3,4-Methylenedioxymetamphetamine (Ecstasy) Induces c-fos-Like Protein and mRNA in Rat Organotypic Dorsal Striatal Slices

DIETER S. SCHATZ,1 WALTER A. KAUFMANN,1 RUFINA SCHULIGOI,2 CHRISTIAN HUMPEL,1 AND ALOIS SARIA*1

1Division of Neurochemistry, Department of Psychiatry, University Hospital Innsbruck, Austria
2Department of Experimental and Clinical Pharmacology, Graz, Austria

KEY WORDS MDMA; ecstasy; immediate early gene; NMDA receptor; dopamine receptor

ABSTRACT 3,4-Methylenedioxymetamphetamine (MDMA, “ecstasy”) is an increasingly abused drug, which has significant effects on the dopamine system in the striatum. The isolated single organotypic slice model allows investigation of the effects of drugs of abuse on the expression of transcription factors in the striatum without dopaminergic and glutamatergic interactions. In this study the effects of MDMA on the expression of c-fos mRNA by in situ hybridization as well as the c-fos-like protein by immunohistochemistry in isolated dorsal striatum was investigated. It was shown that 100 µM MDMA induced c-fos mRNA expression 30 min after treatment. Expression of c-fos-like protein was transiently detected 3 h afterwards. The c-fos expression was inhibited by MK 801 and metoclopramide, indicating the involvement of dopaminergic D2 receptors and glutamatergic NMDA receptors. The dopaminergic D1 receptor antagonist SCH 23390 did not affect c-fos expression. We conclude that MDMA treatment leads to the induction of c-fos expression in isolated rat striatal slices. This effect is independent of extrinsic neuronal circuitry and seems to be associated with direct interactions between MDMA and the dopamine/glutamate receptor system.


INTRODUCTION

3,4-Methylenedioxymetamphetamine (MDMA, “ecstasy”) is a “designer drug” structurally related to both mescaline and amphetamine. The molecular mechanisms underlying behavioral changes after exposure to amphetamine and cocaine are thought to involve the dopaminergic system and modifications in the dopamine (DA)-containing nigrostriatal and mesolimbic systems and their neuronal targets in the striatum (Groves et al., 1987; Koob and Bloom, 1988; Ritz et al., 1987; Wise, 1984). MDMA has been shown to raise DA concentrations in neostriatum after acute administration (Schmidt et al., 1986; Stone et al., 1986; Yamamoto and Spanos, 1988). Moreover, reductions of DA levels in neostriatum were seen after repeated administration (Commins et al., 1987; Gazzara et al., 1989; Johnson et al., 1988). No alteration in the striatal tyrosine hydroxylase (TH) activity, the rate-limiting enzyme in DA biosynthesis, was found (Johnson et al., 1988; Stone et al., 1986). Furthermore, Kelland et al. (1989) reported an influence of MDMA on the basal firing activity of nigrostriatal DA neurons. These data suggest an extra-pyramidal dopaminergic influence of the drug and evidence of a direct functional effect of MDMA on the nigrostriatal DA system. Immediate early genes (IEG) act as transcription factors, which are considered important initial factors for plastic and potential cytoskeletal changes in response to drugs of abuse. Because c-fos is transiently induced by extracellular stimulation in nervous cells and tissue, c-fos induction may in general be a molecular marker for neuronal activity (Morgan and Curran, 1989; Zhang et al., 1992). Its induction has been used as a cellular marker to map neurons and circuits activated by amphetamine and cocaine. It was shown that amphetamine and cocaine cause rapid induction of c-fos in the striatum in different patterns in the striosome-matrix compartments. Both drugs are
sensitive to DA receptor blockade (Graybiel et al., 1990; Moratalla et al., 1993; Young et al., 1991). Striatal immediate early gene expression after administration of amphetamines have been reported (Cole et al., 1995; Konradi et al., 1994; Moratalla et al., 1992; Nguyen et al., 1992), which may couple receptor-mediated effects of drugs to prolonged changes in neuronal function. Dragunow et al. (1991) reported that MDMA induced c-fos and related molecules in the caudate-putamen, nucleus accumbens, and olfactory tuberde in rats in vivo (Dragunow et al., 1991), with was reversed by the noncompetitive NMDA antagonist MK-801.

The aim of this study was to investigate the induction of c-fos-like protein and c-fos mRNA in 14-day-old isolated dorsal striatal slices after exposure to MDMA. We have recently shown that this model represents an isolated system without afferent dopaminergic innervation (Schatz et al. 1999). The effects of NMDA, D1, and D2 receptor antagonists on the possible MDMA induced c-fos expression have therefore been studied in the absence of dopaminergic and glutamatergic interactions from cortical and mesencephalic inputs.

