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3,4-Methylenedioxymethamphetamine (MDMA, ecstasy)-induced egr-1 mRNA in rat brain: pharmacological manipulation

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Abstract

Using in situ hybridization and immunohistochemical techniques, we examined the expression pattern of egr-1 mRNA and Egr-1 protein in several brain regions following administration of 3,4-methylenedioxymethamphetamine (MDMA). Furthermore, we also studied the role of *N*-methyl-D-aspartate (NMDA) receptor, dopamine D_1 receptor, 5-hydroxytryptamine (5-HT) transporter or 5-HT_{2A} receptor in the induction of egr-1 mRNA by MDMA. Basal constitutive levels of egr-1 mRNA were detected in control rat brains. A single administration of MDMA (10 mg/kg) caused marked induction of egr-1 mRNA in the prefrontal cortex, striatum and hippocampal dentate gyrus. However, no changes in the egr-1 mRNA levels were detected in the CA1 region of hippocampus and occipital cortex after administration of MDMA (10 mg/kg). Furthermore, the expression of egr-1 mRNA in the prefrontal cortex, striatum and hippocampal dentate gyrus after administration of MDMA (10 mg/kg) was blocked significantly by pretreatment with NMDA receptor antagonist (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,b]-cyclohepten-5,10-imine ((+)-MK801; 1 mg/kg), dopamine D_1 receptor antagonist SCH 23390 (1 mg/kg) or 5-HT uptake inhibitor paroxetine (5 mg/kg), but not by 5-HT_{2A} receptor antagonist SR46349B (5 mg/kg). However, high basal levels of Egr-1 immunoreactivity in the rat brain were not altered by administration of MDMA (10 mg/kg). These results suggest that MDMA alters the expression of egr-1 mRNA in several regions of rat brain, and that the expression of egr-1 mRNA by MDMA in the prefrontal cortex, striatum and hippocampal dentate gyrus appears to be mediated, at least in part, by NMDA receptor, dopamine D_1 receptor and 5-HT transporter.

Keywords: MDMA (3,4-methylenedioxymethamphetamine); 5-HT (5-hydroxytryptamine, serotonin) transporter; Egr-1 (zif-268); Immediate-early gene

1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA or "Ecstasy") is a recreational drug that produces feelings of euphoria and energy, a desire to socialize, which goes far to explain their current popularity as a "rave drug" (Green et al., 1995; Hegadoren et al., 1999; Ropero-Miller and Goldberger, 1999). Although it is widely claimed that MDMA is used almost exclusively as a dance drug, there are increased reports of toxic reaction and death by MDMA (Henry et al., 1992; Solowij, 1993; Hegadoren et al., 1999; Ropero-Miller and Goldberger, 1999). Furthermore, it is well known that MDMA produces neurotoxic effects of serotonergic neurons in laboratory animals (McKenna and Peroutka, 1990; Hashimoto and Goromaru, 1990, 1992). Moreover, it has been demonstrated that MDMA users show decreased global and regional brain 5-hydroxytryptamine (5-HT, serotonin) transporter binding compared with controls (McCann et al., 1998), and that MDMA users, in the early phase of discontinuation of the drug,

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have blunted prolactin and cortisol responses to D-fenfluramine (Gerra et al., 1998). Recently, it has been shown that deficits in memory performance in MDMA users are primarily associated with past exposure to MDMA, rather than with the other legal and illicit drugs consumed by these individuals, suggesting the reduction in serotonergic modulation of mnemonic function as a result of long-term neurotoxic effects of MDMA in humans (Bolla et al., 1998; Morgan, 1999). Thus, it is likely that abuse of MDMA could cause neurotoxic effects in human brain. However, the precise mechanisms underlying the acute and long-term neurotoxic effects of MDMA remain to be determined.

