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LYSERGIC ACID DIETHYLAMIDE-INDUCED FOS EXPRESSION IN RAT BRAIN: ROLE OF SEROTONIN-2A RECEPTORS

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Abstract—Lysergic acid diethylamide (LSD) produces altered mood and hallucinations in humans and binds with high affinity to serotonin-2A (5-HT_{2A}) receptors. Although LSD interacts with other receptors, the activation of 5-HT_{2A} receptors is thought to mediate the hallucinogenic properties of LSD. The goal of this study was to identify the brain sites activated by LSD and to determine the influence of 5-HT_{2A} receptors in this activation. Rats were pretreated with the 5-HT_{2A} receptor antagonist MDL 100907 (0.3 mg/kg, i.p.) or vehicle 30 min prior to LSD (500 μg/kg, i.p.) administration and killed 3 h later. Brain tissue was examined for Fos protein expression by immunohistochemistry. LSD administration produced a five- to eight-fold increase in Fos-like immunoreactivity in medial prefrontal cortex, anterior cingulate cortex, and central nucleus of amygdala. However, in dorsal striatum and nucleus accumbens no increase in Fos-like immunoreactivity was observed. Pretreatment with MDL 100907 completely blocked LSD-induced Fos-like immunoreactivity in medial prefrontal cortex and anterior cingulate cortex, but only partially blocked LSD-induced Fos-like immunoreactivity in amygdala. Double-labeled immunohistochemistry revealed that LSD did not induce Fos-like immunoreactivity in cortical cells expressing 5-HT_{2A} receptors, suggesting an indirect activation of cortical neurons.

These results indicate that the LSD activation of medial prefrontal cortex and anterior cingulate cortex is mediated by 5-HT_{2A} receptors, whereas in amygdala 5-HT_{2A} receptor activation is a component of the response. These findings support the hypothesis that the medial prefrontal cortex, anterior cingulate cortex, and perhaps the amygdala, are important regions involved in the production of hallucinations.

Key words: amygdala, anterior cingulate cortex, DOI, hallucinogen, LSD, medial prefrontal cortex.

Lysergic acid diethylamide (LSD) administration produces hallucinations and altered perceptions, cognition and mood in humans. There is growing evidence in animal studies supporting the hypothesis that activation of serotonin-2A (5-HT_{2A}) receptors mediates the effect of hallucinogens (Aghajanian and Marek, 1999a,b). Electrophysiological studies demonstrate that application of 5-HT or the 5-HT_2 receptor agonist, 1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane (DOI), produces excitatory postsynaptic currents (EPSCs) in apical dendrites of cortical pyramidal cells and this effect is blocked by selective 5-HT_{2A} receptor antagonists (Aghajanian and Marek, 1997, 1999a,b; Marek et al., 2000). In addition, administration of DOI produces 5-HT_{2A} receptor-dependent expression of the immediate early gene *c-fos* in the cortex of the rat (Leslie et al., 1993; Tilakaratne and Friedman, 1996; Scruggs et al., 2000). Furthermore, 5-HT_{2A} receptors are responsible for many of the hallucinogen-induced behavioral effects in rats (Fiorella et al., 1995; Krebs-Thomson et al., 1998).

Human psychopharmacological studies using normal healthy volunteers demonstrate that the hallucinogen psilocybin (an indoleamine with actions similar to LSD) increases glucose metabolism in frontomedial and frontolateral cortex, anterior cingulate cortex (ACC), and temporomedial cortex (Vollenweider et al., 1997). Furthermore, the psychotomimetic effects of psilocybin are blocked dose-dependently by ketanserin (a 5-HT_2 antagonist) and risperidone (a mixed 5-HT_2/D-2 antagonist), but not by haloperidol (D-2 antagonist) (Vollenweider et al., 1998). These interesting results suggest that 5-HT_2 receptors are involved in the production of drug-induced hallucinations in limbic and frontal cortex and add further support to the hypothesis that stimulation of 5-HT_{2A} receptors may be responsible for the cognitive, affective and perceptual distortions of hallucinogenic drugs.

Rapid and transient expression of the immediate early gene, *c-fos*, occurs following neuronal activation, thereby providing an *in vivo* map of cellular responses to a given stimulus (Chaudhuri, 1997). This approach has been used to examine the effects of drugs that interact with 5-HT_2 receptors. For example, DOI produces a marked increase in Fos-like immunoreactivity (Fos-LI) in various brain regions of rat brain including frontal, parietal, cin-

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Abbreviations: 5-HT, serotonin; ACC, anterior cingulate cortex; DAB, 3,3′-diaminobenzidine tetrahydrochloride; DOI, 1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane; EPSC, excitatory postsynaptic current; Fos-LI, Fos-like immunoreactivity; LSD, lysergic acid diethylamide; mPFC, medial prefrontal cortex; PBS, phosphate-buffered saline.