MATERIALS AND METHODS

Organotypic cultures of striatal slices

Organotypic cultures were established as described previously (Hutter et al., 1996; Stoppini et al., 1991). The dorsal caudate putamen of postnatal day 10 (P10) rats was dissected under aseptic conditions, 400-µm thick slices were cut with a tissue chopper (McIllwain), and the slices placed on a Millicel-CM 0.4 µm culture plate (Millipore, Bedford, MA, 5–6 slices per membrane). Slices were cultured in petri dishes at 37°C and 5% CO2 with 1 ml/petri dish of the following culture medium: 50% minimal essential medium/Hepes (Gibco), 25% heat inactivated horse serum (Gibco, Grand Island, NY), 25% Hank’s solution (Gibco), 2 mM NaHCO3 (Merck, Darmstadt, Germany), 6.5 mg/ml glucose (Merck), 2 mM glutamine (Merck), pH 7.2. After 14 days in culture slices were incubated with vehicle (control), or MDMA (“ecstasy,” Sigma, St. Louis, MO), (+) MK-801 hydrogen maleate (RBI, Natick, MA; Wong et al., 1986), metoclopramide hydrochloride (RBI; Jenner et al., 1978; Liu et al., 1995) and R(+) SCH 23390 hydrochloride (RBI; Billard et al., 1984; Liu et al., 1995). At this time, there was no DA present in the slices (Schatz et al. 1999). The medium was changed three times per week.

In situ hybridization

In situ hybridization was performed as described previously (Sirinathsinghji and Dunnett, 1994). Striatal slices were carefully removed from the membrane, applied onto slides (PobeOn slides, Fisher Biotech, Orangeburg, NY), and frozen on CO2-cooled glass plates. Slices were stored at -20°C until use, thawed to room temperature, air-dried, fixed for 5 min in 4% phosphate-buffered parafformaldehyde, rinsed in PBS for 2 min, dehydrated in a series of alcohol and stored in 95% ethanol at 4°C until analysis. For in situ hybridization procedure, the sections were removed from the alcohols, air-dried at room temperature, then incubated overnight at 42°C in a humidified chamber with the radiolabeled probe in hybridization buffer (Sirinathsinghji and Dunnett, 1994). The c-fos oligodeoxyribonucleotide probe (48 bases long, obtained from British Biotechnology, Oxon, UK) was labeled at the 3’ end with [35S]deoxyadenosine-5’-[(α-thio)triphosphate (1300 Ci/mmol; New England Nuclear, Vienna, Austria) and terminal deoxynucleotidyl transferase enzyme (Boehringer Mannheim, Vienna, Austria). After incubation at 37°C for 15 min, the labeled probe was purified by a spin column procedure using Sephadex G-50, its specific activity being 1.0–1.4 × 109 c.p.m./µg. After application of 100 µl of hybridization buffer with the labeled probe (3 × 109 c.p.m./µl) to each slide, the sections were covered with paraflim and incubated overnight at 42°C in a humidified chamber to enable the hybridization process to take place. The sections were then washed for 1 h with standard saline citrate at 55°C, dehydrated through a series of alcohols and dipped in Ilford K5 autoradiographic emulsion diluted 1:1 with water. After 4–5 weeks of exposure, the autoradiograms were developed and the sections counterstained with Methylene Blue to permit identification of cell nuclei. The specificity of the probe was proven by the absence of any hybridization signal when an excess of cold c-fos oligodeoxyribonucleotide was used in the hybridization process. The autoradiograms were examined with a light microscope (Axioskop, Carl Zeiss, Oberkochen, Germany) and a computerized image analysis system (MCID M4, Imaging Research, St. Catharines, Ontario, Canada).

c-fos-like immunohistochemistry

Immunohistochemistry (IHC) using the avidin-biotin technique was performed as described previously (Humpel et al., 1996; Zhang et al., 1992). Slices were fixed for 3 h at 4°C with a fixative consisting of 2% paraformaldehyde (w/v) and 15% of saturated picric acid (v/v) in 0.1 M PBS (Zamboni and DeMartino, 1967), incubated overnight in 20% sucrose/PBS, and carefully removed from the membrane. All IHC detections were performed on whole mount slices using the free-floating technique. The slices were washed three times in 0.05 M tris-buffered saline (TBS) for 5 min at room temperature and incubated with the c-fos specific antiserum (rabbit polyclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature overnight. The antiserum was diluted (1:1,000) in 0.05 M TBS containing 0.1% Triton X-100. Slices were washed three times in TBS again and incubated with secondary antirabbit biotinylated antibody (1:100, Vectastain) for 1 h at room
temperature. After washing, slices were incubated with Vectastain reagent (AB-complex) for 1 h, washed again, and the signal detected using 3,3'-diaminobenzidine (0.5 mg/ml) as a substrate. Slices were mounted on gelatin-coated glass slides, air-dried, and mounted with Entellan. To control the specificity of the immunostaining, free-floating striatal sections were incubated with c-fos antibody preadsorbed with the synthetic peptide (c-fos peptid-2, residues 4–17, human, Oncogene Science, Cambridge, MA). The preadsorption was carried out with the diluted peptide (500 µg/ml, 1:500) at 4°C overnight. Some sections were incubated without the primary antiserum to test nonspecific staining due to the secondary antibody.