The immediate-early genes c-fos and egr-1 (also known as NGFI-A, Krox-24, or zif268) that code for transcription factors have become popular neurobiological tools for imaging functional activity (reviews by Hughes and Dragunow, 1995; Beckmann and Wilce, 1997; Kaczmarek and Chaudhuri, 1997). The expression of immediate-early genes is rapidly induced by a wide variety of extracellular stimuli, and some immediate-early genes-encoded proteins encode transcription factors coupling short-term cell-surface events to long-term changes in the expression of target genes. We have recently reported that MDMA cause marked expression of Fos protein in several regions of rat brain (Hashimoto et al., 1997), and that the expression of Fos protein by MDMA in the striatum and olfactory tubercle appears to be mediated, at least in part, by dopamine D_1 receptor and *N*-methyl-D-aspartate (NMDA) receptor. While c-fos mRNA and Fos protein have low basal levels of expression, egr-1 mRNA and Egr-1 protein are constitutively expressed at high levels in several brain regions. Furthermore, some lines of evidence suggest that high constitutive expression of Egr-1 protein is thought to be maintained by NMDA receptor-mediated physiological neuronal activity (Worley et al., 1991), and that Egr-1 protein may be involved in the production of long-term synaptic changes such as those occurring during long-term potentiation and other learning-related phenomena. Moreover, it is suggested that immediate-early genes such as Fos protein and Egr-1 protein might be involved in the neuronal death and/or neuronal rescue (Beckmann and Wilce, 1997; Kaczmarek and Chaudhuri, 1997).

It has been shown that dopamine D_1 receptor antagonist SCH 23390 blocked the amphetamine- or methamphetamine-induced expression of egr-1 mRNA in the striatum, cortex and nucleus accumbens, as well as behavioral changes such as hyperlocomotion and stereotypies, suggesting the role of dopamine D_1 receptor in the expression of egr-1 mRNA in the three regions by amphetamine or methamphetamine (Moratalla et al., 1992; Wang and McGinty, 1995). Moreover, it has been reported that the expression of egr-1 mRNA in the striatum by methamphetamine is blocked by a NMDA receptor antagonist antagonist (±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid(CPP), suggesting the role of NMDA receptor in the expression of egr-1 mRNA in the striatum by methamphetamine (Wang and McGinty, 1996).

In this study, we investigated the regional distribution of egr-1 mRNA and Egr-1 protein in rat brain following administration of MDMA. We also examined the effects of NMDA receptor antagonist (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,b]-cyclohepten-5,10-imine ((+)-MK801), dopamine D_1 receptor antagonist SCH 23390, 5-HT uptake inhibitor paroxetine and 5-HT_{2A} receptor antagonist SR46349B on the expression of egr-1 mRNA and Egr-1 immunoreactivity by MDMA.

2. Methods

2.1. Animals

Male Sprague–Dawley rats (300–350 g) were housed under a 12 h light/12 h dark cycle with free access to food and water. All experiments were carried out in accordance with the NCNP Guide for the Care and Use of Laboratory Animals.

2.2. Drugs

The following drugs were obtained from the following sources: (+)-MK801 (dizocilpine maleate) and R(+)-SCH 23390 hydrochloride (Research Biochemicals, Natick, MA, U.S.A.), paroxetine hydrochloride (SmithKline Beecham Pharmaceuticals, Surrey, U.K.), and SR46349B (Sanofi Recherche, France). MDMA hydrochloride was synthesized in our laboratory as reported previously (Hashimoto and Goromaru, 1990, 1992). Other chemicals were purchased commercially.

2.3. In situ hybridization

In the experiment ($n = 6$), vehicle (1 ml/kg) plus vehicle (1 ml/kg), vehicle (1 ml/kg) plus MDMA (10 mg/kg), SR46349B (5 mg/kg) plus MDMA (10 mg/kg), (+)-MK801 (1 mg/kg) plus MDMA (10 mg/kg), SCH 23390 (1 mg/kg) plus MDMA (10 mg/kg), or paroxetine (5 mg/kg) plus MDMA (10 mg/kg) were administrated to rats. Vehicle, SR46349B, (+)-MK801, SCH 23390 or paroxetine were administered intraperitoneally 15 min before injection of vehicle or MDMA. Animals were narcotized with carbon dioxide 1 h after administration of MDMA, and the brains removed and frozen. Frozen coronal sections, 20-μm thick, were cut on a Bright cryostat and thaw-mounted onto silanized slides (Dako, Japan). Sections were frozen at −80°C until use. The in situ hybridization of egr-1 mRNA was performed by the method of previous report (Shirayama et al., 1999). The oligonucleotide (45 mer) used for in situ hybridization of egr-1 mRNA was 5′-CTGTGTGCAGGAGACGGGTAGGTAGAGGAGCCCGGAGAGGAGTAA-3′ complementary to

