in pairs under a 12-h light-dark cycle beginning at 6:00 a.m., and allowed free access to water in their temperature controlled home cages. All training and testing took place during the light cycle. Subjects were fed standard rat chow immediately following experimental sessions. Caloric intake was controlled to maintain a mean body weight of 275 g. Caloric control has been shown to lengthen the life span and decrease the incidence of a variety of pathologies in Fischer 344 rats (Keenan et al.1994). Based on a recent sample of 25 rats from our laboratory, the average life span under these conditions is 34.3 months [S.E.M.=1.1]. Animals used in these studies were maintained in accordance with US Public Health Service Policy on Humane Care and Use of Laboratory Animals as amended August 2002. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the State University of New York at Buffalo.

Apparatus

Six small animal test chambers (MED Associates ENV-008) were used for experiments. These were housed in larger light-proof, sound-insulated boxes, which contained a house light and an exhaust fan. The chambers contained two levers mounted at opposite ends of one wall. Centered between the levers was a dipper that delivered 0.1

ml of sweetened condensed milk diluted 2:1 with tap water. Sessions were managed by a microcomputer using operant control software (MED-PC State Notation, Version IV).

Training Procedures

Subjects were trained to discriminate either LSD (0.1 mg/kg 15 min pretreatment time) or 8-OH-DPAT from saline (0.2 mg/kg, 15-min pretreatment time, intraperitoneal injection) as previously described (Fiorella et al., 1995a). A fixed ratio 10 (FR10) schedule of reinforcement was employed. The two treatment conditions, drug or saline, were alternated on successive days while each training session lasted 10 min. 8-OH-DPAT and LSD-induced stimulus control were established after 25-30 training sessions. The 8-OH-DPAT and LSD training doses produced greater than 99% drug-appropriate responding during training sessions conducted throughout the course of these studies. In contrast, less than 3% drug-appropriate responding was observed in training sessions preceded by injection of saline.

Testing Procedures

Drug-induced stimulus control was assumed to be present when, in five consecutive sessions, 83% or more of all responses prior to the delivery of the first reinforcer were on the appropriate lever. After stimulus control was established, tests were conducted once per week in each animal so long as performance did not fall below the criterion level of 83% correct responding in any one of the previous three training sessions. Half of the test sessions were conducted the day after saline training sessions

with the remainder following 8-OH-DPAT or LSD training sessions. During test sessions, no responses were reinforced and the session was terminated after the emission of ten responses on either lever. The distribution of responses between the two levers was expressed as the percentage of total responses emitted on the drug-appropriate lever. Response rate was calculated for each session by dividing the total number of responses emitted prior to lever selection, that is, prior to the emission of 10 responses on either lever, by elapsed time. Data for any subjects failing to emit 10 responses within the constraints of the 10-min test session were not considered in the calculation of the percent drug-appropriate responding but were included in analysis of response rates.

For purposes of discussion of these data, definitions were according to Winter and colleagues (2000). Complete generalization of the training drug to a test drug is said to be present when (a) a mean of 80% or more of all test responses occurs on the drug appropriate lever; (b) there is no statistically significant difference between the response distributions of the training drug and the test drug; and (c) there is a statistically significant difference between the response distributions of the test drug and saline control sessions. Partial generalization or antagonism is defined as being present when the mean response distribution after a test drug or combination of drugs is less than 80% drug-appropriate responding and is statistically significantly different from both training conditions. Finally, when the response distribution after a test drug is not significantly different from that in saline control sessions, an absence of generalization of the training drug to the test drug is assumed. Similar criteria are applied to the definitions of full, partial and no antagonism. Thus, full antagonism is assumed to be present when (a) less than 20% of all test responses are on the training drug-appropriate lever; (b) there is no

significant difference between the response distributions in the test of antagonism and the saline control, and (c) there is a statistically significant difference between the response distributions of the test drug alone and in combination with an antagonist.

Drugs

Lysergic acid diethylamide [(+)-LSD (+)-tartrate (2:1)] and 2,5- dimethoxy-4-methylamphetamine (DOM) were generously provided by the National Institute on Drug Abuse, Rockville, Md., USA. 8-hydroxy-2-(di-*N*-propylamino)tetralin, WAY-100,635, and buspirone were purchased from Tocris, USA. Gepirone and ipsapirone were gifts from Bristol-Meyers Squibb Company, Wallingford, CT and Miles Pharmaceuticals, West Haven, CT, respectively. M100907 was synthesized at the Laboratory of Medicinal Chemistry, National Institute of Diabetes, Digestive and Kidney Disorders at the National Institutes of Health [Bethesda, MD, USA]. A stock solution of M100907 [0.5 mg/ml] was made by dissolving M100907 in a minimal volume of 0.2% w/v tartaric acid and diluting with water. All other drugs were dissolved in 0.9% saline. Doses are expressed as mg/kg and refer to weights of the salts.

Statistical analysis

The statistical significance of combination tests with a 5-HT_{1A} agonist and LSD were determined using two-way ANOVA with dose of LSD and treatment with the 5-HT_{1A} agonist as factors. Two-way ANOVA was also used to determine the statistical significance of the antagonism of the effects of the 5-HT_{1A} agonists on LSD-induced

stimulus control by WAY-100,635. In combination tests involving WAY-100,635, dose of LSD and treatment with the combination of 5-HT_{1A} ligands were used as factors. For assessment of the statistical significance of antagonism of the stimulus effects of the training dose of LSD, by WAY-100,635 one-way ANOVA was used to compare the two training conditions (saline and 0.1 mg/kg LSD) with the combination of LSD and WAY-100,635.

The statistical significance of tests of antagonism with M100907 and 8-OH-DPAT were determined using two-way ANOVA with dose of 8-OH-DPAT and pretreatment with M100907 as factors. Other behavioral data were assessed for statistical significance using one-way analysis of variance [ANOVA]. Differences were considered statistically significant if the probability of their having arisen by chance was ≤ 0.05. All analyses were conducted using SigmaStat for Windows™ (Jandel Scientific Software, San Rafeal, CA). In all measures of analysis of variance, subsequent multiple comparisons were made by the method of Student-Newman-Keuls. For analysis of individual points in substitution tests, Student's t-test was used.

B. Cellular Studies

Animal Model

Thirty five male Fischer 344 rats (Harlan Sprague-Dawley Inc., Indianapolis, IN), were used in this study, 25 to assess LSD-induced c-Fos dose response effects (5/treatment group), and an additional 10 for determining the cell types within the prefrontal cortex that express c-Fos. At arrival, rats were 6 weeks of age, given food and water ad libitum, housed in pairs, and exposed to a 12-h light-dark cycle. Rats were

handled for 15 min/day, 8 days prior to LSD treatment, to reduce stress-induced c-Fos expression incurred by handling and immobilization (Ceccatelli et al., 1989; de Medeiros et al., 2005). Rats were euthanized at 12 weeks of age when they weighed approximately 300 g. Animal treatment in this study was in accordance with US Public Health Service Policy on Humane Care and Use of Laboratory Animals as amended August 2002. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the State University of New York at Buffalo.

LSD Treatment

Rats were injected with lysergic acid diethylamide [(+)-LSD (+)-tartrate (2:1)] which was generously provided by the National Institute on Drug Abuse (Rockville, MD). LSD was diluted in 0.9 % bacteriostatic saline (w/v) to a total volume of 0.25 ml. Doses of 1.0, 0.5, 0.25 and 0.1 mg/kg LSD were used. Controls were injected with saline. There was a 90 min lapse between injection and perfusion because this duration results in maximal expression of c-Fos (Kovacs, 1998; Chaudhuri et al., 2000).

Perfusion and Tissue Preparation

Rats were anesthetized with 100 mg/kg sodium pentobarbital and perfused through the aorta with an 80 ml bolus of physiological saline followed by 300 ml of 4% paraformaldehyde (w/v) in 0.1 M phosphate buffer (pH = 7.4). Brains were removed immediately after perfusion and postfixed for 24 hours. Brains were cryoprotected in 30% sucrose (w/v) in 0.1 M phosphate buffer (pH = 7.4) solution and coded to prevent investigator bias.

In order to remove the entire rat prefrontal cortex, the forebrain was grossly dissected by making a coronal incision immediately anterior to the optic chiasm. The rat prefrontal cortex is located anterior to the motor region of the frontal lobe (Lewis, 2004) and includes frontal cortex area 2 and the medial prefrontal cortex. The medial prefrontal cortex is further subdivided into the anterior cingulate cortex, the prelimbic cortex, and the infralimbic cortex (Uylings and van Eden, 1990; Mogensen and Holm, 1994). A pilot study with toluidine blue staining confirmed identification of the prefrontal cortex by demonstrating the absence of granule neurons in layer IV cortical areas (Van Eden and Uylings, 1985).

c-Fos Dose Response Study: Immunocytochemistry

For dose response studies, forebrains were completely sectioned in the coronal plane at 40 μm on a rotary microtome with a freezing stage (Hacker Industries, Fairfield, NJ). From the 100 sections obtained from each forebrain, 20 free-floating sections (every fifth section) were selected for staining. The immunocytochemical staining procedure was as follows. Sections were washed 3x in phosphate buffered saline (PBS) and incubated in 0.3% hydrogen peroxide (v/v) in PBS for 30 min to eliminate endogenous peroxidase activity. Sections were blocked for 2 h in 2% bovine serum albumin (BSA) (w/v) and 0.25% Triton X-100 (v/v) in PBS and incubated for 12 h at 3°C with c-Fos primary antibody (1:10,000, Calbiochem, San Diego, CA) in a 1% BSA (w/v) and 0.25% Triton-X-100 solution in PBS (pH = 7.4). Sections were washed 3x in PBS and secondarily labeled with a Vectastain Elite ABC horseradish peroxidase kit (Vector Laboratories, Burlingame, CA). Labeling was visualized with the chromagen 3,3'-

diaminobenzidine (DAB) (Vector Laboratories Burlingame, CA). Stained sections were rinsed with distilled water, mounted on slides, dried, and coverslipped with Permount (Fisher, Fair Lawn, NJ).

Quantitation of c-Fos positive cells

For dose response studies, light microscopic quantitation was performed on 10 c-Fos labeled sections (every other section) with a 20x objective and previously described procedures (Dlugos and Pentney, 2002). The prefrontal cortex including the anterior cingulate cortex, prelimbic cortex, and frontal cortex area 2 was delineated on each slide using stereotaxic coordinates (Paxinos and Watson, 1986). An automatic stage control (Biopoint Keypad, Ludl Electronics Ltd, Hawthorne, NY) moved each mounted tissue section in a raster pattern through the delineated cortex. Approximately 75 microscopic fields/slide were quantitated. Each field was projected onto a Commodore 1084S monitor that displayed a fixed counting frame representing 23,460 μm^2 (797X = total magnification). Standard rules for inclusion and exclusion of particles within the counting frame were used (Gundersen, 1977). C-Fos+ cells were counted only if their nuclei contained dark brown or black particles. The areal density of c-Fos+ cells/slide was obtained by dividing the total number of cells/slide by the area of the counting frame. Values from the 10 slides were averaged to determine the mean areal density of c-Fos+ cells in each rat.

Identification of c-Fos+ cells

A dose of 1.0 mg/kg LSD was used because it produced a robust c-Fos expression. The protocols were similar to those used for dose-response studies except that tissue sections were incubated simultaneously with two primary antibodies, one monoclonal and one polyclonal. The polyclonal antibody was always c-Fos and the monoclonal antibody was distinctive for a cell type. Anti-Neurofilament 160 (1:400, Sigma-Aldrich, St. Louis, MO), anti-neuron specific enolase (NSE) (1:40, Serotec Inc., UK) and NeuN (1:500, Chemicon, UK) antibodies were used to detect neurons. Anti-oligodendrocyte marker O1 antibody (1:50, Sigma-Aldrich, St. Louis, MO) and anti-2',3'-cyclic nucleotide-3'-phosphodiesterase (CNPase) (1:500, Sigma-Aldrich, St. Louis, MO) were used to detect oligodendrocytes. O1 labeling produced more robust staining and was used to colocalize c-Fos and O1+ cells. Anti-glial fibrillary acidic protein antibody (GFAP) (1:400, Sigma-Aldrich, St. Louis, MO) was used to detect astrocytes. OX-42 (1:100, Serotec Inc., UK) was used to detect microglia. Fluorescent secondary antibodies were obtained from Invitrogen (Carlsbad, CA). Alexa Fluor® 568 secondary antibody (Invitrogen, Carlsbad, CA) was used to visualize c-Fos positive cells. Alexa Fluor® 488 secondary antibody (Invitrogen, Carlsbad, CA) was used to react with monoclonal antibodies. Sections were washed (5x) and incubated with both fluorescent secondary antibodies diluted 1:200 in PBS for1 h at room temperature. Sections were washed, dried, and mounted in MOWIOL® (Calbiochem, San Diego, CA).

To determine the percentage of cells in which c-Fos was colocalized with either NSE or O1, a Nikon Eclipse E600 microscope equipped with an Expo-X-Cite™ 120 fluorescence illumination system was used (Nikon Inc., Melville, NY). Ten fields in

each section were chosen in a random, systematic fashion (West, 1993). The field size was 147,400 μm^2 (29,674X = total magnification). The stage was moved in a raster pattern throughout the prefrontal cortex. Individual fields were captured with a digital camera (Diagnostic Instruments Inc. model # 9.4 Slider-6, Sterling Heights, MI) using two different emission filters (603 nm and 519 nm) and SPOT software for Windows© (Diagnostic Instruments Inc. V. 4.0.5., Sterling Heights, MI). Single fields labeled with one antibody were viewed using Adobe Photoshop 5.0. and quantified individually to determine the density of cells labeled for c-Fos, O1 or NSE. The individual fields, one labeled with a monoclonal and one with a polyclonal antibody, were then overlayed to determine the percentage of c-Fos+ cells colocalized for the oligodendrocyte marker, O1 or the marker for neurons, NSE. Cells were considered to be colocalized if the red c-Fos+ nuclei were clearly within the boundaries of green NSE or O1 cytoplasm. Confocal microscopy was used to confirm c-Fos colocalization with NSE, O1, and NeuN positive cells.

Confocal Microscopy

Confocal microscopy was performed using a Zeiss LSM 510 Meta NLO microscope mounted on a Axioimager Z1 upright microscope stand. Images were analyzed using Zeiss LSM Image Examiner 4.0.0.9.1 software.

Statistical Analysis

Means (±SEM) for each dose in c-Fos dose-response studies were determined by averaging the values from the 10 slides in each animal. One-way ANOVA with the

Student-Newman-Keuls post-hoc test was used to detect differences in LSD-induced c-Fos expression. Differences were considered to be statistically significant if the alpha level was ≤ 0.05. All analyses were conducted using SigmaStat 2.03 for Windows (Jandel Scientific Software, San Rafael, CA).

STUDY 1. Potentiation of the stimulus effects of LSD by 5-HT_{1A} agonists

Reissig CJ, Eckler JR, Rabin RA, Winter JC. The 5-HT_{1A} Receptor and the Stimulus Effects of LSD in the Rat. Psychopharmacol 2005; 182(2): 197-204.

Introduction

Currently there are fourteen recognized 5-HT receptor subtypes which fall into 7 families, 5-HT_{1-7} (Raymond et al., 2001). Although serotonergic systems are clearly relevant to the effects of LSD, questions still remain as to the contributions of specific serotonergic receptor subtypes (Winter et al., 1999). The blockade of the stimulus effects of LSD by administration of 5-HT_2 receptor antagonists as well as a correlation between affinity of the 5-HT_2 receptor and hallucinogenic potency in man led Glennon and colleagues (1984) to hypothesize that hallucinogens act as 5-HT_2 agonists. In support of this idea Schreiber and colleagues (1994) found that the 5-HT_{2A} receptor antagonist, MDL 100,907, but not the 5-HT_{2C} receptor antagonist, SB 200,646, blocked the stimulus effects of the phenylalkylamine hallucinogen 1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane (DOI). However, affinity at the 5-HT_{2A} receptor could only account for 56% of the variability in the potency of an antagonist to block the stimulus effects of LSD *in vivo* (Fiorella et al., 1995c). Furthermore, compounds such as quipazine have high affinity for the 5-HT_{2A} receptor yet are not hallucinogenic (Fiorella et al., 1995c; Egan et al., 1998). Thus, while 5-HT_{2A} receptor stimulation is a necessary component of

LSD-induced stimulus control, it is not the only mechanism by which the drug exerts its effects.