gulate and piriform cortex, as well as claustrum, amygdala, nucleus accumbens and dorsomedial striatum (Leslie et al., 1993; Moorman and Leslie, 1996; Abi-Saab et al., 1999; Scruggs et al., 2000). A recent report examining *c-fos* mRNA expression and its correlation to abuse potential qualitatively describes an increase in frontal and parietal cortex, lateral septal area, striatum and limbic regions after a large dose of LSD (Erdtmann-Vourliotis et al., 1999). In the current study, we have quantitatively mapped neuronal activation in rat brain after LSD treatment with particular focus on specific brain regions that are thought be involved in the production of hallucinations and determined the contribution of 5-HT_{2A} receptor activation in LSD-induced Fos-LI expression.

EXPERIMENTAL PROCEDURES

Animals

Adult male Sprague–Dawley rats (225–249 g) from Harlan Sprague–Dawley, Indianapolis, IN, USA) were used. All animals were housed in a colony room (ambient temperature 22–23°C, 12:12-h light:dark cycle) with food and water available *ad libitum*. All animal use procedures are in strict accordance with the NIH Guide to the Care and Use of Laboratory Animals and approved by Vanderbilt University Animal Care Committee.

Drug treatments

Rats received a single i.p. injection of vehicle or 5-HT_{2A} receptor antagonist MDL 100907 (0.3 mg/kg) (Sorensen et al., 1993) 30 min prior to i.p. injection of vehicle or LSD (500 μg/kg; gift from NIDA). Animals were killed 3 h after LSD or vehicle injection. The 3-h time point was based on the maximal Fos-LI induction observed after DOI administration (Leslie et al., 1993).

Immunohistochemistry

Rats were anesthetized (150 mg/kg pentobarbital, i.p.) and perfused transcardially with 300 ml of 0.1 M phosphate-buffered saline (PBS) containing 4% paraformaldehyde. Brains were removed immediately after perfusion, stored in the same fixative for 30 min, transferred to increasing concentrations of sucrose, and frozen in isopentane and stored at −80°C until sectioning. Coronal sections were cut at 50 μm on a vibratome and collected in PBS. Immunohistochemistry was performed on free-floating sections. Sections were treated with 0.3% hydrogen peroxide for 20 min followed by washes in PBS. Sections were then incubated in 3% goat serum/0.2% Triton X-100 for 2 h to block non-specific binding. Sections were incubated for 48 h at 4°C in Fos primary antibody (Oncogene Research Products) diluted in blocking solution at 1:30 000. Following three washes in PBS, a Vectastain Elite ABC horseradish peroxidase kit (Vector Laboratories) was used for the secondary antibody and avidin–biotin complex steps. The colorimetric detection reaction produces 3,3′-diaminobenzidine tetrahydrochloride (DAB) as brown chromogen product. Sections were mounted on slides, air-dried, dehydrated through graded ethanol solutions, treated with xylenes, and coverslipped with Permount.

We also determined whether LSD administration induced Fos-LI in cells expressing the 5-HT_{2A} receptor. Immunohistochemistry was performed to first detect 5-HT_{2A} receptor protein. Free-floating sections were incubated with mouse monoclonal 5-HT_{2A} antibody (PharMingen, San Diego, CA, USA: 2.0 μg/ml) using avidin–biotin method with DAB reaction to produce a brown product as chromogen. Sections were thoroughly washed and Fos-LI was detected as described above but with heavy metal intensification to yield a blue-black DAB reaction localized to the nucleus of the cell.

Analysis of Fos-LI positive cells

Brain sections containing medial prefrontal cortex (mPFC), dorsal striatum, nucleus accumbens, ACC, and amygdala were examined (Fig. 1). Bright-field video images were captured using Openlab 2.2.5 software (Improvision) with a 3CCD video camera system (Zeiss) mounted on a Zeiss Axiovert S100 microscope. All microscope, camera and software settings were constant throughout image collection for a given brain region. Analysis and quantification of images was performed by using Image 1.61 (Wayne Rasband, NIH). In brief, a 1.25-mm × 1.13-mm area was analyzed for number of Fos-LI positive cells. Cells containing nuclear brown-black reaction product were considered positive Fos-LI. This was determined by setting the pixel density threshold four times background and using the particle count macro in Image 1.61. Montage assemblies of representative images were created using Adobe Photoshop 5.0.2.