bp 1666–1710 of rat egr-1 (Milbrandt, 1987). Furthermore, control sections hybridized with a sense oligonucleotide probe showed no evidence of specific hybridization. The oligonucleotide probe was labeled at the 3′ end with [^{35}S]dATP (> 30 TBq/mmol, Amersham, UK) using oligonucleotide 3′-end labeling system (DuPont/New England Nuclear, MA, USA) and purified over NENSORB™ 20 cartridge (DuPont/New England Nuclear). The sections were fixed in 4% paraformaldehyde-0.1 M phosphate buffer (PB) for 30 min at 4°C, rinsed twice in 0.1 M phosphate buffer saline (PBS), acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0), rinsed twice in 2 × saline–sodium citrate solution (SSC, 0.15 M NaCl, 15 mM sodium citrate), dehydrated and delipidated through a graded series of ethanol and chloroform. After being air dried, the sections were hybridized overnight at 42°C with $0.5–1 \times 10^6$ cpm of the labeled probe in 100 μl of the hybridization solution (Oncor, Gaithersburg, MD, USA) including 0.1 M dithiothreitol. After hybridization, each slide was washed twice for 5 s in 1 × SSC at 55°C, four times for 15 min in 1 × SSC at 55°C, for 1 h in 1 × SSC at room temperature, and twice in deionized water for 5 min. Sections were dipped in 60%, 80%, 90%, 95%, 100% ethanol and air dried. The slides were exposed to Hyperfilm βmax (Amersham, UK) for 2 weeks before being developed. Densitometric analysis of sections was done using Macintosh-based image analysis software (NIH image 1.61). The statistical evaluation of the data was performed by a one-way analysis of variance (ANOVA), followed by the Fisher PLSD test for multiple comparison. The criterion for significance was $P < 0.01$.

2.4. Immunohistochemistry

In the experiment ($n = 4$), vehicle (1 ml/kg) plus vehicle (1 ml/kg), vehicle (1 ml/kg) plus MDMA (10 mg/kg), SR46349B (5 mg/kg) plus MDMA (10 mg/kg), (+)-MK801 (1 mg/kg) plus MDMA (10 mg/kg), SCH 23390 (1 mg/kg) plus MDMA (10 mg/kg), or paroxetine (5 mg/kg) plus MDMA (10 mg/kg) were administered