It has been suggested that functional interactions exist among the different populations of 5-HT receptors and that stimulation of one receptor subtype may influence the activity of another. 5-HT_{2A} mediated behaviors have been shown to be influenced by the 5-HT_{1A} receptor in a variety of experimental paradigms. For example, the head-twitch response, a behavior typically associated with 5-HT_{2A} receptor stimulation (Green et al., 1983; Schreiber et al., 1995a) has been shown to be variably affected by 5-HT_{1A} agonism. Prior research has found that quipazine-induced head twitches are increased by the administration of the 5-HT_{1A} agonist, gepirone (Eison et al., 1986; Yocca et al., 1991). However the effects of 5-HT_{1A} agonism on this behavioral outcome are unclear as other investigations have shown that 8-hydroxy-2-(di-*N*-propylamino)tetralin (8-OH-DPAT) is able to significantly decrease DOI mediated head twitches (Darmani et al., 1990a). 8-OH-DPAT is the prototypical 5-HT_{1A} receptor agonist and has an affinity for the 5-HT_{1A} receptor which is several hundred-fold greater than for the 5-HT_2 receptor (Hamon et al., 1984; Winter and Petti, 1987; Gozlan et al., 1988; Winter and Rabin, 1988). Further complicating matters is a report showing that 8-OH-DPAT is able to increase 5-methoxy- N,N-dimethyltryptamine (5-MeO DMT)-induced, but not 5-hydroxytryptophan-induced, head-twitch response in rats (Darmani et al., 1990a). In addition isobolographic analysis suggests that 5-HT_{1A} and 5-HT_{2A} receptors act antagonistically with regards to their locomotor suppressing effects (Krebs-Thomson and Geyer, 1998). While complex, the relationship between 5-HT_2 and 5-HT_{1A} receptors seems to be of a reciprocal nature. This is evident from studies showing that 8-OH-

DPAT-induced forepaw treading is increased 20 fold by administration of DOI (Arnt and Hyttel, 1989).

The 5-HT_{1A} receptor has been implicated in a variety of CNS responses and may play a role in depression and the formation of memory (Winter and Petti, 1987; Sarnyai et al., 2000; Gingrich and Hen, 2001; Hoyer et al., 2002) It has also been suggested that 5-HT_{1A} receptors mediate the behavioral effects of the anxiolytics buspirone and ipsapirone (Cunningham et al., 1987). The present study sought to characterize the effects of the 5-HT_{1A} ligands 8-OH-DPAT, buspirone, gepirone, (Eison et al., 1986) and ipsapirone (Glaser and Traber, 1983; Fanelli et al., 1990)on the discriminative stimulus effects of LSD.

Results

Initial experiments determined the effects of 8-OH-DPATon the stimulus effects of LSD and DOM, when administered to LSD and DOM-trained animals. Figure 1 shows an orderly, dose related increase in DOM-appropriate responding, in animals trained and tested with DOM. The data of figure 2 show the partial substitution of DOM to the 8-OH-DPATstimulus cue. A maximum of 97% DOM-appropriate responding was achieved with the highest dose of 8-OH-DPAT tested (1.0 mg/kg) although significant impairment of test subjects resulted in only 3 of 8 animals completing the test session. One-way ANOVA revealed that the highest dose of 8-OH-DPAT yielded a level of drug-appropriate responding which was not significantly different from the training dose of DOM (i.e., complete substitution) [$F(2,16)=1208.243$; $p=0.001$]. Rate suppressant effects and technical issues resulted in discontinuation of DOM testing.

The data of figure 4 are a confirmation of our previous findings showing the partial substitution of LSD to the 8-OH-DPAT stimulus cue (Winter and Rabin, 1988). A maximum of 53.2% LSD-appropriate responding was achieved with the highest dose of 8-OH-DPAT tested (1.0 mg/kg) although significant impairment of test subjects resulted in only 9 of 12 animals completing the test session. Rate suppression precluded us from testing higher doses. One-way ANOVA revealed that the highest dose of 8-OH-DPAT yielded a level of drug-appropriate responding which was significantly different from both the training dose of LSD and saline (i.e., intermediate substitution) [$F(2,30)=52.331$; $p=0.001$]. The effects of the highest dose of 8-OH-DPAT on rate of responding and LSD substitution were blocked by the selective 5-HT_{1A} receptor antagonist WAY-100,635 [Student's t-test, $p=.044$ and $p=.002$, respectively] (Forster et al., 1995; Fletcher et al., 1996). However, when combined with the training dose of LSD, one-way ANOVA revealed that WAY-100,635 had no effect on drug-appropriate responding although there was a significant rate suppressant effect [$F(2,34)=9.978$; $p<0.001$]. A dose of 0.05 mg/kg 8-OH-DPAT was chosen for subsequent experiments as this dose yielded a degree of LSD-appropriate responding which did not differ significantly from that following the injection of saline. The rate of responding was not significantly decreased at this dose, and all subjects were able to complete the test session.

Figure 5 shows a dose-related increase in LSD-appropriate responding in rats trained and tested with LSD. When the same doses were tested in rats pretreated with a fixed dose of 8-OH-DPAT (0.05 mg/kg), LSD-appropriate responding increased for all doses of LSD less then the training dose (0.01 mg/kg and 0.03 mg/kg). Two-way ANOVA showed a significant increase in LSD-appropriate responding following the

combination of LSD and 8-OH-DPAT compared with LSD alone [$F(1,47)=9.057$; $p=0.004$]. Neither the effect of dose nor the interaction term were significant. A significant decrease in the rate of responding was also seen with the combination of LSD and 8-OH-DPAT [$F(1,47)=13.67$; $p<0.001$] although the effects of dose and the interaction term did not reach significance. Although displaying a much higher affinity for the 5-HT_{1A} receptor versus other subtypes, 8-OH-DPAT has been shown to be a partial agonist at the 5-HT_7 receptor and have affinity for the α_2-adrenoceptor (Winter and Rabin, 1992; Ruat et al., 1993; Wood et al., 2000). To rule out the possibility of effects caused by administration of 8-OH-DPAT other then 5-HT_{1A} receptor stimulation, WAY-100,635 (0.3 mg/kg) was used. Upon administration of the combination of 8-OH-DPAT, LSD, and WAY-100,635 drug-appropriate responding returned to levels which were not significantly different from LSD administered alone as measured by two-way ANOVA. However, a suppression of rate of responding was still seen with the combination of 8-OH-DPAT, LSD, and WAY-100,635 [$F(1,47)=16.25$; $p<0.001$]. In a manner similar to drug-appropriate responding, neither the effect of dose, nor the interaction term were significant.

A similar potentiation of LSD-appropriate responding caused by administration of the clinically effective anxiolytic buspirone (Riblet et al. 1982; Goa and Ward 1986) is seen in figure 6. Buspirone has been shown to possess agonist activity and be relatively specific for receptors of the 5-HT_{1A} receptor subtype (Riblet et al., 1982; Dourish et al., 1986). A dose of buspirone of 0.3 mg/kg was chosen for combination tests as this dose resulted in a level of LSD-appropriate responding which was not significantly different from that achieved with the injection of saline. When a dose-response curve was

performed with doses of LSD less then the training dose (0.01 mg/kg and 0.03 mg/kg), the addition of buspirone (0.3 mg/kg) resulted in an increase in drug-appropriate responding. Two-way ANOVA revealed that the increase in LSD-appropriate responding was significant in comparison to LSD given alone [$F(1,43)=23.46$; $p<0.001$] while the effects of dose of LSD were significant [$F(1,43)=5.865$; $p=.020$] the interaction term was not. A significant decrease in rate of responding was also seen with the combination of LSD and buspirone [$F(1,47)=16.28$; $p<.001$], although neither the effect of dose nor the interaction term were significant. However, when WAY-100,635 (0.3 mg/kg) was added to the combination of LSD and buspirone two-way ANOVA determined that drug-appropriate responding and rate of responding returned to levels which were not significantly different from LSD administered alone.

Figure 7 shows an orderly dose-related increase in LSD-appropriate responding in rats trained and tested with LSD. Substitution tests revealed that a 0.3 mg/kg dose of gepirone resulted in a level of LSD-appropriate responding which did not differ from that following the administration of saline. As was true for buspirone and 8-OH-DPAT, when doses of LSD less then the training dose of LSD were administered in the presence of a fixed dose of gepirone (0.3 mg/kg) an increase in drug-appropriate responding occurred. This increase was statistically significant as measured by two-way ANOVA [$F(1,45)=4.227$; $p=0.046$]; the effects of dose were significant [$F(1,45)=7.6$; $p=0.008$] although the interaction term was not. A significant decrease in the rate of responding was also observed [$F(1,47)=31.80$; $p<0.001$] with a non-significant effect of dose and interaction term. The effects of gepirone on LSD-appropriate responding were reversed by administration of WAY-100,635 (0.3 mg/kg); however gepirone's effects on rate

suppression remained unchanged and were significantly reduced in comparison to LSD given alone [$F(1,47)=28.72$; $p<0.001$].

Based upon its high affinity for the 5-HT_{1A} receptor (Dompert et al., 1985), ipsapirone was also screened for potential interactions with the LSD stimulus cue. A dose of 0.3 mg/kg was chosen for combination tests, as this dose resulted in a level drug-appropriate responding which did not differ from that following the injection of saline. Results of the combination of ipsapirone (0.3 mg/kg) with a range of LSD doses are shown in figure 8. For LSD doses of 0.01 and 0.03 mg/kg, two-way ANOVA revealed a significant increase in LSD-appropriate responding following the combination of LSD and ipsapirone compared with LSD alone [$F(1,38)=4.488$; $p=0.041$] with a significant effect of dose [$F(1,38)=4.25$; $p=0.047$] and non-significant interaction term . An additional, significant, rate suppressing effect was also seen by administration of the combination of ipsapirone and LSD [$F(1,43)=24.05$; $p<0.001$], although neither dose, nor the interaction term were significant. Two-way ANOVA determined that the effects of ipsapirone on the LSD stimulus cue and rate suppression were eliminated by pretreatment with the 5-HT_{1A} antagonist WAY-100,635 (0.3 mg/kg).

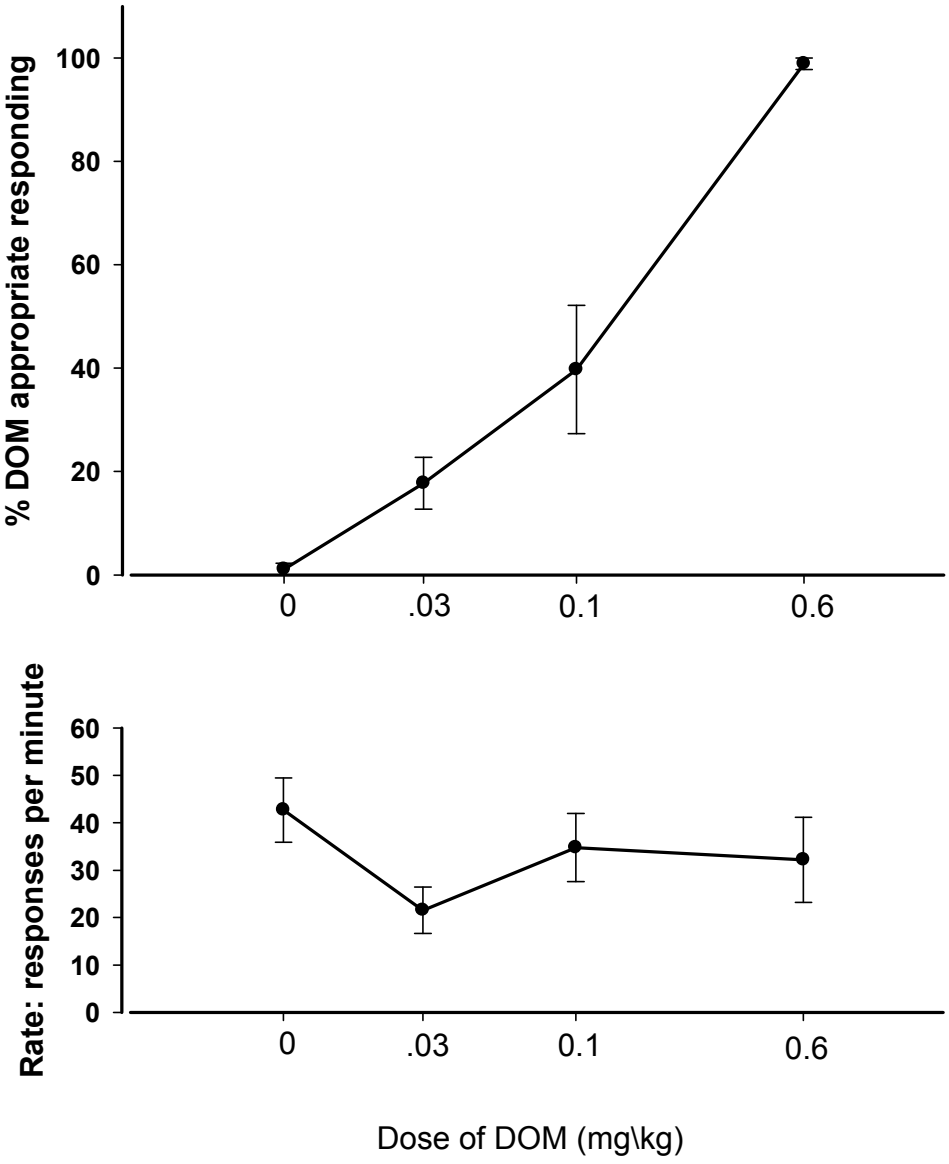

Fig. 1 Dose-response relationship for DOM alone. *Circles* represent the effects of DOM alone in rats trained with DOM as a discriminative stimulus (0.6 mg/kg) n=8.

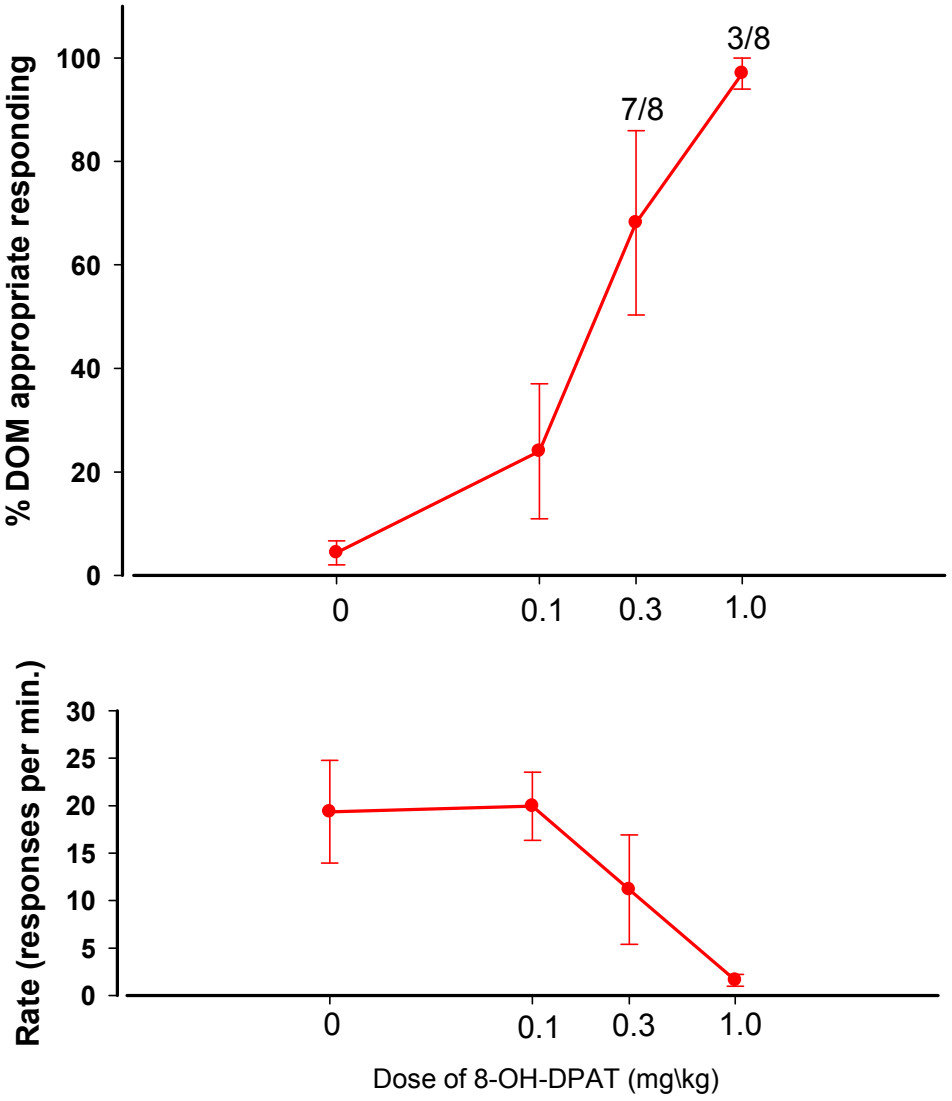

Fig. 2 Effects of 8-OH-DPAT in rats trained to discriminate DOM (0.6 mg/kg) from saline. n=8