RESULTS

Administration of LSD significantly increased the Fos-LI positive expressing cells in the mPFC compared to vehicle-injected rats (Fig. 2). Pretreatment with the 5-HT_{2A} receptor antagonist MDL 100907 attenuated the LSD-induced Fos-LI expression in mPFC (Fig. 3A). There was no significant difference between MDL 100907 treatment alone and MDL 100907 plus LSD administration (Fig. 3A). LSD administration also significantly increased the Fos-LI positive expressing cells in the ACC compared to vehicle-injected rats (Fig. 4). Pretreatment with the 5-HT_{2A} receptor antagonist MDL 100907 completely eliminated the Fos-LI expression produced by LSD treatment in ACC (Fig. 3B). There was no statistically detectable difference between MDL 100907 treatment and MDL 100907 plus LSD treatment (Fig. 3B).

In the central nucleus of the amygdala, LSD induced a significant increase in the number of cells expressing Fos-LI compared to vehicle-injected rats (Fig. 5). In contrast to the cortical areas, pretreatment with the 5-HT_{2A} receptor antagonist MDL 100907 only partially attenuated the Fos-LI expression produced by LSD treatment in central amygdala (Fig. 3C). LSD administration did not significantly induce Fos-LI expression in the dorsal medial striatum or nucleus accumbens (Table 1).

Double staining for Fos-LI and 5-HT_{2A} receptors demonstrated that LSD administration did not induce Fos-LI in 5-HT_{2A} receptor positive pyramidal cells in parietal cortex or mPFC (Fig. 6). The majority of Fos-LI positive cells were located in layer IV–III. There was the rare occurrence of double-labeled pyramidal cell.

DISCUSSION

Numerous studies have examined the induction of Fos-LI after administration of the 5-$HT_{2A/2C}$ agonist, DOI. The frontal cortex, cingulate cortex, somatosensory cortex, nucleus accumbens and piriform cortex are

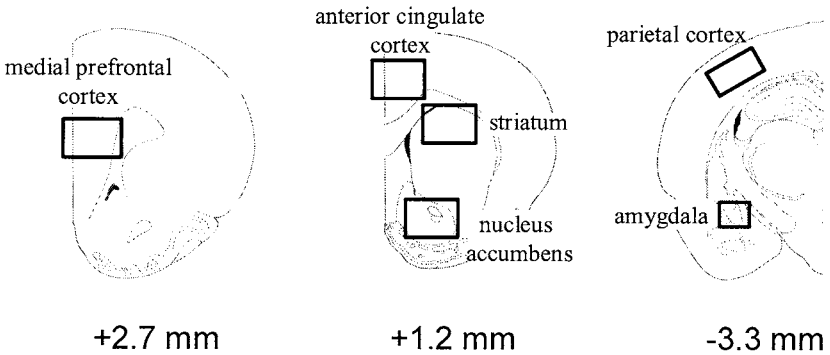

Fig. 1. Schematic representation of regional analysis; boxes highlight areas of quantification. All measurements are relative to bregma (Paxinos and Watson, 1986).

all activated by DOI administration (Leslie et al., 1993; Scruggs et al., 2000). In addition, subcortical regions such as amygdala, bed nucleus stria terminalis, dorsal striatum and hippocampus display a DOI-induced Fos-LI expression. In this study, we demonstrate that administration of LSD produces profound activation of cortical regions and amygdala but not dorsal striatum or nucleus accumbens, as indicated by increased expression of the immediate early gene product, Fos. Importantly, there was no Fos-LI activation in the dorsal–medial striatum and nucleus accumbens in the LSD-treated rats. The lack of Fos-LI activation in the nucleus accumbens is consistent with the suggestion that LSD is not reinforcing in animals (for discussion, see Parker, 1996). Our findings of a more localized increase in Fos-LI expression in cortical regions and amygdala after LSD treatment suggests that the pattern of neuronal activation is more discrete even though DOI is a more specific