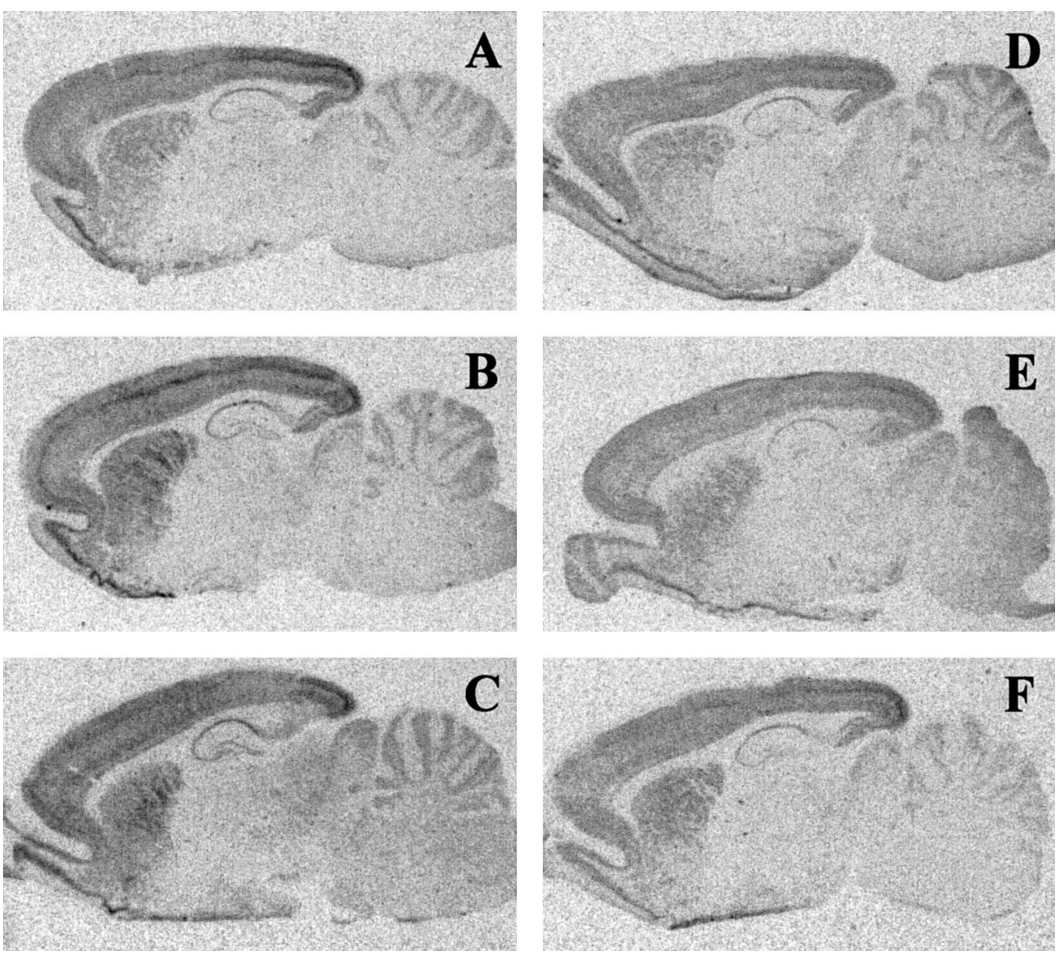

Fig. 1. The effects of pretreatment with SCH 23390, paroxetine, SR46349B or (+)-MK801 on the expression of egr-1 mRNA in the rat brain 1 h after administration of MDMA. (A) Vehicle (1 ml/kg) plus vehicle (1 ml/kg). (B) Vehicle (1 ml/kg) plus MDMA (10 mg/kg). (C) SR46349B (5 mg/kg) plus MDMA (10 mg/kg). (D) (+)-MK801 (1 mg/kg) plus MDMA (20 mg/kg). (E) SCH 23390 (1 mg/kg) plus MDMA (10 mg/kg). (F) Paroxetine (5 mg/kg) plus MDMA (10 mg/kg). Vehicle or drugs were administered i.p. 15 min before injection of MDMA.

into rats. Vehicle, SR46349B, (+)-MK801, SCH 23390 or paroxetine were administered intraperitoneally 15 min before injection of vehicle or MDMA. The rats were deeply anesthetized with pentobarbital sodium, 3 h after administration of vehicle or MDMA. The rats were then transcardially perfused with 100 ml of isotonic saline followed by 500 ml of ice-cold, 4% paraformaldehyde in 0.1 M PB (pH 7.4). Brains were removed, postfixed overnight at 4°C in the same fixative, and 100-μm thick sagittal sections (approximately lateral 2.40 mm according to the rat brain atlas of Paxinos and Watson, 1997) were cut on a Microslicer® (DTK-3000, Dosaka EM, Kyoto, Japan), and placed in ice-cold 0.01 M PBS (pH 7.4), which were then washed in ice-cold PBS. Free-floating sections were placed in 0.01 M PBS buffer containing 2% normal goat serum, 0.2% Triton X-100 and 0.1% bovine serum albumin (GS-PBST) for 1 h at room temperature. Then, sections were incubated for 36–48 h at 4°C with primary antibody (Egr-1 rabbit polyclonal antibody, Santa Cruz Biotechnology, CA, USA) diluted 1:1000 in GS-PBST. Sections were then washed twice in PBS, incubated for 1 h in second antibody (biotinylated goat anti-rabbit IgG adsorbed against rat serum)