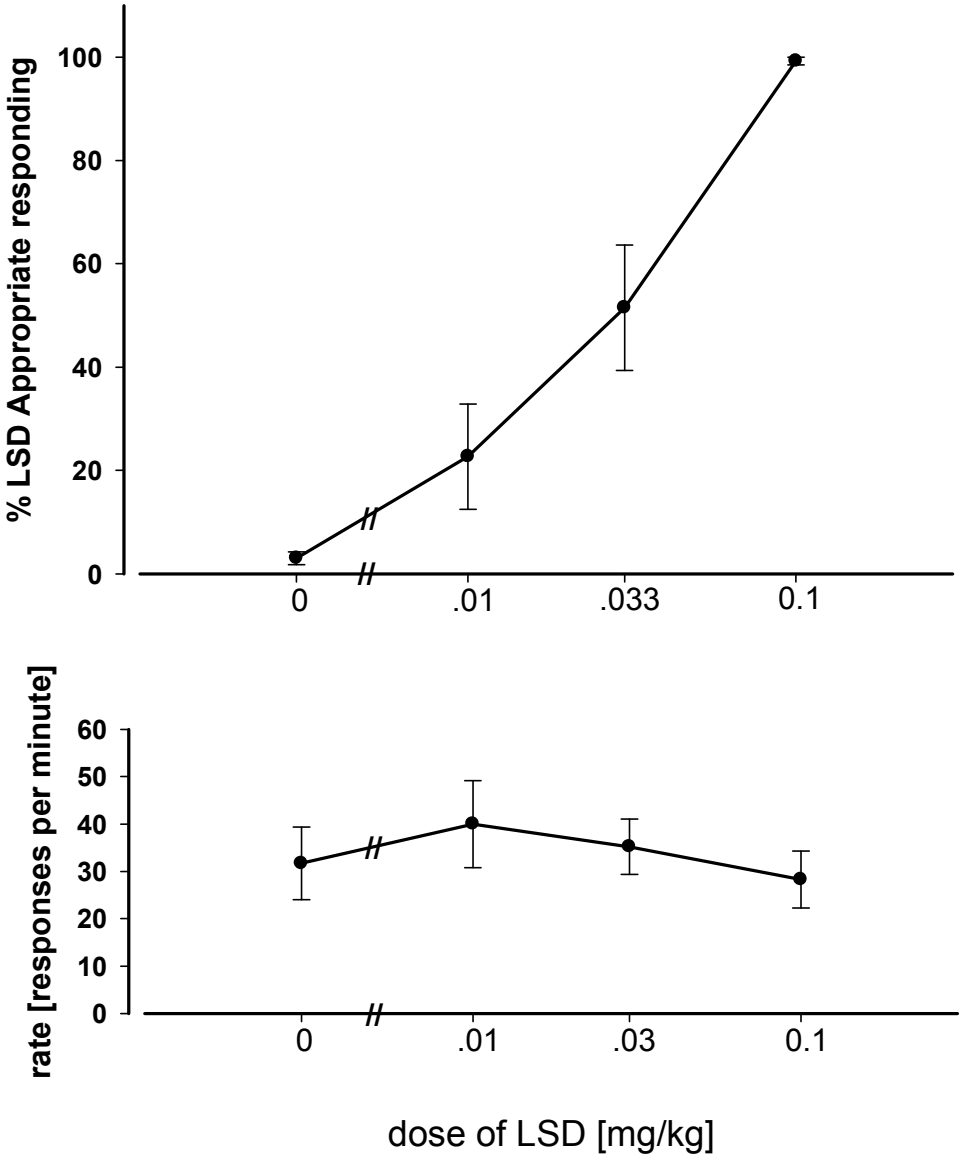

Fig. 3 Dose-response relationship for LSD alone. *Circles* represent the effects of LSD alone in rats trained with LSD as a discriminative stimulus (0.1 mg/kg 15 min pretreat).

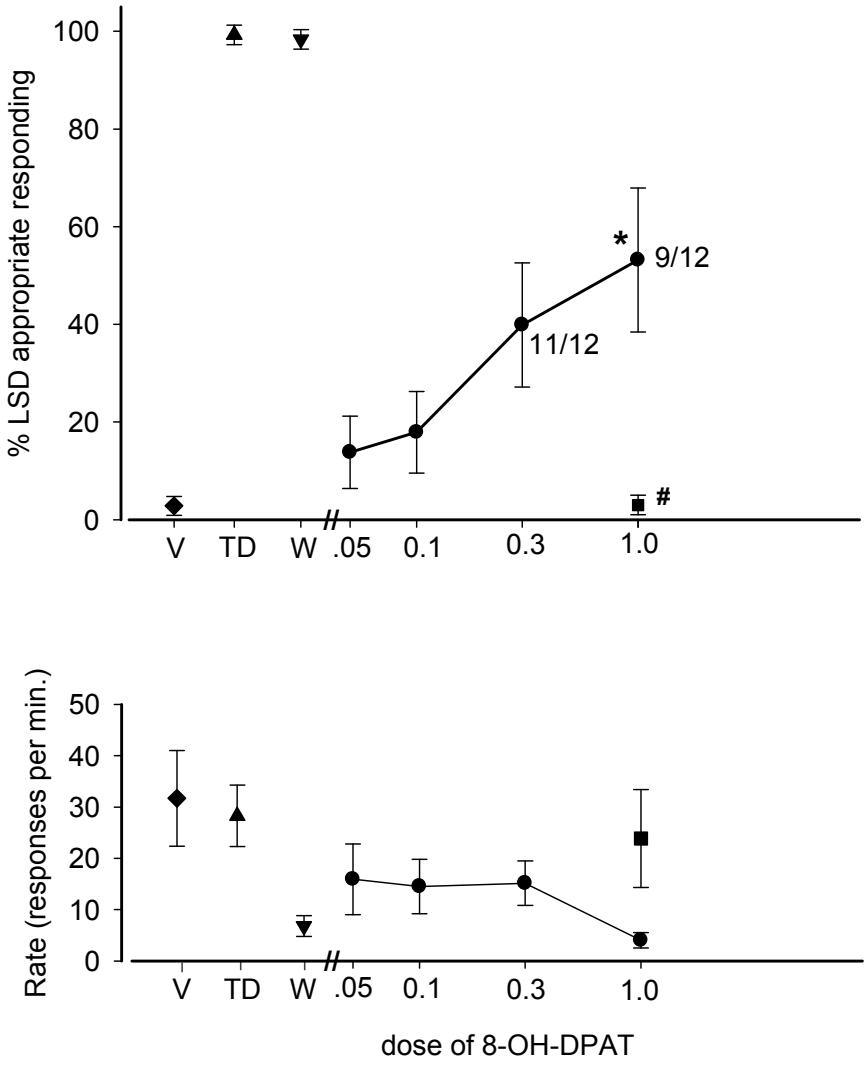

Fig. 4 Effects of 8-OH-DPAT, the selective 5-HT_{1A} antagonist WAY-100,635, and their combination in rats trained to discriminate LSD (0.1 mg/kg) from saline. The *diamond* represents the effects of water administered IP 15 min before testing. *Circles* represent the effects of 8-OH-DPAT administered IP 15 min. before testing. The *Square* represents the effects of 8-OH-DPAT in the presence of WAY-100,635 (0.3 mg/kg, SC, 30 min pretreatment). The *Triangle* represents the training dose of LSD. The *Inverted Triangle* represents the effects of the training dose of LSD given in the presence of WAY-100,635. Each point represents the mean of one determination in each of 12 rats. Standard errors of the mean are shown. * Significantly different from both training conditions. # Significantly different from 8-OH-DPAT (1.0 mg/kg).

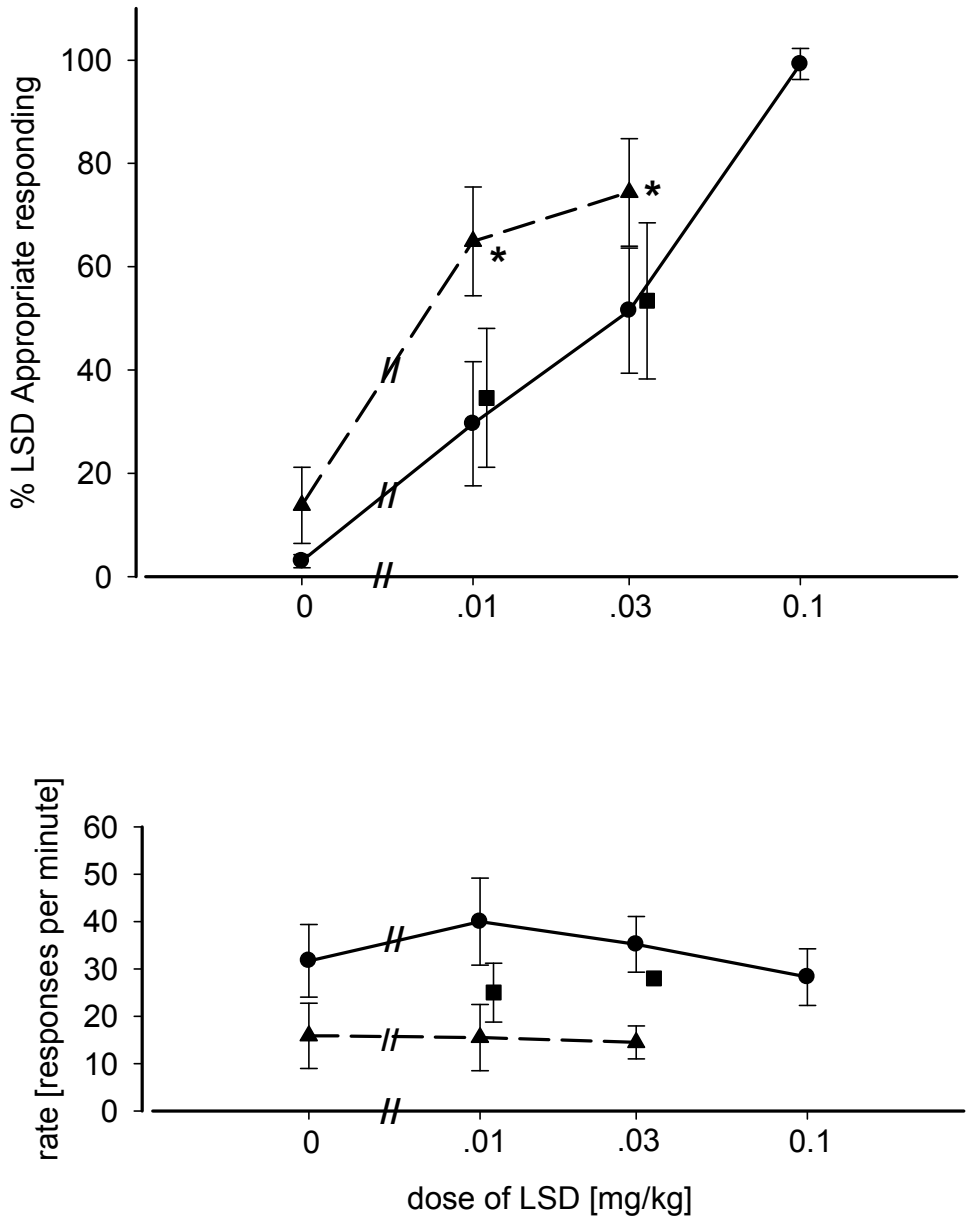

Fig. 5 Dose-response relationship for LSD alone and in combination with 8-OH-DPAT. *Circles* represent the effects of LSD alone in rats trained with LSD as a discriminative stimulus (0.1 mg/kg). *Triangles* represent the effects of LSD in combination with 8-OH-DPAT (0.05 mg/kg). *Squares* represent the effects of LSD following treatment with 8-OH-DPAT and WAY-100,635 (0.3 mg/kg). LSD was administered IP 15 min. before testing. 8-OH-DPAT and WAY-100,635 were administered SC 15 min and 30 min respectively, before testing. * Significantly different from LSD given alone.

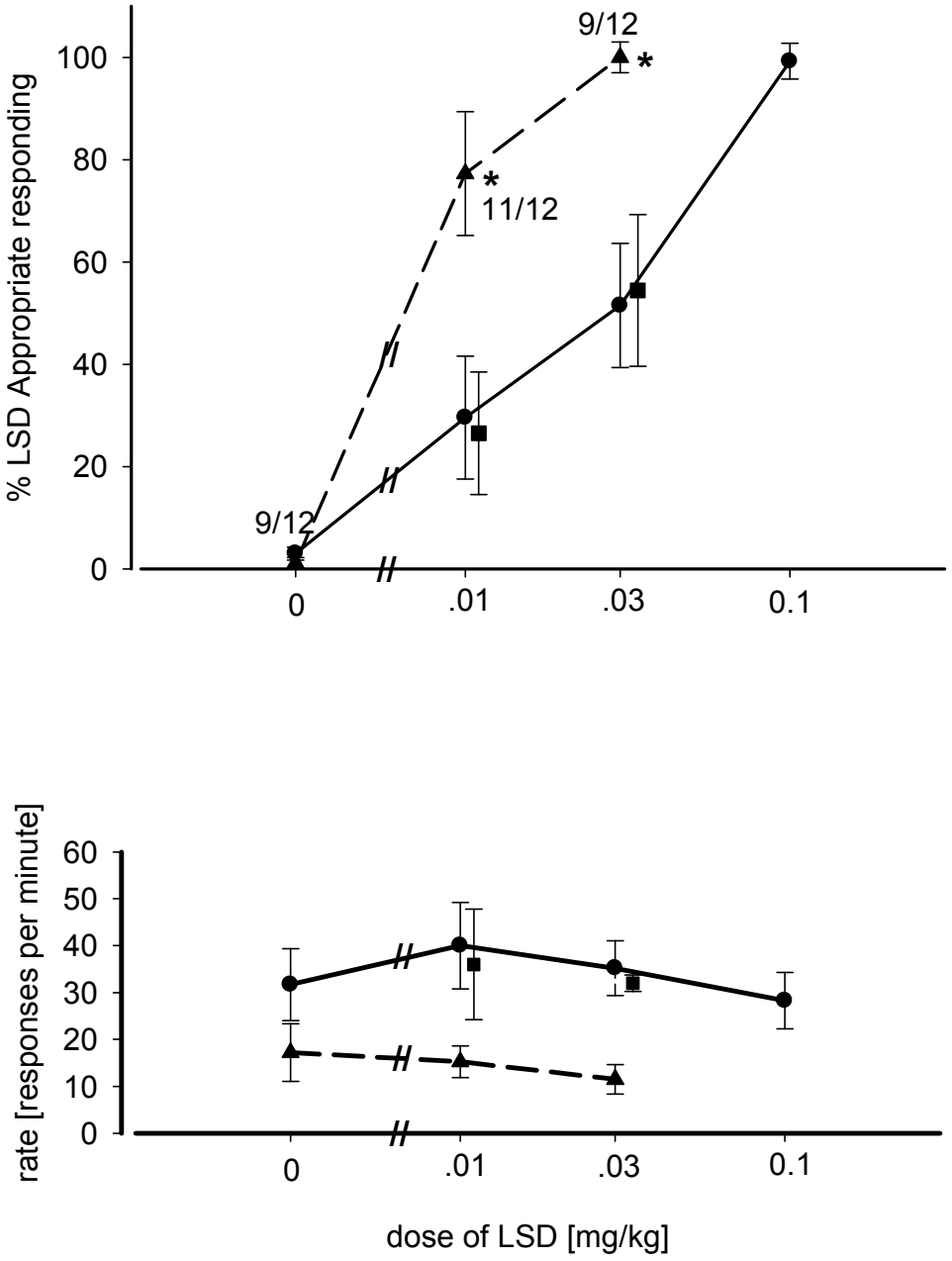

Fig. 6 Dose-response relationship for LSD alone and in combination with buspirone. *Circles* represent the effects of LSD alone in rats trained with LSD as a discriminative stimulus (0.1 mg/kg). *Triangles* represent the effects of LSD in combination with buspirone (0.3 mg/kg). *Squares* represent the effects of LSD in combination with buspirone and WAY- 100,635. Number of subjects completing each session are indicated. Other details are as described in figure 5.