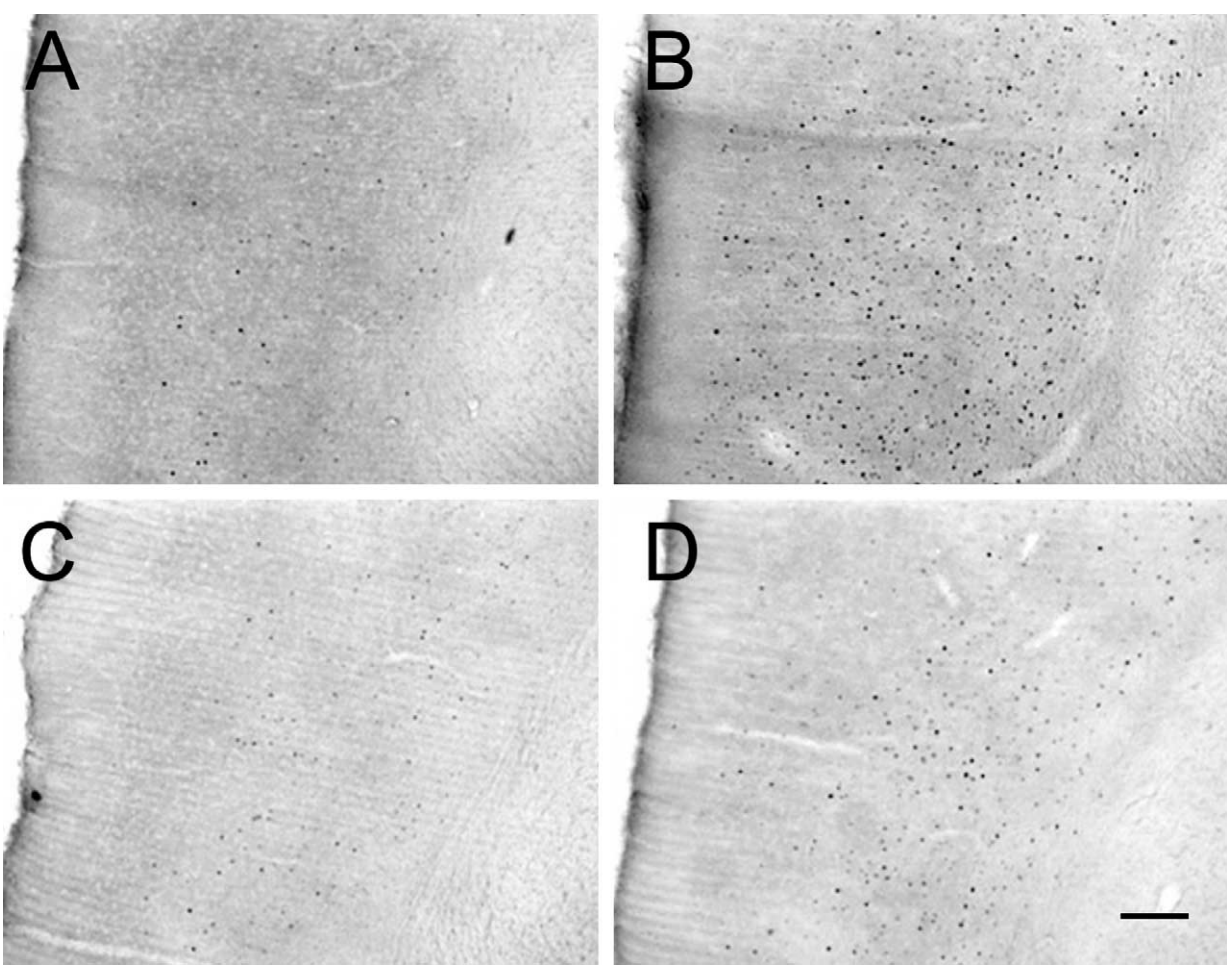

Fig. 2. Representative images showing Fos-LI containing cells in mPFC of rats treated with vehicle (A), LSD (B), MDL 100907 (C), and MDL 100907 prior to LSD (D). Acute administration of LSD (500 μg/kg) increased the number of Fos-LI containing cells in mPFC that appears to be attenuated by MDL 100907 (0.3 mg/kg) pretreatment. Scale bar = 200 μm.