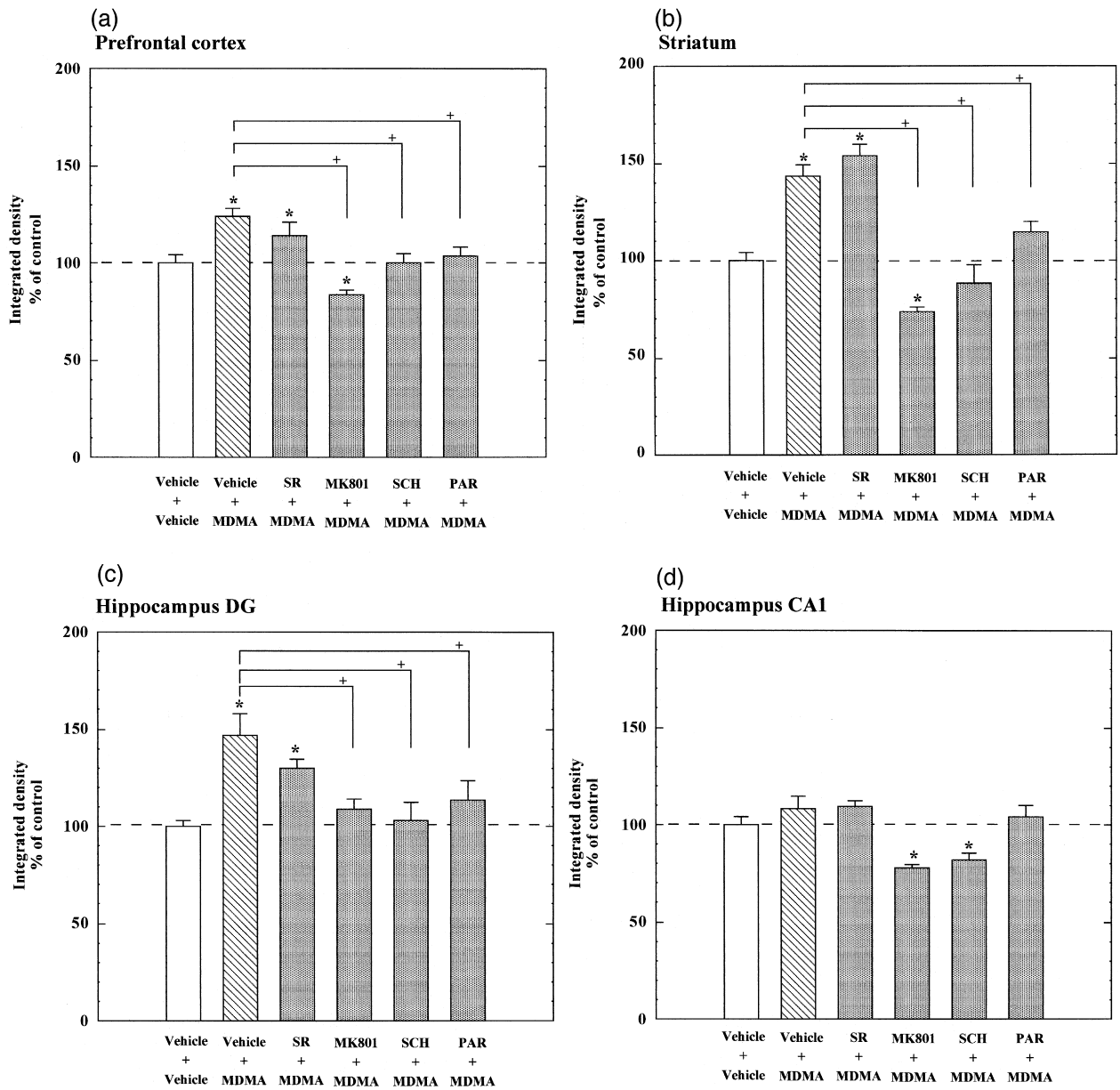

Fig. 2. The effects of pretreatment with SR46349B, (+)-MK801, SCH 23390 or paroxetine on the expression of egr-1 mRNA in the rat brain 1 h after administration of MDMA in the prefrontal cortex, striatum, hippocampus dentate gyrus (DG), hippocampus CA1 and occipital cortex. Vehicle (1 ml/kg), SR46349B (SR, 5 mg/kg), (+)-MK801 (MK801, 1 mg/kg), SCH 23390 (SCH, 1 mg/kg) or paroxetine (PAR, 5 mg/kg) were administered i.p. 15 min before injection of MDMA (10 mg/kg). The values are the mean ± SEM of six rats. $^{*}P < 0.01$ when compared to vehicle plus vehicle injected controls. $+P < 0.01$ between the two groups linked with the solid line. ANOVA followed by Fisher PLSD test.

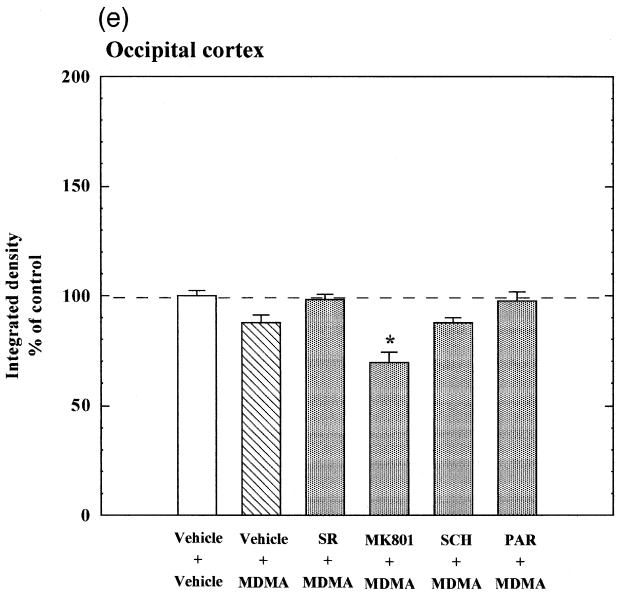

Fig. 2 (*continued*).