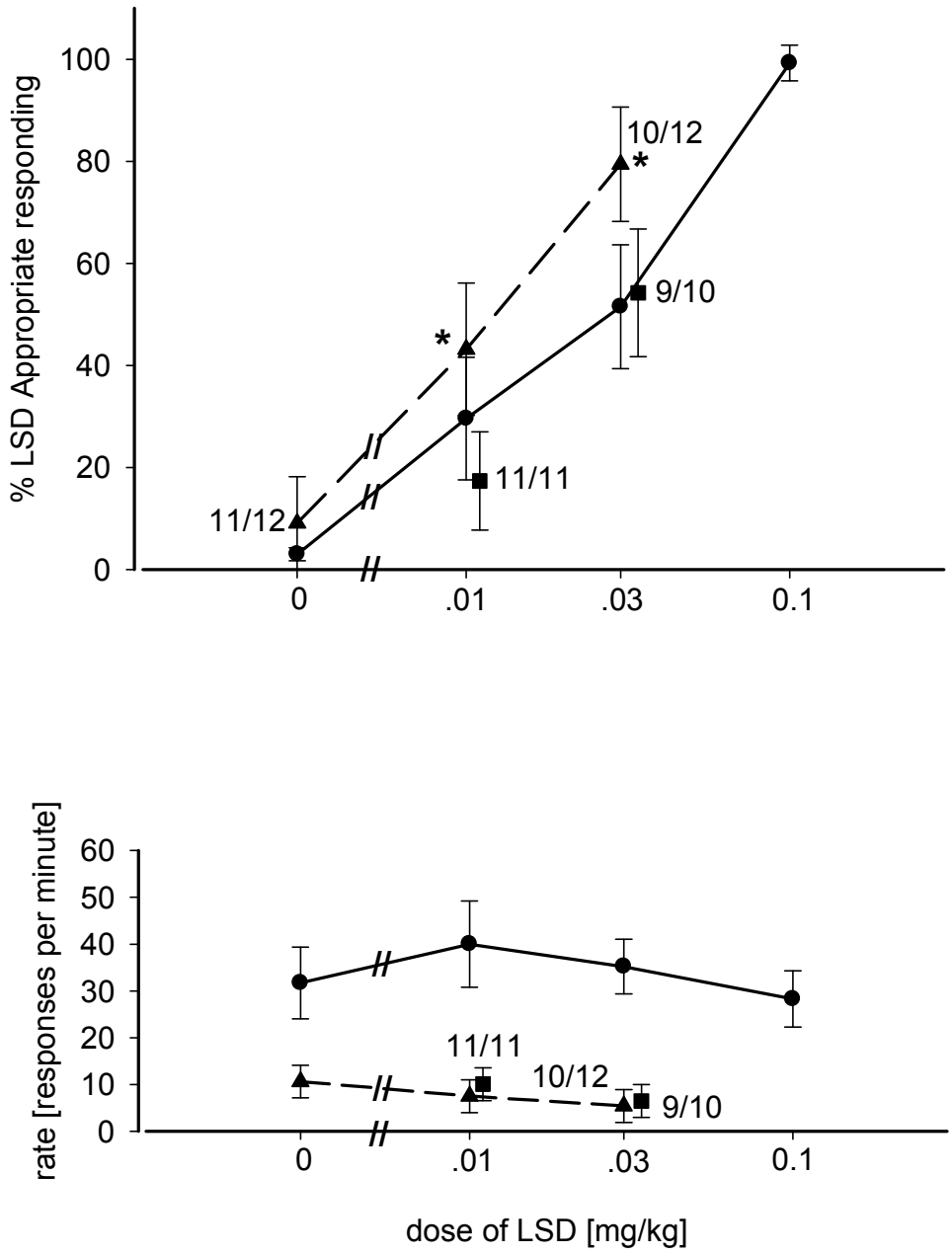

Fig 7. Dose-response relationship for LSD alone and in combination with gepirone. *Circles* represent the effects of LSD alone in rats trained with LSD as a discriminative stimulus (0.1 mg/kg). *Triangles* represent the effects of LSD given in combination with gepirone (0.3 mg/kg). *Squares* represent the effects of LSD in combination with gepirone and WAY-100,635. Other details are as described in figure 5.

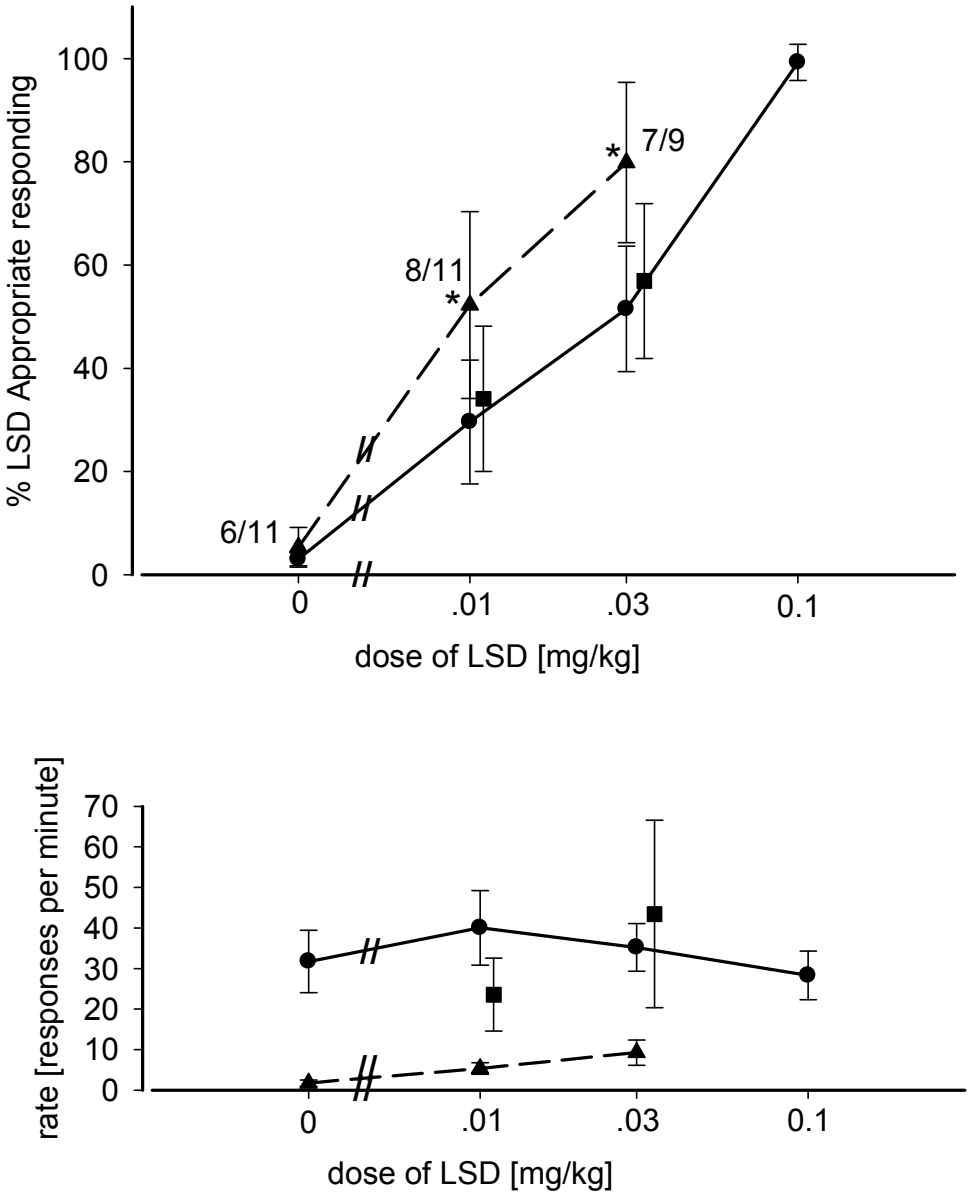

Fig 8. Dose-response relationship for LSD alone and in combination with ipsapirone. *Circles* represent the effects of LSD alone in rats trained with LSD as a discriminative stimulus (0.1mg/kg). *Triangles* represent the effects of LSD given in combination with ipsapirone (0.3 mg/kg). *Squares* represent the effects of LSD in combination with ipsapirone and WAY-100,635. Other details are as described in figure 5.

Discussion

Figure 4 shows the partial substitution of LSD for the 5-HT_{1A} agonist 8-OH-DPAT**.** Cunningham and Appel (1987) failed to produce substitution between these two compounds. Procedural differences may account for this discrepancy, as the latter study utilized a different strain of rat, lower dose of the training agent (0.08 mg/kg LSD) and higher FR schedule (FR 20). The intermediate level of LSD substitution achieved with 8-OH-DPAT in the present investigation indicates that 8-OH-DPAT and LSD share a common stimulus component. However, 5-HT_{1A} receptor stimulation seems to be a non-essential component of the LSD stimulus cue because the training dose of LSD was unaffected by WAY-100,635. Thus it would seem that LSD produces effects on 5-HT_{1A} receptors which become apparent in drug discrimination studies when drugs which are active at 5-HT_{1A} receptors are tested. Although the salient characteristics of LSD-induced stimulus control are mediated via agonist actions at 5-HT_{2A} receptors (Fiorella et al., 1995a; Fiorella et al., 1995c), the data shown in Fig. 4 suggest that an additional, albeit smaller role is played by the 5-HT_{1A} receptor.

The 5-HT_{1A} receptor is found throughout the brain, with high concentrations in dorsal raphe nucleus (DRN), medial raphe nucleus (MRN), hippocampus, lateral septum, entorhinal cortex, and central amygadala. The raphe nuclei are the major source of serotonergic cell bodies in the brain and send projections to cortical and limbic areas (Aghajanian et al., 1968). In general, projections from DRN and MRN overlap one another with the former sending projections to the frontal cortex (Molliver, 1987), an area containing a high density of 5-HT_{2A} receptors (Pazos et al., 1985; Pazos and Palacios, 1985) and thought to play a significant role in hallucinogenesis and psychosis (Arvanov

et al., 1999; Gewirtz and Marek, 2000). Studies in our laboratory support a role of the medial raphe nucleus in hallucinogenesis as systemically administered 2,5-dimethoxy-4-methylamphetmine (DOM) generalized completely to DOM infused into this area (Doat et al., 2003). Indeed, LSD is known to produce a complete inhibition of neuronal activation within the raphe nucleus (Aghajanian GK and Haigler, 1975) which would likely affect the activity of downstream cortical neurons and contribute to the drug's effects. Although suppression of neurons within the raphe nucleus does not seem to be a tenable hypothesis for the primary mechanism of hallucinogenesis, it may be important for the overall psychopharmacology of psychotropic compounds (Nichols, 2004). A similar conclusion was reached by Penington and Fox (1994) who suggested that inhibition of 5-HT release resulting from 5-HT_{1A} receptor activation may play a role in the hallucinogenic actions of LSD.

A previous investigation has found a potentiation of the phenethylamine hallucinogen DOM by pretreatment with 8-OH-DPAT (Glennon, 1991). Prior research examining the head twitch response and its interaction with 5-HT_{1A} agonists has found that quipazine-induced head twitches were increased by the administration of gepirone (Darmani et al., 1990b; Yocca et al., 1990). DOI-induced ear scratch stereotypy (another behavior thought to be mediated via 5-HT_{2A} receptor stimulation) was also increased by administration of 8-OH-DPAT (Darmani et al., 1990b). These data suggest a potentiation of 5-HT_{2A} function caused by 5-HT_{1A} agonism and is fully in keeping with the results in Fig. 5-8. The precise mechanism by which this potentiation occurs however remains obscure.

Several other investigations have been made into the complex mechanism of action of LSD. Considering its relatively non-selective binding profile, it is not surprising that numerous pharmacological stimuli are able to affect the stimulus properties of LSD. Studies in our laboratory have demonstrated that the stimulus effects of LSD are modulated by 5-HT_{2C} receptors (Fiorella et al., 1995c; Fiorella et al., 1995b), significantly reduced by the antipsychotic clozapine (Palumbo and Winter, 1994), and potentiated by selective serotonin reuptake inhibitors (SSRI's) (Fiorella et al., 1996). The last observation is interesting in light of data suggesting that the efficacy of antidepressant therapies and the azapirone anxiolytics may be due to region specific changes in 5-HT_{1A} receptor function (Hensler, 2002). In addition the decrease in the subjective effects of LSD following chronic treatment with serotonergic antidepressants may involve changes in 5-HT_{1A} receptor sensitivity (Bonson et al., 1996; Bonson and Murphy, 1996). These findings suggest that manipulation of serotonergic neurotransmission can affect the behavioral outcome of LSD administration. To our knowledge, this is the first report to show the potentiation of the stimulus properties of LSD with multiple compounds having agonist actions at the 5-HT_{1A} receptor.

Schreiber and colleagues (1995b) have correlated the ability of a drug to substitute for the 8-OH-DPAT stimulus cue with its affinity for the 5-HT_{1A} receptor. 8-OH-DPAT has previously been shown to generalize to ipsapirone, buspirone, and gepirone (Winter, 1988; Rabin and Winter, 1993; Winter and Rabin, 1993) all of which have appreciable selectivity for the 5-HT_{1A} receptor with pK_D values ranging from 8.90-7.49 (Eison et al., 1986; Traber and Glaser, 1987; Rabin and Winter, 1993). These data suggest that the primary stimulus component of these compounds is mediated via 5-HT_{1A}

stimulation and that all have similar stimulus properties. The fact that the observed potentiation of the LSD stimulus cue by 5-HT_{1A} agonists was completely reversed by WAY-100,635 further supports this hypothesis.

In vitro studies have also demonstrated similarities among the 5-HT_{1A} ligands tested. Electrophysiological experiments have shown that application of 8-OH-DPAT, buspirone, or gepirone all result in the suppression of firing of neurons within the DRN through activation of $G_{i/o}$ proteins (Innis and Aghajanian, 1987; Blier et al., 1993a; Blier et al., 1993b). Suppression of raphe firing and second messenger systems may be a potential mechanism by which the ligands studied are able to potentiate the stimulus effects of LSD. Microdialysis studies have shown that buspirone and ipsapirone mimic one another in their ability to enhance dopamine outflow within the prefrontal cortex of the rat (Wedzony et al., 1996). Although it appears buspirone produced the largest amount of potentiation of the LSD stimulus cue**,** the sum of these data indicates that the agonists used in this study have similar pharmacological properties. Subtle differences in the mechanisms of action of these compounds may account for the slight differences in the levels of potentiation observed with each compound.

In summary, it has been demonstrated that 5-HT_{1A} receptor agonists are able to increase the stimulus effects of LSD. These findings support the idea that the 5-HT_{1A} receptor plays a modulatory role in the stimulus effects of LSD. The exact mechanism of this increase is unknown, although it likely involves modulation of serotonergic neurotransmission and 5-HT_{2A} receptor function. Further study of this receptor subtype may offer a greater understanding of its functional role with respect to hallucinogens and the etiology of numerous psychiatric disorders. A suggestive role for the 5-HT_{1A} receptor

for increasing the efficacy of current antipsychotic medications has been proposed (Meltzer, 1999). Indeed, it has been suggested that the atypical profile of the antipsychotic aripiprazole may be derived in part, from its 5-HT_{1A} agonist effect (Marona-Lewicka and Nichols, 2004). Further investigation is needed to determine the precise role of this receptor in the mechanism of action of LSD and other psychotomimetic substances.

STUDY 2. Attenutation of the stimulus effects of 8-OH-DPAT by 5-HT_{2A} antagonists

Reissig CJ, Eckler JR, Rabin RA, Winter JC. The Discriminative Properties of 8-OH-DPAT: Evidence for a 5-HT_{2A} component.