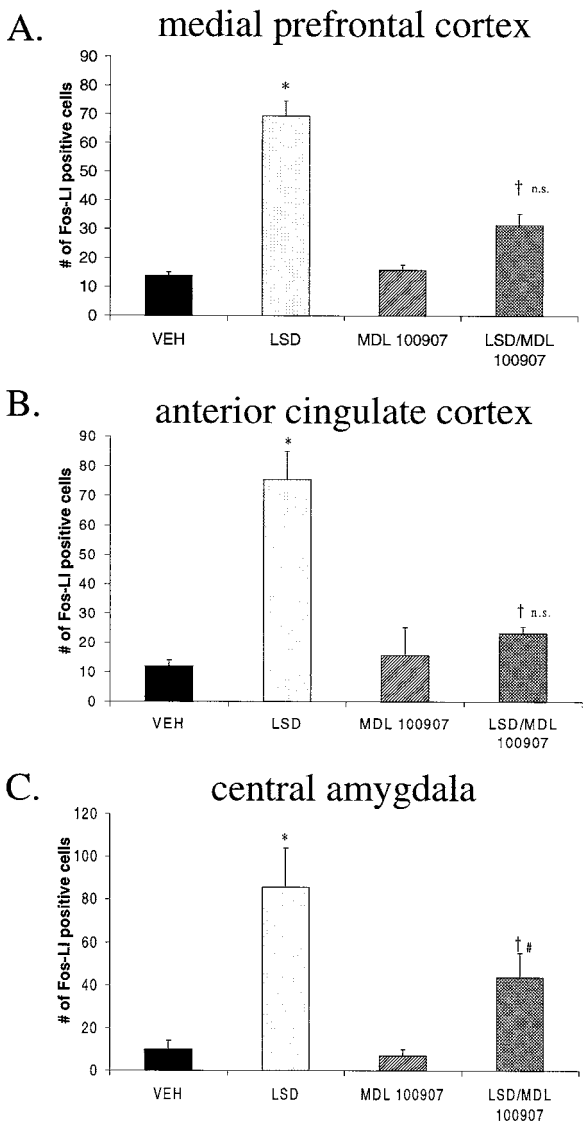

Fig. 3. Quantitative analysis of number of Fos-LI positive cells in the mPFC, ACC, or central amygdala after vehicle, LSD, MDL 100907, and MDL 100907 pretreatment prior to LSD. Values are number of Fos-LI positive cells (mean ± S.E.M. within area of analysis; $n = 4$). (A) The LSD-induced Fos-LI expression in mPFC is attenuated by MDL 100907 pretreatment ($F_{3,12} = 49.09$; $P < 0.0001$). (B) The LSD-induced Fos-LI expression in ACC is attenuated by MDL 100907 ($F_{3,12} = 16.18$; $P < 0.0001$). (C) The increase in number of Fos-LI positive cells after LSD treatment in central amygdala is attenuated by MDL 100907 pretreatment ($F_{3,12} = 11.154$; $P < 0.01$). *Significantly different from vehicle-injected; †significantly different from LSD-treated (Tukey's HSD test; $P < 0.05$); n.s., not significantly different from MDL 100907/vehicle-treated rats; #significantly different from MDL 100907/vehicle-treated rats.

agonist than LSD. Taken together, these data suggest that despite the high affinity of LSD for multiple 5-HT and other neurotransmitter receptors, the Fos-LI signal is highly localized in cortical areas previously suggested to be involved in hallucinations.

We sought to determine the relative role of 5-HT_{2A} receptors in the activation of Fos-LI expression by LSD administration. The 5-HT_{2A} receptor is widely expressed throughout the rat brain, with high expression in cortical pyramidal cells, septum, hippocampus, basal ganglia, and amygdala (Willins et al., 1997; Jakab and Goldman-Rakic, 1998; Cornea-Hebert et al., 1999). Blockade of the 5-HT_{2A} receptor 30 min prior to LSD treatment completely attenuated the Fos-LI response in mPFC and ACC. However, in the amygdala there was only a partial attenuation of the LSD response by 5-HT_{2A} receptor antagonism. These data suggest that LSD primarily acting on 5-HT_{2A} receptors causes profound neuronal activation in the mPFC, ACC and amygdala. The observed partial contribution of 5-HT_{2A} receptors in the LSD activation of the central amygdala suggests another receptor-mediated component in this region. We are currently investigating whether 5-HT_{2C} receptors have a role in the LSD activation of the central amygdala since this region expresses high levels of 5-HT_{2C} receptors (J.R. Backstrom, unpublished observations).

Additional experiments were designed to determine whether the LSD-induced Fos-LI positive cells in the cortex also express 5-HT_{2A} receptors. Our results from double-labeled experiments revealed that the majority of Fos-LI positive cells were not 5-HT_{2A} receptor positive either in the mPFC or the somatosensory cortex. This observation is consistent with previous reports that the DOI-induced Fos-LI expression in the cortex does not coincide with 5-HT_{2A} receptor immunoreactivity (Mackowiak et al., 1999; Scruggs et al., 2000). This finding that the Fos-LI expression is not localized to 5-HT_{2A} receptor positive cells suggests a possible indirect influence of LSD on cortical activation. Growing evidence supports the hypothesis of an indirect action of 5-HT_{2A} receptors on cortical pyramidal cell activity via alterations in glutamatergic neurotransmission (Aghajanian and Marek, 1999a,b; Marek et al., 2000). Using isolated brain slices of frontal cortex that preserve local circuitry, Marek et al. (1997, 2000) demonstrated that ionophorectic applied DOI or 5-HT produced EPSCs in apical dendrites of pyramidal cells that were inhibited by AMPA receptor antagonist as well as 5-HT_{2A} receptor antagonism. The finding that a metabotrophic glutamate receptor II/III agonist, which inhibits glutamate release via an autoreceptor, also attenuates the 5-HT_{2A} receptor-mediated excitation supports the model that 5-HT_{2A} receptors on glutamate nerve terminals release glutamate that in turn activates pyramidal cells. The evidence of an indirect action of 5-HT_{2A} receptors on cortical activation is further supported by the findings that thalamocortical lesions decreased 5-HT_{2A}-mediated pyramidal cell EPSCs

Table 1. Effect of LSD (500 μg/kg) on Fos-LI expression in the dorsal striatum and nucleus accumbens

Region	Vehicle	LSD
Striatum	8.87 ± 4.41	14.10 ± 9.03
Nucleus accumbens	5.67 ± 2.85	3.60 ± 1.82

Values are numbers of Fos-LI positive cells detected in given region. There was no significant change in the number of Fos-LI positive cells after LSD treatment in dorsal striatum ($F_{3,12} = 0.336$; $P = 0.799$) or nucleus accumbens ($F_{3,12} = 2.562$; $P = 0.104$).