and incubated in the avidin–horseradish peroxidase solution prepared from the Vectastain® elite ABC kit (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Sections were washed twice in ice-cold PBS and the antibody reaction was developed with 3,3′-diaminobenzidine (0.015%) and 0.001% hydrogen peroxidase in 50 mM Tris HCl (pH 7.4). After several rinses in PBS, sections were mounted on gelatinized slides, dehydrated, and cover slipped with Permount® (Fisher Scientific, Fair Lawn, NJ, USA).

3. Results

Basal levels of egr-1 mRNA in the rat brain were detected after administration of vehicle (1 ml/kg, i.p.). A single administration of MDMA (10 mg/kg, i.p.) caused the induction of egr-1 mRNA in the prefrontal cortex, striatum and hippocampal dentate gyrus (Figs. 1 and 2). However, administration of MDMA (10 mg/kg) did not alter egr-1 mRNA levels in the CA1 region of hippocampus and occipital cortex (Figs. 1 and 2).

The increase in egr-1 mRNA in the prefrontal cortex, striatum and hippocampal dentate gyrus by administration of MDMA (10 mg/kg, i.p.) was blocked significantly by pretreatment with a non-competitive antagonist of NMDA receptor (+)-MK801 (1 mg/kg), a dopamine D_1 receptor antagonist SCH 23390 (1 mg/kg) or a 5-HT uptake inhibitor paroxetine (5 mg/kg) but not by pretreatment with a 5-HT_{2A} receptor antagonist SR46349B (5 mg/kg) (Figs. 1 and 2).

Basal constitutive levels of Egr-1 immunoreactivity in several regions of the control rat brain were detected. No changes in the Egr-1 immunoreactivity in the prefrontal cortex, striatum, CA1 region of hippocampus, dentate gyrus of hippocampus or occipital cortex of rat brain, were detected after a single administration of MDMA (10 mg/kg, i.p.) (Fig. 3).

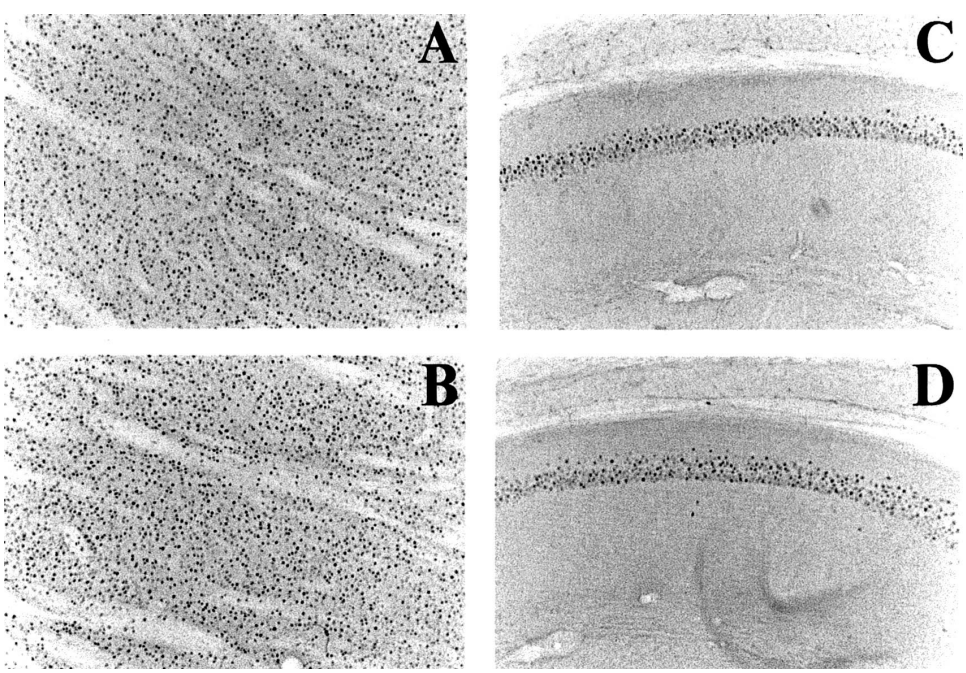

Fig. 3. The effects on the expression of Egr-1 immunoreactivity in the striatum (A, B) and CA1 region of hippocampus (C, D) 3 h after administration of MDMA. (A, C) Vehicle (1 ml/kg) plus vehicle (1 ml/kg), (B, D) vehicle (1 ml/kg) plus MDMA (10 mg/kg). Vehicle or drugs were administered i.p. 15 min before injection of MDMA.