Introduction

The 5-HT_{1A} receptor may be the most well characterized of the fourteen serotonergic receptor subtypes (Pucadyil et al., 2005). 5-HT_{1A} receptors have been implicated in psychiatric disorders such as depression (Celada et al., 2004) and schizophrenia (Millan, 2000; Yasuno et al., 2004) as well as the formation of memory (Winter and Petti, 1987) and neural development (del Olmo et al., 1998; Pazos et al., 1998). The 5-HT_{1A} receptor has been proposed as the pharmacological target of the azapirone anxiolytics (Cunningham et al., 1987) and may contribute to the therapeutic effects of the novel antipsychotic aripiprazole (Marona-Lewicka and Nichols, 2004). Thus, insight concerning 5-HT_{1A} function will offer knowledge applicable across a wide spectrum of research interests.

Dysfunction of a second serotonergic receptor subtype, 5-$HT_{2A,}$ may also underlie a variety of central nervous system disorders including impulsive behavior (Nomura et al., 2006), Alzheimer's disease (Lorke et al., 2006), schizophrenia (Dean, 2003), and depression (de Angelis, 2002). Although selective 5-HT_{2A} antagonists have yielded

disappointing results in the treatment of schizophrenia (de Paulis, 2001), 5-HT_{2A} antagonism may contribute to the lower side effect profile and increased efficacy of atypical antipsychotic drugs (Meltzer, 1999; Meltzer et al., 2003) and improve the efficacy of selective serotonin reuptake inhibitors (SSRI's) (Boothman et al., 2006). In light of these observations, manipulation of 5-HT_{2A} receptor function and the resultant behavioral effects have considerable therapeutic potential.

There is ample evidence supporting functional interactions between 5-HT_{1A} and 5-HT_{2A} receptors. *In vivo* analyses have examined these interactions using a variety of behavioral paradigms including the head twitch response (Arnt and Hyttel, 1989; Yocca et al., 1990), forepaw treading (Arnt and Hyttel, 1989; Backus et al., 1990), production of the serotonin syndrome (Backus et al., 1990), and locomotor activity (Krebs-Thomson and Geyer, 1998). However, conflicting results have emerged, as some of these studies have shown additive, or potentiating interactions between 5-HT_{1A} and 5-HT_{2A} receptors (Arnt and Hyttel, 1989; Backus et al., 1990), while others have shown functional opposition, or antagonistic interactions (Yocca et al., 1990; Krebs-Thomson and Geyer, 1998).

As many of the aforementioned psychiatric disorders manifest themselves as alterations of cognitive function, it is important to employ behavioral measures utilizing similarly complex behaviors. To this end, drug discrimination has proven useful in determining the neuropharmacological mechanisms underlying a diverse array of psychoactive substances (Winter, 1974; Koek et al., 1992; Winter, 1994). Of particular interest are drug discrimination studies showing that hallucinogens whose stimulus effects are mediated primarily by 5-HT_{2A} receptors (Fiorella et al., 1995a; Fiorella et al.,

1995c; Winter et al., 2004) are potentiated by 5-HT_{1A} agonists (Figs 5,6,7,8). This finding suggests that 5-HT_{1A} stimulation enhances 5-HT_{2A} receptor function in the drug discrimination paradigm.

Since drug discrimination studies have found that 5-HT_{1A} receptor agonists potentiate the stimulus effects of hallucinogens whose effects are mediated by actions at 5-HT_{2A} receptors (Figs 5,6,7,8) we hypothesized that 5-HT_{2A} receptors may have a modulatory effect on the stimulus effects of 8-OH-DPAT. Thus, in the present study, the 5-HT_{2A} antagonist M100907 was evaluated for its ability to modulate the 8-OH-DPAT-trained discriminative stimulus. This will further characterize functional interactions between 5-HT_{1A} and 5-HT_{2A} receptors using drug discrimination, a technique used to model the subjective outcome of receptor-receptor interactions.

Results

8-OH-DPAT Dose Effect Relationship

Fig. 9 shows an orderly, dose-related increase in 8-OH-DPAT-appropriate responding in rats trained and tested with 8-OH-DPAT. When the training dose of 8-OH-DPAT (0.2mg/kg) was tested in rats pretreated with WAY-100,635 (0.3 mg/kg), 8-OH-DPAT appropriate responding was completely blocked. Pretreatment with WAY-100,635 caused a significant decrease in drug appropriate responding ($p=<0.001$), although response rate was not altered.

Substitution and Combination tests with buspirone, M100907 and WAY-100,635.

Fig. 10 shows the results of substitution tests with buspirone and combination tests using the selective 5-HT_{2A} antagonist M100907 (Sorensen et al., 1993; Marek and Aghajanian, 1994). M100907 attenuates the stimulus effects of the training dose (0.2 mg/kg), and an intermediate dose (0.1 mg/kg), but not the lowest dose (0.05 mg/kg) of 8-OH-DPAT tested. Two-way ANOVA revealed that the overall level of 8-OH-DPAT appropriate responding was decreased by pretreatment with M100907 ($F_{2,118}$=9.006;p=0.003). Pretreatment with M100907 also produced a significant increase in subject's rate of responding ($F_{2,118}$=5.534;p=0.003).

A 1.0 mg/kg dose of buspirone substituted completely for 8-OH-DPAT. This substitution was significantly decreased by pretreatment with M100907 and WAY-100,635 as revealed by one-way ANOVA ($F_{2,46}$=99.743;p<0.001).

Substitution and Combination tests with DOM, LSD, and serotonergic antagonists.
In Fig 11 are shown the results of substitution test performed with the hallucinogens LSD and DOM as well as combination tests using M100907 and WAY-100,635. In substitution tests a maximum of 48.8% 8-OH-DPAT appropriate responding was achieved with a 0.1 mg/kg dose of LSD. One-way ANOVA revealed that this dose of LSD resulted in a level of drug-appropriate responding which was significantly different from both the training dose of 8-OH-DPAT and saline (partial generalization) ($F_{2,53}$=83.082;p<0.001). Rate of responding was also significantly decreased as determined by one-way ANOVA ($F_{2,55}$=4.228;p=0.020). In a manner similar to LSD, a 0.6 mg/kg dose of DOM resulted in a level of 38.7% 8-OH-DPAT appropriate responding. One-way ANOVA revealed that this dose of DOM resulted in a level of

drug-appropriate responding which was significantly different from both the training dose of 8-OH-DPAT and saline (partial generalization) ($F_{2,47}$=130.384;$p<0.001$). This dose of DOM also decreased rate of responding as determined by one-way ANOVA ($F_{2,48}$=5.93;$p=0.005$). Combination tests using either 0.3 mg/kg WAY-100,635 or 0.1 mg/kg M100907 in conjunction with LSD or DOM produced modest, non-significant changes in 8-OH-DPAT-appropriate responding.

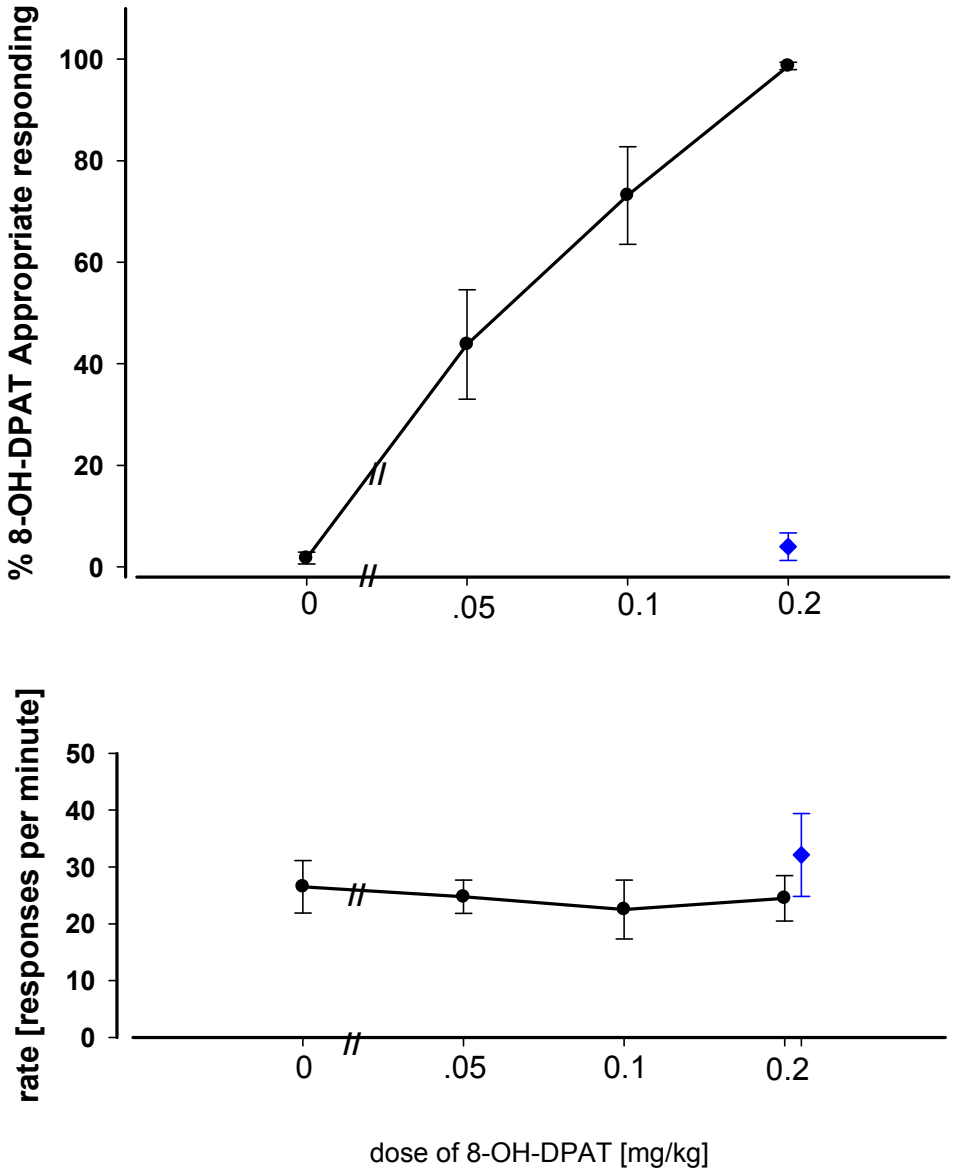

Fig. 9 Dose-response relationship for 8-OH-DPAT alone and in combination with WAY-100,635. *Circles* represent the effects of 8-OH-DPAT alone in rats trained with 8-OH-DPAT as a discriminative stimulus (0.2 mg/kg; 15 min pretreatment time). Each point represents the mean of two determinations in each of 10 rats, with the exception of WAY-100,635 where a single determination is shown. *Diamonds* represent the effects of WAY-100,635 (0.3 mg/kg 30 min. pretreatment) in combination with the training dose 8-OH-DPAT.

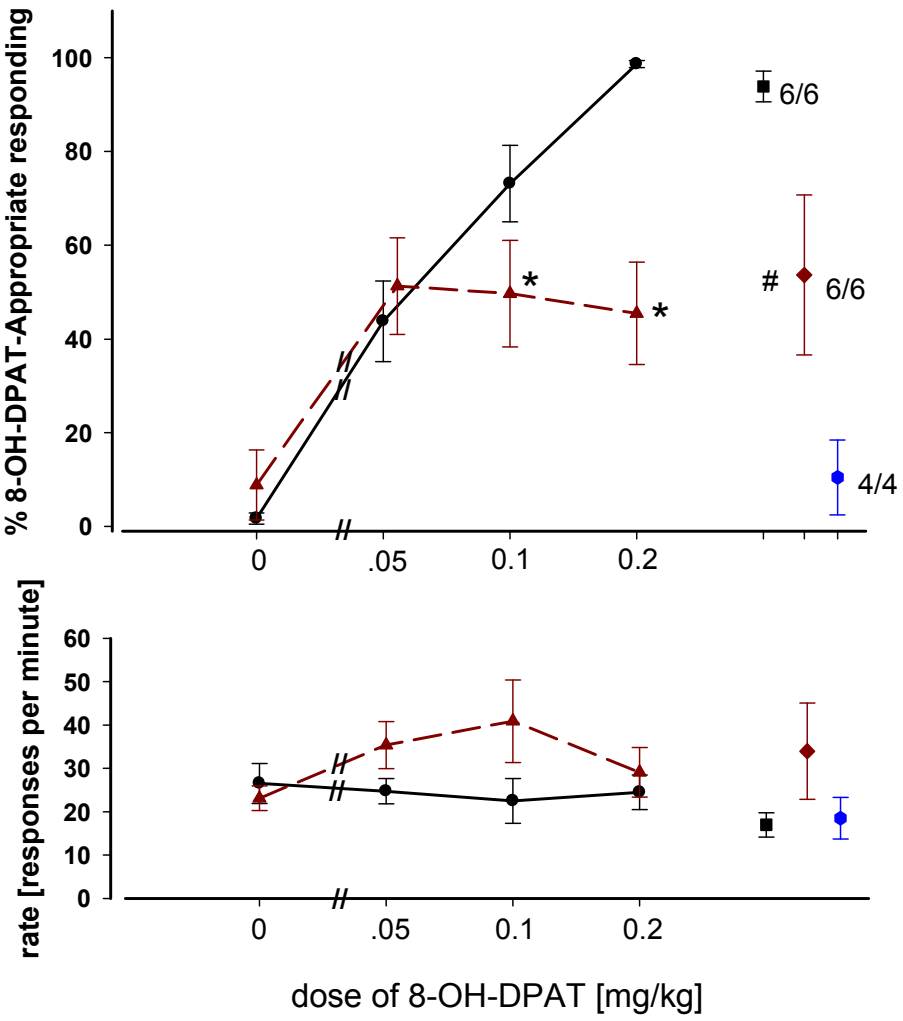

Fig. 10 Dose-response relationship for 8-OH-DPAT alone and in combination with M100907. *Circles* represent the effects of 8-OH-DPAT alone in rats trained with 8-OH-DPAT as a discriminative stimulus (0.2 mg/kg; 15 min pretreatment time). Each point represents the mean of two determinations in each of 10 rats, unless otherwise noted. *Triangles* represent the effects of M100907 (0.1 mg/kg 30 min. pretreatment) in combination with 8-OH-DPAT. The *square* represents the effects of buspirone (1.0 mg/kg 15 min pretreatment). The *diamond* represents the effects of buspirone given in combination with M100907. The *hexagon* represents the effects of buspirone in combination with WAY-100,635. An asterisk indicates a statistically significant difference from 8-OH-DPAT given alone. A numeric symbol indicates a significant difference from buspirone given alone, and in combination with WAY-100,635.