Double-labeling experiments

Incubation procedures on free-floating slices using the indirect immunofluorescence method were performed as described by Marksteiner et al. (1996) with slight modifications. Specifically, slices were rinsed in 50 mM TBS, pH 7.2, containing 0.2% Triton X-100 for 30 min at room temperature. Afterwards, nonspecific binding was blocked with 10% normal swine serum (Dakopatts, Copenhagen, Denmark) in TBS for 30 min at room temperature. Slices were subsequently incubated with a mixture of rat substance P-antiserum (1:200; Seralab, Sussex, England) and rabbit c-fos antiserum (1:500) in TBS for 48 h at 4°C. Then, slices were parallel-incubated with Texas red conjugated swine antirat antibodies (1:50; Amersham, Buckinghamshire, UK) and fluorescein-conjugated donkey antirabbit antibodies (1:50; Amersham) for 3 h at room temperature in the dark. All antisera were diluted in TBS containing 0.2% Triton X-100 and 0.1% bovine serum albumin. Incubations were performed in humid chambers. The sections were embedded in Glycergel (Dakopatts, Copenhagen, Denmark) and examined with an Olympus BX60F microscope equipped for fluorescence microscopy, using a WB (BP450–480) and a WG (BP510–550) filter (Olympus, Hamburg, Germany). For the investigation and quantification of a possible substance P and c-fos colocalization, 100 substance P-like immunopositive nuclei were uniformly distributed throughout the dorsal striatum even though a patchy distribution could sometimes be detected. The incubation of slices with c-fos antibody preabsorbed overnight with a specific peptide shows the specificity of the IHC (Fig. 1C).

Time-dependent c-fos-like protein expression after incubation with MDMA

Slices were incubated with 100 µM MDMA and the c-fos-like immunoreactivity determined by IHC at various time points. The c-fos-like protein showed a transient increase 3 h after stimulation and a rapid decrease to baseline after 4 h. The maximum density of c-fos-like immunoreactivity was found 3 h after MDMA addition (353 ± 52% of control, n = 21) with a highly significant difference vs. the control (P < 0.001). No significant difference was obtained after 42 ± 15% of control, n = 9). 8 h (137 ± 27% of control, n = 16), and 24 h (77 ± 19% of control, n = 15) compared to control (100 ± 18%, n = 18, Fig. 2B).

c-fos mRNA in striatal slice cultures after incubation with MDMA

Striatal slice cultures were incubated with 100 µM MDMA for 30 min and the c-fos mRNA was determined by in situ hybridization. Silver grains were found over Methylene Blue-counterstained sections, indicating the specificity of the method. Statistical analysis showed a significant increase of c-fos mRNA in MDMA-stimulated slices (207 ± 15% of control, n = 12, P < 0.001) compared to control (100 ± 3.4% of control, n = 12, Fig. 3).

Effects of the NMDA and dopamine receptor antagonists on the MDMA-induced c-fos

Striatal slices were pretreated for 1 h with the selective noncompetitive NMDA antagonist MK-801,
Fig. 1. c-fos-like immunoreactivity in 2-week-old dorsal striatal slices. **A**: Two-week-old slices were incubated for 3 h with vehicle, (**B** and **D**, arrows in **B** indicate c-fos-like immunopositive nuclei) or with 100 µM MDMA. **C**: To test the specificity of the IHC, slices were incubated with c-fos antibody preabsorbed overnight with a specific synthetic peptide (bar = 21 µm in **A**, **B**, **C**, and 4.2 µm in **D**).
the D1 selective antagonist SCH 23390, or the D2 selective antagonist metoclopramide. Neither MK-801 (100 µM: 133 ± 35% of control, n = 17) nor metoclopramide (10 µM: 115 ± 24% of control, n = 17; 100 µM: 143 ± 25% of control, n = 19) nor SCH 23390 (1 µM: 93 ± 31% of control, n = 11) induced c-fos-like immunoreactivity above the background level (100 ± 11% of control, n = 15, Table 1). After pretreatment, slices were incubated with 100 µM MDMA for 3 h and the c-fos-like immunoreactivity was determined by IHC. The NMDA antagonist MK-801 and the D2 antagonist metoclopramide completely inhibited MDMA-induced c-fos-like immunoreactivity. The c-fos induction after pretreatment with MK-801 (100 µM: 130 ± 25% of control, n = 19, P < 0.01) and metoclopramide (10 µM: 191 ± 29% of control, n = 18, P < 0.05; 100 µM: 127 ± 16% of control, n = 16, P < 0.001) was significantly reduced compared to MDMA without pretreatment (388 ± 83% of control, n = 10). The D1 antagonist SCH 23390 did not change the MDMA response (1 µM: 361 ± 42% of control, n = 19).