4. Discussion

The present results suggest that administration of MDMA alters the basal levels of egr-1 mRNA in many cortical, limbic and striatal brain regions, and that the expression of egr-1 mRNA in the prefrontal cortex, striatum and hippocampal dentate gyrus by MDMA is mediated partly by NMDA receptor, dopamine D_1 receptor or 5-HT transporter.

It has been reported that antagonists for NMDA receptor or dopamine D_1 receptor block the expression of Fos protein in rat striatum by psychostimulants such as amphetamine, methamphetamine, MDMA and cocaine (Graybiel et al., 1990; Berretta et al., 1992; Hashimoto et al., 1997), suggesting roles of NMDA receptor and dopamine D_1 receptor in the expression of Fos protein in the striatum by administration of these abused drugs. It has also been reported that morphine induces c-fos and junB mRNAs in the striatum and nucleus accumbens via dopamine D_1 and NMDA receptors (Liu et al., 1994). It has also been shown that a dopamine D_1 receptor antagonist SCH 23390, blocked the amphetamine- or methamphetamine-induced expression of egr-1 mRNA in the striatum, cortex and nucleus accumbens, as well as behavioral changes such as hyperlocomotion and stereotypies, suggesting the role of dopamine D_1 receptor in the expression of egr-1 mRNA in the three region by amphetamine or methamphetamine (Moratalla et al., 1992; Wang and McGinty, 1995). Moreover, it has been reported that the expression of egr-1 mRNA in the striatum by methamphetamine is blocked by a NMDA receptor antagonist CPP, but not by a kainate/AMPA receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX), suggesting the role of NMDA receptor in the expression of egr-1 mRNA in the striatum by methamphetamine (Wang and McGinty, 1996). Our data is consistent with previous reports demonstrating that NMDA receptor and dopamine D_1 receptor play a role in the expression of egr-1 mRNA in the cortex and striatum by amphetamine or methamphetamine. Taken together, it is likely that the expression of egr-1 mRNA by MDMA is mediated, at least partly, by NMDA receptor and dopamine D_1 receptor in the prefrontal cortex, striatum and hippocampal dentate gyrus of rat brain.

The present study showed that the pretreatment with a NMDA receptor antagonist (+)-MK801, prior to MDMA, significantly reduced the expression of egr-1 mRNA below the basal levels in the prefrontal cortex, striatum, hippocampal CA1 region and occipital cortex, and that the pretreatment with a dopamine D_1 receptor antagonist SCH23390 significantly decreased the egr-1 mRNA only in the hippocampal CA1 region compared to the control (Fig. 2). Taken together with the fact that NMDA receptor mediates tonic excitation of dopamine neural transmission in the prefrontal cortex, striatum and other regions (Hata et al., 1990; Karreman et al., 1996; Wolf, 1998), it may be conceivable that tonic glutamatergic activity is essential for egr-1 mRNA activity. It has been reported previously that (+)-MK801 decreases the constitutive basal expression of egr-1 mRNA or Egr-1 protein in the rat neocortex (Worley et al., 1991; Gass et al., 1993). Therefore, the fact that (+)-MK801 inhibits MDMA-induced egr-1 mRNA below basal levels may suggest that it has no role on MDMA effects. However, the present study showed that the pretreatment with (+)-MK801 or SCH23390 did not alter the Egr-1 protein expression in the areas examined (Fig. 3). Further studies are needed to elucidate the relationships among the egr-1 mRNA, dopamine D_1 receptor and NMDA-type glutamate receptor.