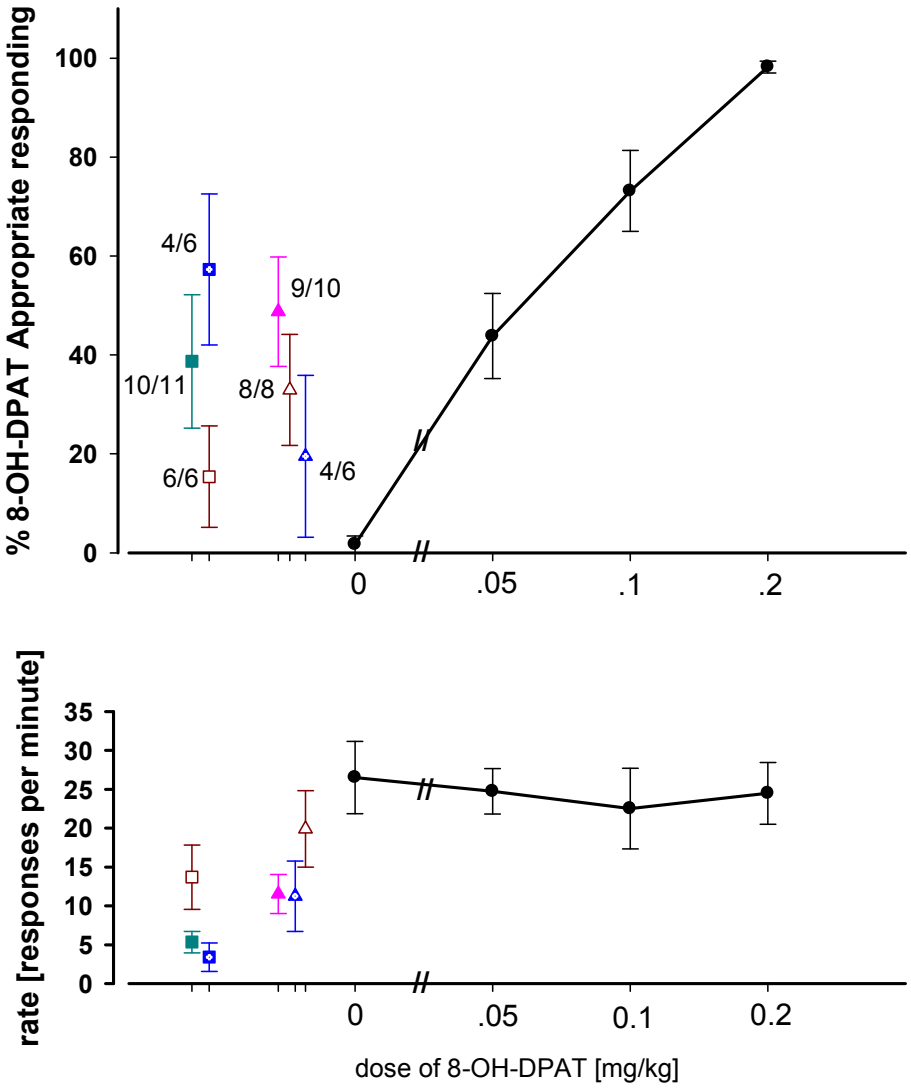

Fig. 11 Substitution and combination tests with serotonergic hallucinogens and antagonists in 8-OH-DPAT trained animals. *Circles* represent the effects of 8-OH-DPAT alone in rats trained with 8-OH-DPAT as a discriminative stimulus (0.2 mg/kg; 15 min pretreatment time). Each point represents the mean of two determinations in each of 10 rats, unless otherwise noted. The *triangle* represents the effects of LSD (0.1 mg/kg). The *open Triangle* represents the effects of LSD in combination with M100907 (0.1 mg/kg). The *hatched Triangles* represent the effects of LSD (0.1 mg/kg) in combination with WAY-100,635 (0.3 mg/kg, S.C.). The *square* represents the effects of DOM (0.6 mg/kg). The *open square* represents the effects of DOM given in combination with M100907. The *hatched square* represents the effects of DOM in combination with WAY-100,635.

Discussion

The data in Fig. 9 demonstrate that the stimulus effects of 8-OH-DPAT are completely blocked by the selective 5-HT_{1A} antagonist WAY-100,635 (Fletcher et al., 1993; Forster et al., 1995; Fletcher et al., 1996). These results are in agreement with previous reports suggesting that 5-HT_{1A} stimulation is the primary component of the 8-OH-DPAT stimulus cue (Cunningham et al., 1987; Tricklebank et al., 1987; Sanger and Schoemaker, 1992; Celada et al., 2004).

Fig. 10 shows that antagonism of 5-HT_{2A} receptors attenuates the stimulus effects of the training dose (0.2 mg/kg) and an intermediate dose (0.1 mg/kg), but not the lowest dose (0.05 mg/kg) of 8-OH-DPAT tested. These results are interesting in light of a previous investigation which failed to attenuate the 8-OH-DPAT stimulus cue using the 5-HT_2 antagonist ketanserin (Cunningham et al., 1987). However, the latter study used a higher dose of the training agent (0.4 mg/kg), different strain of rat (Sprague Dawley), and different FR schedule (FR=20). Additionally, ketanserin may exhibit affinity for other brain receptors including 5-HT_{2C} receptors, α1 receptors, and dopamine receptors (Leysen et al., 1985). The present study employed M100907, a more selective 5-HT_{2A} antagonist, which has a > 100-fold higher affinity for 5-HT_{2A} versus 5-HT_{2C} receptors (Johnson et al., 1996; Kehne et al., 1996). Our finding that 8-OH-DPAT generalizes completely to the 5-HT_{1A} agonist buspirone (Riblet et al., 1982; Peroutka, 1985) and that this substitution is blocked by WAY-100,635 demonstrates that this is not an idiosyncratic event unique to 8-OH-DPAT.

Fig 11 shows the partial generalization of 8-OH-DPAT to the hallucinogens LSD and DOM. In agreement with these results, Cunningham and colleagues (1987) produced approximately 38% 8-OH-DPAT appropriate responding with LSD in animals trained to discriminate 8-OH-DPAT from saline. These results are surprising because 5-HT_{2A} receptor stimulation is the salient component of both DOM and LSD-induced stimulus control (Winter, 1994; Fiorella et al., 1995a; Fiorella et al., 1995c; Winter et al., 2004). Because LSD displays affinity for 5-HT_{1A} receptors (Pauwels et al., 1993; Rabin and Winter, 1993), the partial generalization of 8-OH-DPAT to LSD may be through stimulation of this receptor subtype. This seems unlikely though, because pretreatment with WAY-100, 635 does not completely block 8-OH-DPAT appropriate responding produced by LSD. In addition, M100907 decreases the partial generalization of 8-OH-DPAT to LSD, suggesting a 5-HT_{2A} component in LSD's mimicry of the 8-OH-DPAT stimulus. A role for 5-HT_{2A} receptors in the 8-OH-DPAT stimulus cue is further supported by our results with DOM, a compound more selective for 5-HT_{2A} versus 5-HT_{1A} receptors (Leysen et al., 1989; Rabin et al., 2000). 8-OH-DPAT appropriate responding produced by DOM is blocked by M100907 and pretreatment with WAY-100,635 actually increase the amount of substitution produced by DOM.

The data in figures 10 and 11 suggests that 5-HT_{2A} stimulation mimics the 8-OH-DPAT interoceptive cue to a level which results in partial generalization. It should be noted that 8-OH-DPAT produces a high degree of substitution in LSD (Fig 4) (Winter, 1988; Reissig et al., 2005) and DOM trained animals (Fig 2). Taken together, the data of Fig 9, 10 and 11 suggest that although the 8-OH-DPAT stimulus cue is mediated primarily by stimulation of 5-HT_{1A} receptors, there exists an underlying 5-HT_{2A}

component. Thus, it appears that 5-HT_{2A} and 5-HT_{1A} receptors exert bidirectional modulatory influences upon one another in drug discrimination studies.

Previous behavioral studies have yielded inconclusive results regarding the complex interaction between 5-HT_{2A} and 5-HT_{1A} receptor subtypes. For example, the head-twitch response, a behavior typically associated with 5-HT_{2A} receptor stimulation (Green et al. 1983; Schreiber et al. 1995b) is variably affected by 5-HT_{1A} agonists. While quipazine induced head twitches are increased by administration of the 5-HT_{1A} agonist gepirone (Eison et al. 1986; Yocca et al. 1991), DOI mediated head twitches (Darmani et al. 1989) are decreased by 8-OH-DPAT. Further complicating matters is a report demonstrating the ability of 8-OH-DPAT to increase 5-MeO-DMT-induced, but not 5-hydroxytryptophan-induced, head-twitches in rats (Goodwin and Green, 1985). Thus, it appears that the interaction between 5-HT_{1A} and 5-HT_{2A} receptors on the head-twitch response is dependent upon the compounds used to investigate this interaction.

Other behavioral analyses have yielded similar, contradictory results regarding 5-HT_{2A} and 5-HT_{1A} receptor interactions. Isobolographic analysis suggests that 5-HT_{1A} and 5-HT_{2A} receptors act antagonistically with regards to their locomotor suppressing effects (Krebs-Thompson and Geyer 1998). An antagonistic 5-HT_{1A}/5-HT_{2A} interaction is also seen in the regulation of body temperature in rats (Gudelsky et al., 1986). However, 8-OH-DPAT induced forepaw treading is increased by the 5-HT_{2A} agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) suggesting additive effects between 5-HT_{1A} and 5-HT_{2A} receptors (Arnt and Hyttel 1989). It may be argued however, that all of these behavioral measures are components of the serotonin

syndrome, a condition thought to be caused by excessive serotonin at 5-HT_{1A} (Darmani and Ahmad, 1999) and 5-HT_2 receptors (Pranzatelli and Pluchino, 1991).

In vitro evidence also suggests an interaction between 5-HT_{1A} and 5-HT_{2A} receptors. Electrophysiological studies have shown that 5-HT_{1A} and 5-HT_{2A} receptors mediate opposite responses on pyramidal neuron membrane excitability (Araneda and Andrade, 1991; Ashby et al., 1994). This antagonistic interaction can be extended to include 5-HT release, where stimulation of 5-HT_{2A} receptors causes 5-HT release in mice (Bortolozzi et al., 2003) and rats (Martin-Ruiz et al., 2001), an effect that is reversed by 5-HT_{1A} agonism.

Considering the above data, our discrimination results do not lend themselves to easy interpretation. Our results showing that M100907 attenuates the stimulus effects of 5-HT_{1A} agonists suggests the engagement of a complex synaptic circuit in the stimulus effects of 5-HT_{1A} agonists. It would appear that 5-HT_{2A} receptors are involved in producing this cue; however their exact role remains to be determined. In light of the previous data demonstrating the potentiating effects of 5-HT_{1A} agonists on LSD-induced stimulus control (Figs 5,6,7,8); it appears that 5-HT_{2A} and 5-HT_{1A} agonists have a bidirectional, modulatory influence upon one another in the drug discrimination paradigm. As it has been shown that M100907 can function as a discriminative stimulus (Dekeyne et al., 2002), it would be interesting to determine the effects of 8-OH-DPAT and WAY-100,635 on this stimulus cue.

STUDY 3. LSD-induced c-Fos Expression

Reissig CJ, Rabin, RA, Winter JC, and Dlugos CA. LSD-induced c-Fos expression occurs in a population of oligodendrocytes in rat prefrontal cortex.

Introduction

The psychotropic effects of lysergic acid diethylamide (LSD) include alterations of mood, perceptual changes, and cognitive impairment (Hofmann, 1959; Abraham et al., 1996; Halpern and Pope, 2003; Nichols, 2004). A role for 5-HT_2 receptors in the mechanism of action of LSD was established when LSD's binding affinity at the 5-HT_2 receptor was correlated with its hallucinatory effects in humans (Glennon et al., 1984) and selective 5-HT_{2A} antagonists were found to block LSD's discriminative effects (Fiorella et al., 1995c; Fiorella et al., 1995b; Winter et al., 2004). Despite extensive research on LSD (Dyck, 2005) and its effects on serotonin receptors, the biochemical and signaling pathways responsible for producing LSD's effects remain largely unknown. LSD has been shown to induce the expression of c-Fos, an immediate early gene (IEG) that is rapidly and transiently induced in the central nervous system. Immediate early genes are normally involved in translating extracellular signals and stimuli into alterations of neuronal and cellular function by regulating the expression of other genes (known as late or target response genes) (Morgan and Curran, 1991; Chaudhuri, 1997b; Chaudhuri, 1997a; Kovacs, 1998).

Previous studies have investigated the time course (Frankel and Cunningham, 2002; Gresch et al., 2002) and brain regions (Frankel and Cunningham, 2002; Gresch et

al., 2002) in which LSD-induced c-Fos expression occurs, although dose-related effects have not been examined. Studying the dose-related effects of LSD induced c-Fos expression is essential in view of the fact that c-Fos activates the transcription of genes involved in long-term morphological and biochemical changes including: apoptosis (Walker and Carlock, 1993; Kasof et al., 1995) and neuronal plasticity (Chiasson et al., 1997; Kaczmarek and Chaudhuri, 1997). If LSD-induced c-Fos expression is dose-dependent, any long term changes associated with this expression are likely to be affected by dose as well. The effect of dosage on LSD-induced c-Fos expression has not been determined. Determination of dose effects will serve as the basis for future experiments in which the target genes affected by c-Fos expression will be determined and correlated with the phenotypic results of this expression.

Analysis of *c-fos* expression (and its protein product c-Fos) has also been used to provide an anatomical map of changes in neuronal activity caused by administration of psychoactive drugs, such as LSD (Chaudhuri, 1997b; Chaudhuri et al., 2000; Gresch et al., 2002; Nichols et al., 2003). LSD-induced c-Fos expression has been shown in several cortical areas, including the anterior cingulate cortex, and the medial prefrontal cortex (Frankel and Cunningham, 2002; Gresch et al., 2002; Nichols et al., 2002; Nichols et al., 2003). These results are expected as cortical brain areas contain a high density of 5-HT_{2A} receptors (Pazos et al., 1985) which are thought to have a functional role in hallucinogenesis and psychosis (Arvanov et al., 1999; Gewirtz and Marek, 2000).

A relationship between 5-HT_{2A} receptor stimulation and c-Fos expression has been established because 5-HT_{2A} receptor antagonists attenuate LSD mediated increases in c-Fos expression (Gresch et al., 2002). An intuitive supposition stemming from these

results is that c-Fos expression occurs in 5-HT_{2A} containing neurons. Doubling labeling experiments have shown, however, that LSD-induced c-Fos expression does not occur on 5-HT_{2A} receptor containing neurons (Gresch et al., 2002). This finding suggests that although LSD initiates c-Fos production via a 5-HT_{2A} receptor dependent mechanism, the final expression of c-Fos occurs in a population of cells synaptically connected to 5-HT_{2A} containing neurons. These c-Fos expressing cells, whether neurons or glia have yet to be identified.

The purpose of the present study was to determine whether LSD-induced c-Fos expression is dose-dependent. Determining the influence of dose on c-Fos expression may increase the utility of c-Fos mapping by providing a correlate between dose and changes in gene expression which may be associated which the acute and chronic effects of a drug. The second goal of this study was to identify the specific cells in which LSD-induced c-Fos expression occurs. These cells may represent the convergence point for the effects of a variety of hallucinogens, and play a significant role in endogenous psychotic disorders.

Results

Dose response studies

Injection of LSD resulted in an increase in c-Fos immunoreactivity in the prefrontal cortex and several other cortical areas. C-Fos labeled cells were detected in all cortical layers, but were most numerous in layers III, IV and V (Fig. 12). C-Fos+ cells were small and round, suggestive of small neurons or glial cells. One-way ANOVA showed a dose related increase in the number of c-Fos+ cells after LSD injection. The

number of c-Fos + cells was increased relative to saline at LSD doses of 0.5 and 1.0 mg/kg [$F(4,24)=12.315$; $p<0.001$] (Fig. 13 & 14).

Identification of c-Fos+ cells

To determine the specific cell types responsible for LSD-induced c-Fos expression, the prefrontal cortex was examined. We focused on neurons, astrocytes, oligodendrocytes and microglia. In this region, neurons labeled with NF160 were clearly pyramidal as showed by their soma and processes. NSE labeled neurons had small and round soma but no visible processes. NeuN produced extensive labeling of neuronal nuclei. Astrocytes had clearly labeled soma and extensive, radiating processes. Microglia had very small oval soma, and spiderlike processes. Oligodendrocytes had round nuclei and less extensive processes.

Colabeling of c-Fos, with GFAP, OX-42 or NF160 labeled cells did not occur (Fig 15). Quantitative results (Table 1) showed colabeling of c-Fos+ cells in NSE+ neurons and O1+ oligodendrocytes. Of the total cells labeled with NSE, 22.3 % were found to be colocalized with c-Fos, while 14.2% of O1+ oligodendrocytes colocalized with c-Fos (Table 1). To confirm the possibility of c-Fos expression in oligodendrocytes, nuclear measurements were performed on 100 c-Fos+ cells in dose-response studies. The value of 9.1 μm (SEM ± 0.132 μm) was similar to the maximum diameter of oligodendrocyte nuclei (6.8 ± 0.8 μm) reported in previous studies (Wolswijk, 2000).