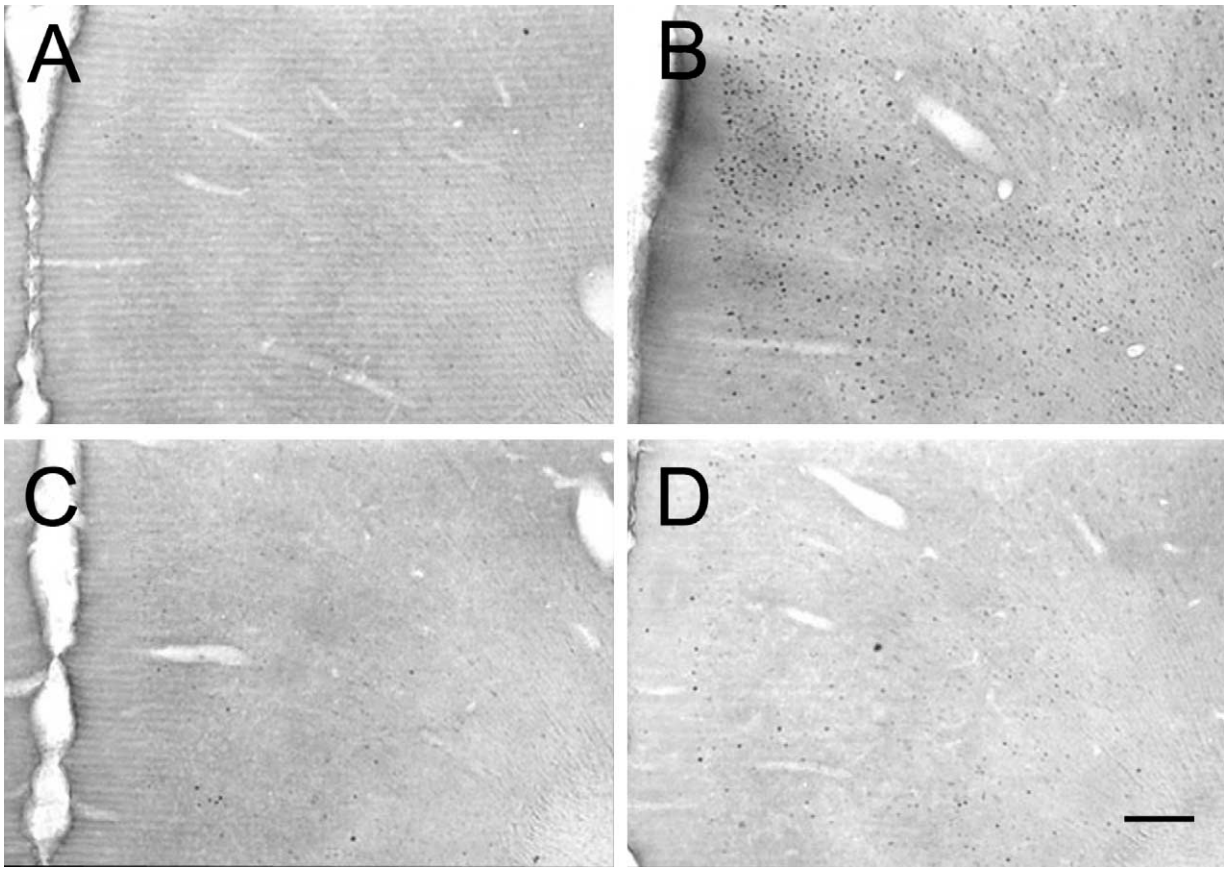

Fig. 4. Representative images showing Fos-LI containing cells in ACC from rats treated with vehicle (A), LSD (B), MDL 100907 (C), and MDL 100907 prior to LSD (D). Acute administration of LSD (500 μg/kg) increased the number of Fos-LI containing cells in ACC that appears to be attenuated by MDL 100907 (0.3 mg/kg) pretreatment. Scale bar = 200 μm.