It is well known that MDMA causes release of 5-HT from the nerve terminals, and that acute effects of MDMA in rats results in the decline of 5-HT content in several regions of rat brain after a single administration of MDMA (Schmidt, 1987; McKenna and Peroutka, 1990; Green et al., 1995). Furthermore, it has been shown that the acute 5-HT depleting effects of MDMA can be blocked by administration of 5-HT uptake inhibitors such as fluoxetine, paroxetine or 6-nitroquipazine, and that MDMA causes long-term neurodegeneration of serotonergic neurons on rat brain, and that the neurotoxicity of MDMA can be blocked by treatment with 5-HT uptake inhibitors (Schmidt, 1987; McKenna and Peroutka, 1990; Hashimoto and Goromaru, 1990). Thus, 5-HT transporter plays an important role in the acute and long-term neurotoxic effects of MDMA in the brain. In this study, the induction of egr-1 mRNA in the rat frontal cortex and striatum by MDMA was blocked significantly by pretreatment with paroxetine. Taken together, it is likely that 5-HT transporter may play, in part, a role in the expression of egr-1 mRNA in rat brain by MDMA. Furthermore, it is well known that NMDA receptor and dopamine D_1 receptor play a role in the expression of egr-1 mRNA in rat brain following administration of the psychostimulants such as amphetamine, methamphetamine and cocaine. The present study suggests that 5-HT transporter as well as NMDA receptor and dopamine D_1 receptor play a role in the expression of egr-1 mRNA in rat brain following administration of MDMA.

MDMA releases dopamine and 5-HT in vivo and stimulates locomotor activity. It is reported that the high selective 5-HT_{2A} receptor antagonist MDL 100,907 blocked MDMA-stimulated dopamine synthesis in vivo without affecting basal synthesis, and that the long-term deficits in 5-HT levels believed to be a consequence of MDMA-induced dopamine release were also blocked by MDL 100,907 (Schmidt et al., 1992). Furthermore, MDL 100,907 significantly reduced MDMA-stimulated locomotion without affecting basal levels of locomotion, suggesting that 5-HT released onto 5-HT_{2A} receptor contributes to MDMA-stimulated locomotion (Kehne et al., 1996). In this study, selective 5-HT_{2A} receptor antagonist SR46349B did not alter the expression of egr-1 mRNA in the brain by MDMA. Therefore, it is unlikely that 5-HT_{2A} receptor

plays a role in the expression of egr-1 mRNA in the brain by MDMA.

High basal expression of Egr-1 immunoreactivity in rat brain was found in neurons of the cerebral cortex, hippocampus, thalamus and striatum, and the basal expression of Egr-1 immunoreactivity markedly exceeds that of Fos protein, indicating a different transcriptional control of these genes in non-stimulated conditions (Herdegen et al., 1995). Furthermore, the alteration of egr-1 mRNA and Egr-1 immunoreactivity by neurotransmitter analogue stimulation, physiological mimetic or brain injury paradigms have been shown (Beckmann and Wilce, 1997). In this study, there is a difference between the expression of egr-1 mRNA and Egr-1 protein in rat brain following administration of MDMA. This difference may be explained as follows: (i) The in situ hybridization is a more sensitive technique than the immunohistochemistry. (ii) The Egr-1 protein is rapidly degraded post-translationally and therefore, cannot be detected by immunohistochemistry.

Although the exact mechanisms of regulation of egr-1 expression in response to MDMA are far from clear, the following possibilities should be taken into consideration. First, glutamate, as a major excitatory transmitter in the brain, may generally serve to maintain the excitability of dopaminergic and serotonergic neurons. Second, the interactions of glutamate-dopamine-5-HT may facilitate presynaptic release of transmitters such as dopamine and 5-HT. Third, the interactions of glutamate-dopamine-5-HT on the intracellular signaling pathways might play a role in the regulation of gene expression in response to MDMA. Further detailed studies to clarify this working hypothesis will be necessary.

In summary, these findings demonstrate that an abused drug, MDMA, induces the expression of egr-1 mRNA in the prefrontal cortex, striatum and hippocampal dentate gyrus of rat brain. Furthermore, the expression of egr-1 mRNA in the prefrontal cortex, striatum and hippocampal dentate gyrus of rat brain was blocked by a selective NMDA receptor antagonist (+)-MK801, a selective dopamine D_1 receptor antagonist SCH 23390 or a 5-HT uptake inhibitor paroxetine, but not by a selective 5-HT_{2A} receptor antagonist SR46349B. These data suggest that the expression of egr-1 mRNA by MDMA is mediated at least partly by NMDA receptor, dopamine D_1 receptor and 5-HT transporter in the prefrontal cortex, striatum and hippocampal dentate gyrus of rat brain.

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