Analysis of a single animal found that 33.8% of cells labeled for c-Fos colocalized with NeuN (Table 2). Confocal microscopy was used to confirm these results and produced a weighted coefficient of colocalization of 0.923 in area 1 (Fig 16). By

contrast, analysis of the adjacent background area produced a weighted colocalization coefficient of 0.169 (Fig 16). These results indicatean overlap of green NeuN labeled cells and red c-Fos+ nuclei, suggesting a high probability of colocalization

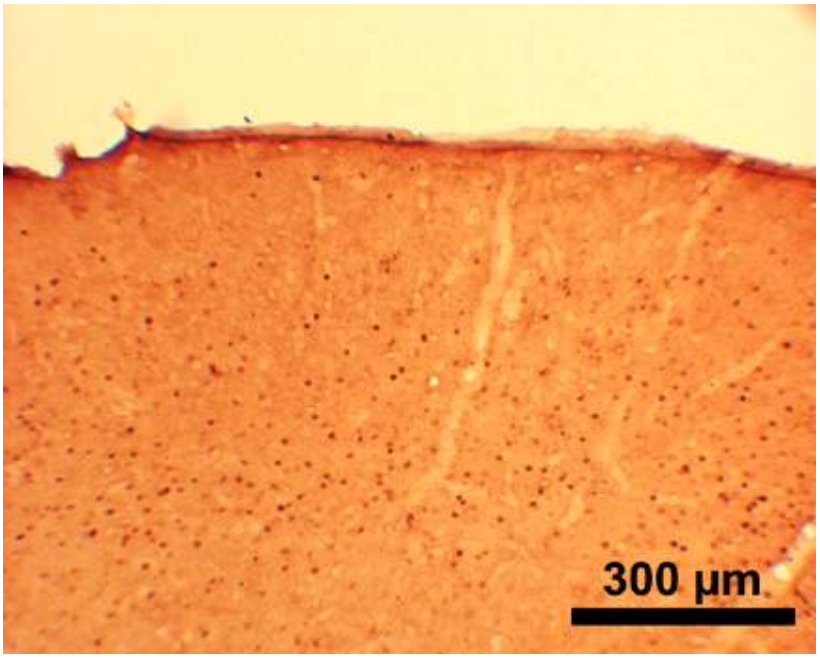

Fig. 12 C-Fos immunoreactivity in the PFC of rats treated 1.0 mg/kg LSD. Rats were sacrificed 90 min. after injection with LSD or vehicle. Immunohistochemistry was performed on free-floating sections.

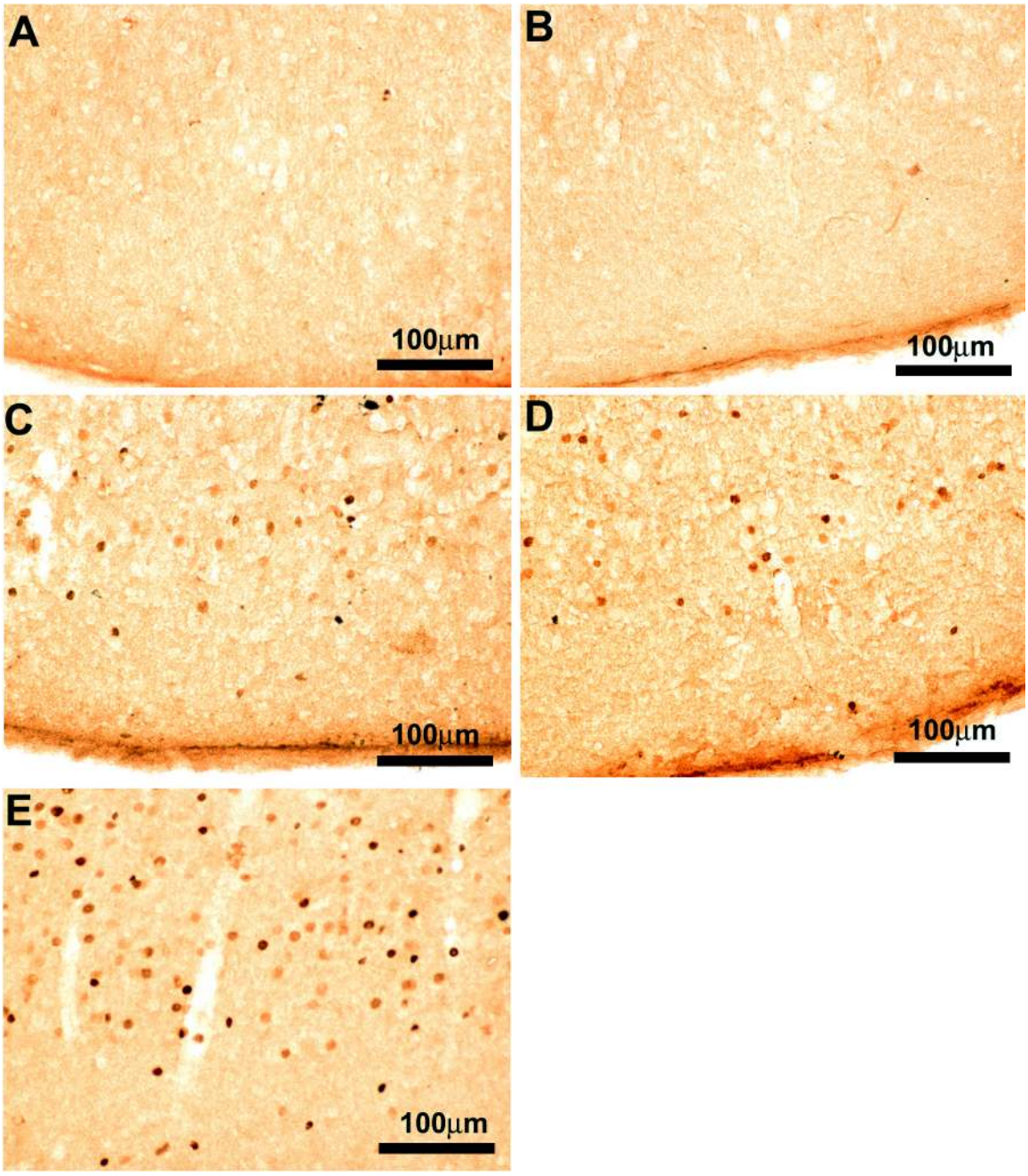

Fig. 13 Representative images showing c-Fos immunoreactivity in the PFC of rats treated with (A) vehicle (B) 0.1 mg/kg LSD (c) 0.25 mg/kg LSD (D) 0.5 mg/kg LSD and (E) 1.0 mg/kg LSD. Rats were sacrificed 90 min. after injection of either LSD or vehicle.

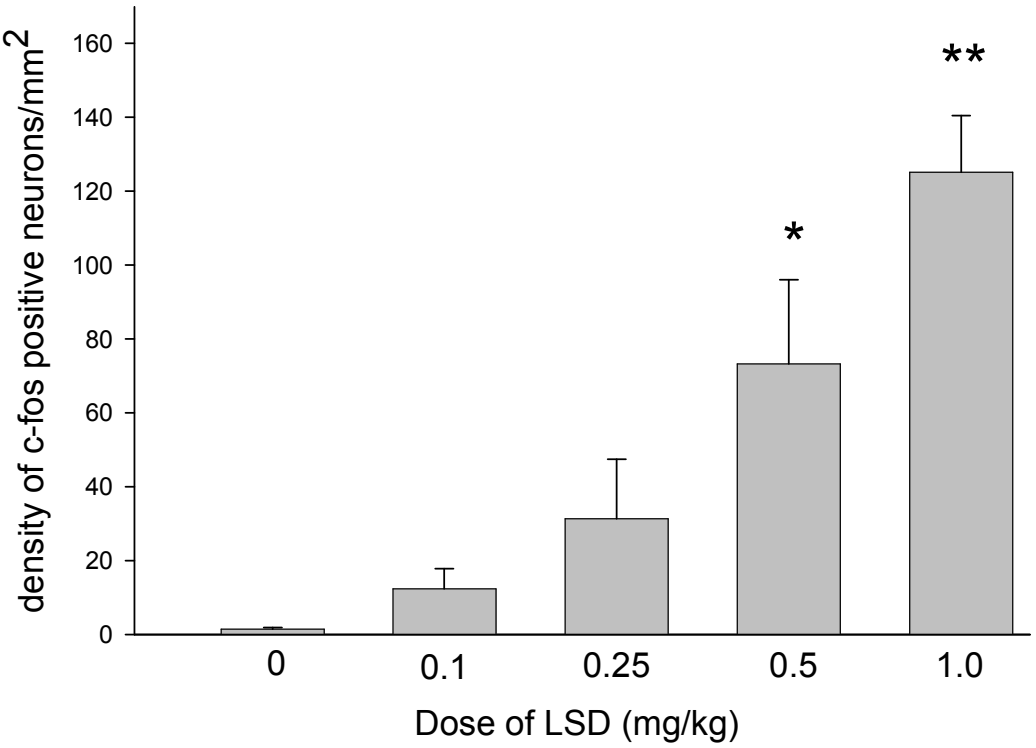

Fig. 14 Areal density of c-Fos-positive nuclei in rat PFC following systemic administration of LSD or vehicle. Each dose represents the mean (± S.E.M) of 5 animals (n=5). Rats were sacrificed 90 min after injection of either LSD or vehicle.

* Significantly different from vehicle and 0.1 mg/kg as measured by one-way ANOVA.

** Significantly different from all doses.

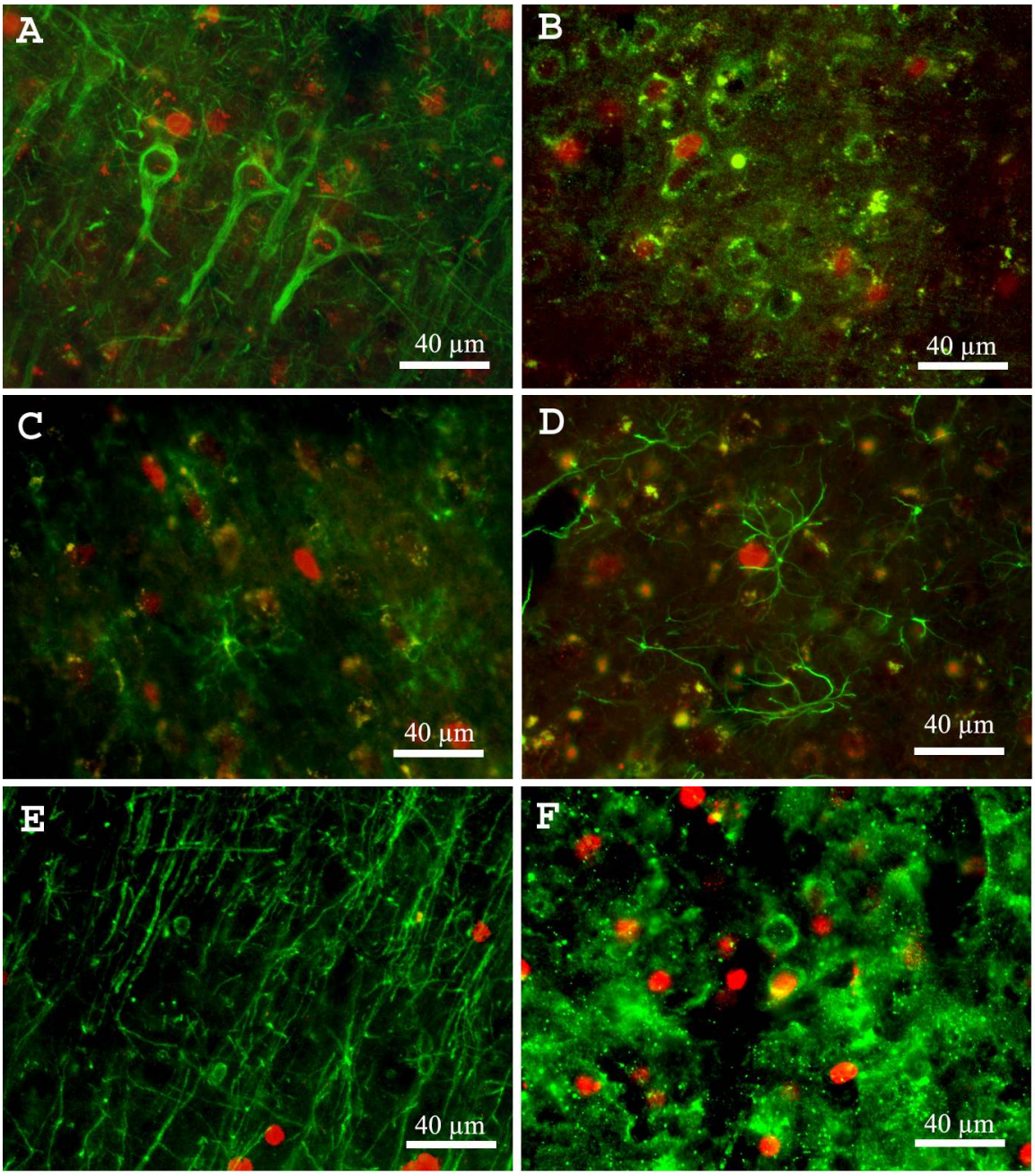

Fig. 15 Double labeling for c-Fos-positive nuclei and a cellular marker in the PFC of rats. In each section, c-Fos-positive nuclei are labeled in red and the following antibodies are labeled in green: (A) NF160 (B) NSE (C) OX-42 (D) GFAP (E) CNPase. (F) O1

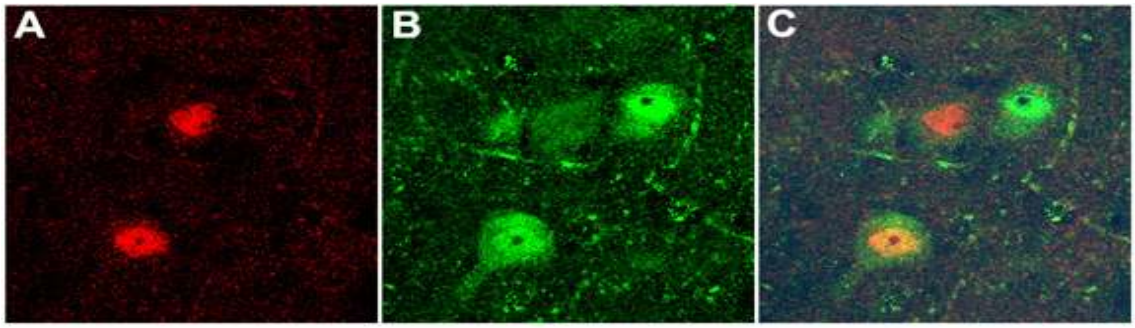

Fig. 16 Confocal microscopy images showing double labeling for c-Fos-positive nuclei and NeuN. (A) Red c-Fos+ nuclei (B) Green NeuN labeled neurons (C) overlay

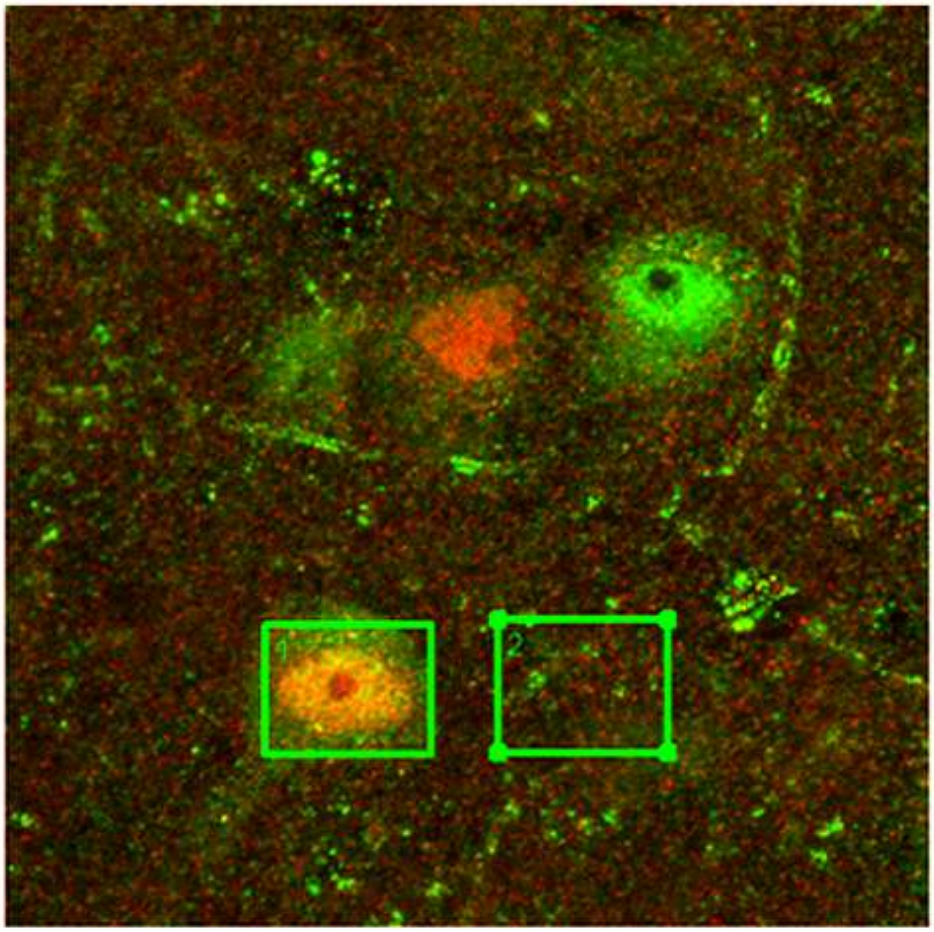

Fig. 17 Confocal microscopy images showing double labeling for c-Fos-positive nuclei and NeuN. Box 1 represents an area of high colocalization between c-Fos and NeuN. Box two represents the area analyzed for background staining.