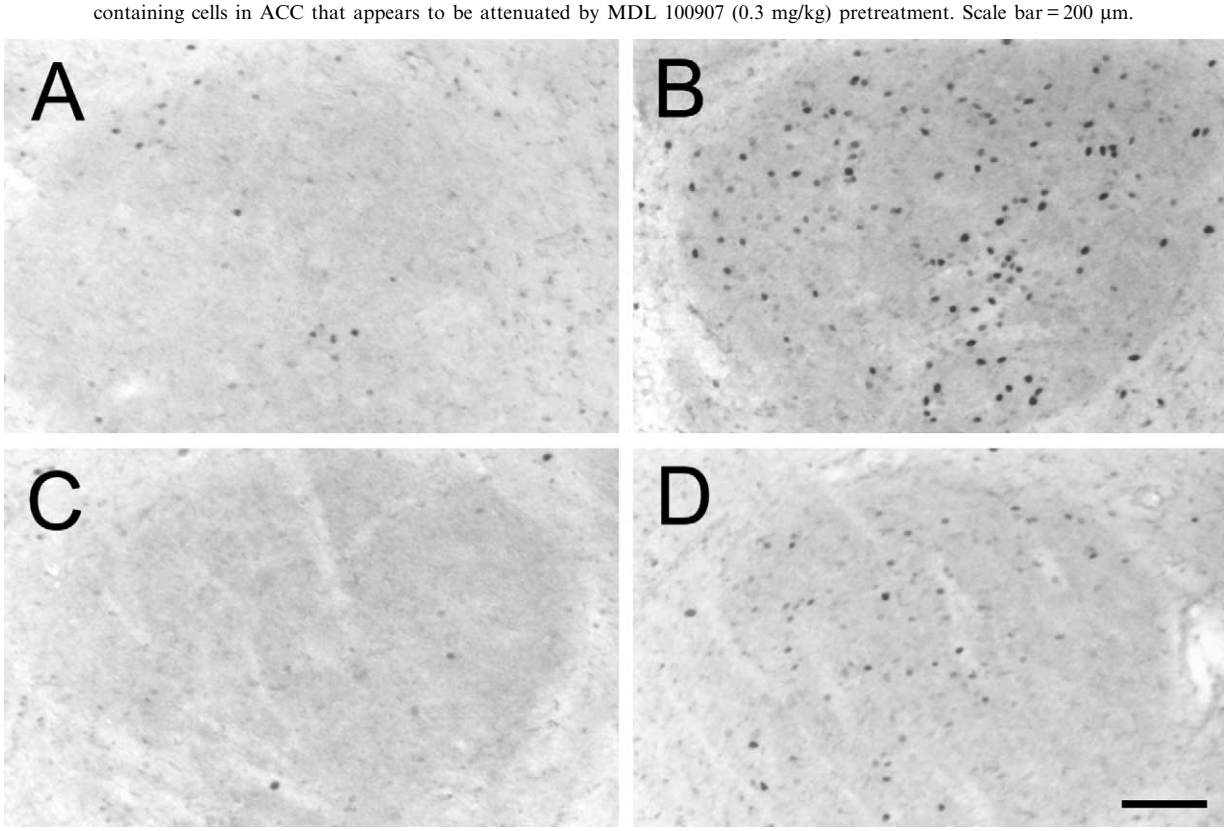

Fig. 5. Representative images showing Fos-LI containing cells in central amygdala from rats treated with vehicle (A), LSD (B), MDL 100907 (C), and MDL 100907 prior to LSD (D). Acute administration of LSD (500 μg/kg) increased the number of Fos-LI containing cells in central amygdala that appears to be attenuated by MDL 100907 (0.3 mg/kg) pretreatment. Scale bar = 100 μm.