Table 1.

Animal	O1 cells/mm^2	c-Fos cells/mm^2	Colocalized cells/5 mm^2	% of c-Fos cells colabeled	% of O1 cells colabeled
1	9.2	28.9	2.7	1.88	5.88
2	10.4	33.6	7.5	4.44	14.29
3	7.3	130.0	2.7	0.42	7.41
4	3.9	190.8	6.1	0.64	31.03
5	4.3	158.9	2.7	0.34	12.50
		MEAN	4.34 (±1.0)	1.54 (±0.78)	14.2 (±4.48)

Animal	NSE cells/mm^2	c-Fos cells/mm^2	Colocalized cells/5 mm^2	% of c-Fos cells colabeled	% of NSE cells colabeled
1	36.2	56.2	19.0	6.76	10.49
2	48.0	59.7	40.7	13.64	16.95
3	25.5	206.5	43.4	4.20	34.04
4	10.0	163.2	12.2	1.50	24.32
5	8.4	140.2	10.9	1.55	25.81
		MEAN	25.2 (±7.0)	5.53 (±2.2)	22.3 (±4.01)

Table 1. Colocalization of c-Fos-positive nuclei and a specific cellular marker following systemic administration of a 1.0 mg/kg dose of LSD. Animals were sacrificed 90 min after LSD administration. Data for O1 and NSE represent the mean of five (mean ± S.E.M) animals (n=5).

Table 2.

Animal	NeuN cells/mm^2	c-Fos cells/mm^2	Colocalized cells/mm^2	% NeuN colabeled	% c-Fos colabeled
	378.7	**56.6**	**19.1**	**5.0**	**33.8**

Table 2. Colocalization of c-Fos-positive nuclei and NeuN following systemic administration of a 1.0 mg/kg dose of LSD. Animals were sacrificed 90 min after LSD administration.

Table 3.

Total c-Fos	colocalized	% colabeled
8025	355	4.4

Table 3. Total percentage of c-Fos nuclei which were colocalized in a specific cell type. Data were totaled from tables 1-2.

Discussion

The present study found that LSD-induced c-Fos expression in the prefrontal cortex is dose dependent and that both neurons and oligodendrocytes express c-Fos. Although previous studies have examined LSD-induced increases in c-Fos (Frankel and Cunningham, 2002; Gresch et al., 2002), this is the first report to demonstrate the effect of dose on c-Fos expression (Fig 14).

Our finding that c-Fos expression is dose dependent suggests dose may be an important factor in predisposing individuals to adverse effects associated with LSD and other drugs of abuse. C-Fos affects the transcription of genes which are involved in long term cellular and morphological changes. Therefore, upregulation of c-Fos may contribute to some of the chronic effects associated with LSD use (Nichols et al., 2003; Nichols and Sanders-Bush, 2004) including hallucinogen persisting perceptual disorder or "flashbacks" (Strassman, 1984; Halpern and Pope, 2003) and the 5-HT_{2A} receptor downregulation observed in animals after repeated LSD exposure (Buckholtz et al., 1990).

The effect of dose on c-Fos expression has implications for its use in traditional dose-response studies in addition to identifying activated cells and neurons (Chaudhuri, 1997b; Chaudhuri et al., 2000). Sumner and colleagues (Sumner et al., 2004) have suggested utilizing c-Fos expression profiles in the classification of a variety of psychoactive agents such as: antidepressants, antipsychotics, and psychostimulants. The data presented in this report demonstrate the importance of dose in such classification schemes. Taken together, c-Fos mapping may be a valuable and versatile tool for characterizing the acute and chronic effects of psychoactive substances.

We found that c-Fos expression occurred in NSE and Neu-N labeled neurons but there were also c-Fos+ cells that did not colocalize with any of the antibodies used (Tables 1 and 3). None of the NF160 labeled pyramidal neurons colocalized with c-Fos (Fig 15), which is consistent with previous studies demonstrating that LSD induced c-Fos expression does not occur on 5-HT_{2A} containing neurons (Mackowiak et al., 1999; Scruggs et al., 2000; Gresch et al., 2002).

Interestingly, several other reports have shown that c-Fos protein and mRNA are induced in non-neuronal cells (Morgan and Curran, 1991; Pyykonen and Koistinaho, 1991; Simpson and Morris, 1994; Mesner et al., 1995; Bokesch et al., 1996; Herkenham et al., 1998; Hilgenberg et al., 1999), although to our knowledge, this is the first report to demonstrate that LSD induces glial c-Fos expression *in vivo*. The morphological appearance and nuclear diameter of the c-Fos expressing cells suggests they are oligodendrocytes. Oligodendrocytes have in fact, been shown to produce c-Fos (Cohen et al., 1996; Chiasson et al., 1997; Richter-Landsberg and Vollgraf, 1998). Table 1 shows that positively labeled oligodendrocytes express c-Fos in response to LSD injection. A larger proportion of oligodendrocytes may be expressing c-Fos, however, the expression of known surface markers such as O1 and O4 antigens varies throughout oligodendrocyte development, making positive identification difficult (Poduslo et al., 1985). In light of the psychotomimetic properties of LSD (Abraham et al., 1996; Aghajanian and Marek, 2000), it is interesting to note that others have suggested the involvement of oligodendroglial cells in human psychosis (Christensen et al., 2004; Uranova et al., 2004). These oligodendrocytes may be associated with the transient psychosis induced by administration of LSD. Although speculative, it is possible that these

oligodendrocytes have been stimulated to initiate myelination in response to altered neurotransmission caused by LSD (Coman et al., 2005). This increased myelination may be a mechanism of activity dependent neuronal plasticity (Fields, 2005) caused by LSD exposure. Changes in brain structure resulting from environmental experience have been known for some time (Bennett et al., 1964), including changes in oligodendrocyte number and function (Szeligo and Leblond, 1977; Sirevaag and Greenough, 1987).

In summary, we have presented evidence showing that LSD-induced c-Fos expression is dose dependent. Further work is needed to determine additional changes in gene expression that may occur as a consequence of c-Fos upregulation. In addition, it appears that LSD induces c-Fos expression in a non-neuronal cell population which consists (at least partially) of oligodendrocytes. These oligodendrocytes may be involved in producing hallucinogenesis and endogenous psychosis.

General Discussion

Study 1. Indoleamine and phenethylamine hallucinogens are drugs of abuse and mimic some aspects of idiopathic psychosis. To investigate the mechanisms of action of the indoleamine hallucinogen lysergic acid diethylamide, animals were trained to discriminate LSD (0.1 mg/kg 15 min pretreatment) from saline. We found that LSD-induced stimulus control was potentiated by a series of compounds with agonist properties at the 5-HT_{1A} receptor. The mechanism by which this potentiation occurs is unclear, although inhibition of neuronal activation within the raphe nucleus (Aghajanian GK and Haigler, 1975) may contribute to this effect. As noted in an earlier section, suppression of raphe firing was the basis for the first hypotheses regarding the mechanism of action of LSD and other hallucinogens (Aghajanian et al., 1970). This hypothesis could not be extended to the phenethylamine hallucinogens, however, as application of mescaline to the raphe nuclei did not produce a similar inhibition (Haigler and Aghajanian, 1973). Nonetheless, suppression of raphe firing would likely affect the activity of downstream cortical neurons and contribute to the potentiating effects of the 5-HT_{1A} agonists tested.

LSD has affinity for 5-HT_{1A} receptors, as do several other hallucinogens including 5-MeO-DMT (McKenna et al., 1990), psilocin (Blair et al., 2000), dipropyltryptamine (DPT) (Thiagaraj et al., 2005), and dimethyltryptamine (DMT) (Deliganis et al., 1991). Our finding the 8-OH-DPAT mimics the LSD stimulus (Fig.4) is a confirmation of previous findings (Winter and Rabin, 1988) and has been extended to include the phenethylamine hallucinogen DOM (Fig 2). Our findings with DOM are interesting in light of the fact that the phenethylamine hallucinogens lack affinity for 5-

HT_{1A} receptors (Titeler et al., 1988). This substitution of LSD and DOM for 8-OH-DPAT is symmetrical as 8-OH-DPAT produces partial generalization to both LSD and DOM (Fig10). This finding suggests that 8-OH-DPAT can mimic the stimulus effects of both indoleamine and phenethylamine hallucinogens to some degree and that the functional outcome of 5-HT_{1A} receptor stimulation may play a role in hallucinogenesis. To our knowledge however, there are no reports of the subjective effects of 8-OH-DPAT in humans.

It is also noteworthy that the 5-HT_{2A} antagonists ketanserin and pirenperone failed to block the LSD cue in LSD-trained monkeys (Nielsen, 1985). These results prompted the authors to conclude that there may be "preferential role for 5-HT_1 sites in mediating the LSD stimulus effect". This hypothesis is supported by the finding that 5-MeO-DMT, a drug whose stimulus effects are mediated primarily by 5-HT_{1A} receptors (Winter et al., 2000) does substitute in monkeys.

Although previous studies have suggested that 5-HT_{2A} receptor activation is a necessary component of hallucinogenesis, the current report suggests there may be a more substantial role of 5-HT_{1A} receptors than previously thought. While 5-HT_{1A} receptor stimulation is not a tenable hypothesis for the primary mechanism of hallucinogenesis, our findings demonstrate that it has a significant modulatory role.

Study 2. 5-HT mediated behaviors are important tools for examining the effects of drugs on 5-HT receptor function. The discovery of multiple populations of 5-HT receptors has stimulated efforts to determine whether functional relationships exist among the fourteen currently identified 5-HT receptor subtypes (Raymond et al., 2001). Of relevance to the current proposal are functional interactions between 5-HT_{1A} and 5-HT_{2A} receptor subtypes. To investigate the interaction between 5-HT_{1A} and 5-HT_{2A} receptors, animals were trained to discriminate 8-OH-DPAT from saline (0.2 mg/kg 15 min pretreatment). The training dose, and an intermediate, but not the lowest dose of 8-OH-DPAT were attenuated by pretreatment with the 5-HT_{2A} antagonist M100907 (0.1 mg/kg, 30 min. pretreat) (Fig10). 8-OH-DPAT substituted completely for the 5-HT_{1A} agonist buspirone, which is in agreement with previous results (Rabin and Winter, 1993) and this substitution was also attenuated by M100907 (Fig 10).

A frequently used model of 5-HT_{2A} function is the head twitch response, a behavior associated with 5-HT_{2A} receptor stimulation (Green et al., 1983; Schreiber et al., 1995a). Studies have shown that 8-OH-DPAT decreases head twitches induced by a number of compounds with agonist activities at 5-HT_2 receptors. These studies suggest functional opposition between these two receptor subtypes (Arnt and Hyttel, 1989; Darmani et al., 1990a). This conclusion is supported by studies using locomotor activity, which also suggest antagonistic interactions between 5-HT_{2A} and 5-HT_{1A} receptors (Krebs-Thomson and Geyer, 1998). Other 5-HT_2 mediated behaviors such as ear-scratch stereotypy, seem to be increased by administration of 5-HT_{1A} agonists (Darmani et al., 1990b). Taken together, the nature of the interaction between 5-HT_{2A} and 5-HT_{1A} receptors seems to be dependent upon the behavioral paradigm used to investigate the

interaction, and the compounds used induce and modulate the behaviors. Thus, our discrimination data demonstrating an attenuation of the 8-OH-DPAT stimulus cue by M100907 do not lend themselves to simple interpretation. It appears that 8-OH-DPAT produces an interoceptive cue in part, through stimulation of 5-HT_{2A} receptors.

Serotonin has been implicated in the regulation of various physiological functions including appetite, sleep, body temperature, blood pressure and sexual behavior (Lucki, 1998). As the modulation of one 5-HT receptor mediated behavior may be influenced by another 5-HT receptor subtype, the ramifications for drug development are vast. For example, a lower dose of a given agent may be needed to produce a specific therapeutic effect if that compound simultaneously targets and additional receptor subtypes. In a similar manner, the adverse effects of a drug may be reduced by modifying an existing compound to create a more specific binding profile. As many of the current serotonergic compounds are somewhat non-specific, this concept is worthy of additional study. Indeed, this idea has already been employed in the study of SSRI's, where 5-HT_2 antagonists have shown promise in increasing the efficacy of existing SSRI treatments (Boothman et al., 2006).

Study 3 LSD is able to alter human perception, mood and behavior although its mechanism of action remains elusive. While the behavioral effects of LSD are thought to involve 5-HT_{2A} (Titeler et al., 1988), 5-HT_{2C} (Krebs-Thomson et al., 1998) and 5-HT_{1A} (Burris et al., 1991) receptor subtypes, the biochemical and signaling pathways which result in the behavioral effects are unknown. Although serotonergic receptors are relevant to the actions of LSD, the signaling pathways and second messenger systems activated by

hallucinogens are clearly different from those of the endogenous ligand serotonin (Backstrom et al., 1999).

The effects of LSD on c-Fos expression were also investigated. *C-Fos* in an immediate early gene which gives rise to the protein c-Fos. The c-Fos protein is an inducible transcription factor which regulates the expression of "late" or "target-genes" involved in more long-term cellular and biochemical changes. Because receptor mediated intracellular signaling cascades influence gene expression, we hypothesized that LSD-induced changes in gene expression may alter the functional state of neurons, leading to changes in cognitive function. Thus, characterizing LSD-induced changes in gene expression was deemed important in determining LSD's mechanism of action.

LSD has previously been shown to induce the expression of c-Fos in the rat brain (Frankel and Cunningham, 2002; Gresch et al., 2002), however the effects of dose on this expression had not yet been characterized. In view of the fact that c-Fos activates the transcription of genes involved in long-term morphological and biochemical changes (Chaudhuri, 1997b; Kovacs, 1998), it is likely that changes in gene expression resulting from c-Fos expression are affected by dose as well. The results of Fig 14 demonstrate that c-Fos expression is dose-related. Whether this c-Fos expression can be correlated with the dose-related subjective effects in humans and the target genes affected by this expression, remain to be determined. These results suggest that c-Fos can provide a correlate between dose and changes in gene expression which may be associated with the chronic effects of a drug.

C-Fos is also used as an anatomical mapping tool, as rapid upregulation occurs in response to cellular and neuronal stimulation (Chaudhuri, 1997b; Chaudhuri et al., 2000).

The next experiments in this study employed c-Fos to determine the specific cell types which LSD-induced c-Fos expression occurs. Based on the fact that 5-HT_{2A} receptor antagonists attenuate LSD mediated increases in c-Fos expression, and that LSD-induced c-Fos expression does not occur on 5-HT_{2A} receptor containing neurons (Gresch et al., 2002) we hypothesized that LSD-induced c-Fos expression occurs in a population of cells other than 5-HT_{2A} containing neurons. Our results Fig 15 and 16 indicate the c-Fos expression occurs in neurons and a population of oligodendrocytes. It is interesting to note, however, that of the total population c-Fos positive nuclei quantified, approximately 95% did not colocalize within any of the neurons labeled (NSE, NF160 and NeuN labeled neurons). Moreover, of the 8025 c-Fos+ cells counted (Table 3), only 359 were colocalized with a specific cell type (4%). This finding suggests that LSD-induced c-Fos expression occurs in a cell type characterized by a unique epitope.

The present study also found that c-Fos expression occurred in oligodendrocytes (Fig 15). In light of the psychotomimetic properties of LSD (Abraham et al., 1996; Aghajanian and Marek, 2000), it is interesting to note that others have suggested the involvement of oligodendroglial cells in human psychosis (Christensen et al., 2004; Uranova et al., 2004). These oligodendrocytes may be associated with the transient psychosis induced by administration of LSD, hallucinogenesis and endogenous psychosis. Alternatively, these oligodendrocytes may be undergoing the initial phases of myelination as a result of changes in neuronal firing and neurotransmitter release caused by LSD.

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