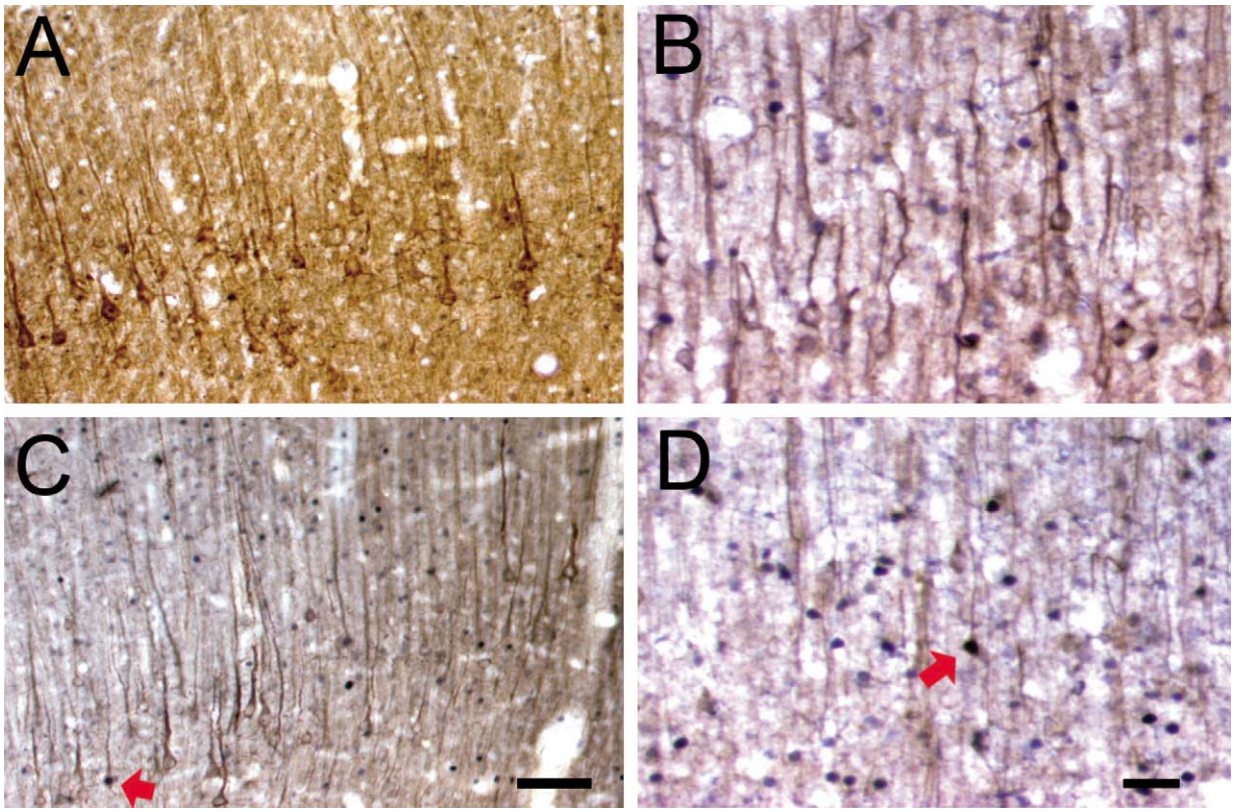

Fig. 6. Images showing double staining for 5-HT_{2A} receptor (brownish stain) and Fos-LI (blue-black stain) immunoreactivities in parietal cortex of vehicle (A) or LSD (C) and mPFC of vehicle (B) or LSD (D) treated rats. Dual staining reveals that LSD does not induce Fos-LI in 5-HT_{2A} positive cells in the parietal or mPFC. On rare occasions, a 5-HT_{2A}-LI pyramidal cell is also Fos-LI positive (arrow). Scale bar = 200 μm (partial cortex), 100 μm (mPFC).

and cortical Fos-LI activation (Scruggs et al., 2000, Marek et al., 2001). It remains to be determined whether the Fos-LI induction in cortical regions after LSD administration is also mediated indirectly by glutamate transmission.

The Fos-LI activation observed in the amygdala is consistent with previous studies examining effects of 5-HT_2 receptor agonists on brain Fos-LI induction. DOI administration produces a significant increase in Fos-LI in the central nucleus of the amygdala that is blocked by MDL 100907 and ritanserin (Leslie et al., 1993; Van de Kar et al., 2001). It remains to be determined if the hallucinogen-induced Fos-LI positive cells in the central amygdala are also 5-HT_{2A} receptor positive. Since the amygdala integrates cortical and hypothalamic inputs and has been implicated in numerous functions such as fear response, anxiety and affect, it may be important in the affective components of LSD's actions.

Our results support the hypothesis that cortical activation may underlie the neuroanatomical substrate mediating drug-induced hallucinations. Vollenweider et al. (1997) demonstrated that the hallucinogenic drug psilocybin produced activation of ACC, frontal cortex and other brain regions in humans as measured by increased metabolic activity. There was a strong correlation between the psychopathology score of hallucinations and an increase in metabolic activity in the frontomedial cortex (Vollenweider et al., 1997). Also, scores of Ego pathology (loss of autonomy and self control) were associated with activation in the ACC. These clinical findings are consistent with a corresponding Fos-LI activation in the rat mPFC and ACC. Furthermore, the psychotomimetic effects of psilocybin in human subjects are blocked by the administration of ketanserin or risperidone which antagonizes the 5-HT_{2A} receptor (Vollenweider et al., 1998). Thus, our findings that 5-HT_{2A} receptor antagonism blocks LSD-induced Fos-LI in the frontal cortex and ACC parallel these clinical observations. We are currently performing experiments to determine the specific role of these brain regions in the discriminative stimulus cue of LSD by direct microinjection of drugs into these brain regions.

CONCLUSION

Our results identified specific brain regions that are activated by the administration of the hallucinogen LSD. In the rat mPFC and ACC, the LSD activation is mediated via 5-HT_{2A} receptors, whereas in the central nucleus of the amygdala 5-HT_{2A} receptors apparently have only a partial role in the LSD activation. In the cortex, LSD activation of neurons is mediated by an indirect influence of 5-HT_{2A}, possibly via glutamatergic thalamocortical inputs. The anatomical identification of brain regions activated by LSD in this study may lead to the further understanding of the neuronal substrates mediating the behavioral effects of LSD.

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