**Co-localization of c-fos and substance P**

Substance P (SP)-immunostaining of nonstimulated as well as MDMA-stimulated striatal slices revealed single distributed immunoreactive perikarya (Fig. 4A) and a dense network of immunoreactive fibers, which
obscured labeled cells for the most part. Immunostaining for c-fos displayed a base-expression of c-fos-like immunoreactive nuclei in nonstimulated slices and a high density of immunoreactive nuclei, present in a clustered arrangement, in MDMA-stimulated slices (Fig. 4B). Double-labeling experiments on stimulated striatal slices revealed c-fos expression in SP-like immunoreactive cells (Fig. 4C, D). Quantification of c-fos and SP-colocalization revealed $32 \pm 6$ (n = 5) SP-positive perikarya out of 100 counted SP-ir cells per slice that exhibited c-fos immunoreactive nuclei.

**DISCUSSION**

In this study, the effect of MDMA on the expression of c-fos in isolated rat dorsal striatum was investigated. The effects of MDMA on c-fos expression could be blocked by the NMDA receptor antagonist MK-801 and DA D2 receptor antagonist metoclopramide, but not by the DA D1 receptor antagonist SCH 23390.

Up until now, a direct MDMA-induced c-fos expression within the striatum without peripheral inputs has not been addressed. The organotypic slice model used in this study has thoroughly been characterized and was proven to be an excellent model system for studying isolated brain regions in vitro. It has recently been described as a powerful model (Humpel et al., 1996; Stoppini et al., 1991) and is suitable to study the expression of transcription factors (Liu et al., 1995). Therefore, this model offers the chance to investigate neuronal activity in the isolated rat striatum after exposure to MDMA without mesostriatal and cortico-striatal circuitry, thereby distinguishing direct striatal from indirect effects.

It was recently shown that in 14-day-old isolated striatal slices the tyrosine-hydroxylase immunoreactivity was dramatically reduced (Humpel et al., 1996) and that DA levels were not detectable after 7 days or longer (Schatz et al. 1999). As the remaining tyrosine-hydroxylase staining cannot be interpreted quantitatively, and is no proof of active tyrosine hydroxylase, it may represent small fragments of inactive enzyme, since DA fell below the detection limit after 7 days in

---

**TABLE I. c-fos like immunoreactivity in dorsal striatal slices**

<table>
<thead>
<tr>
<th>Substance (µM)</th>
<th>c-Fos LI ± SE [% of CO]</th>
<th>n</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO</td>
<td>100 ± 11</td>
<td>15</td>
<td>n.s.</td>
</tr>
<tr>
<td>MK (100)</td>
<td>133 ± 35</td>
<td>17</td>
<td>n.s.</td>
</tr>
<tr>
<td>SCH (1)</td>
<td>93 ± 31</td>
<td>11</td>
<td>n.s.</td>
</tr>
<tr>
<td>ME (10)</td>
<td>115 ± 24</td>
<td>17</td>
<td>n.s.</td>
</tr>
<tr>
<td>ME (100)</td>
<td>143 ± 25</td>
<td>19</td>
<td>n.s.</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E (100)</td>
<td>388 ± 83</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>MK (100) + E</td>
<td>130 ± 25</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>SCH (1) + E</td>
<td>361 ± 42</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>ME (10) + E</td>
<td>191 ± 29</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>ME (100) + E</td>
<td>127 ± 16</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

(A) 2 week old striatal slices were incubated for 4 h with the selective noncompetitive NMDA antagonist MK-801 (MK, 100 µM), the D1 selective antagonist SCH-23390 (SCH, 1 µM) and the D2 selective antagonist metoclopramide (ME, 10 and 100 µM). (B) Slices were pre-treated for 1 h with the respective antagonists or saline and then stimulated for 3 h with 100 µM MDMA (E). Values are given in % of control. Multistatistical analysis was carried out by ANOVA and subsequent Scheffe post hoc test. n.s. = not significant; n = number of slices; *p < 0.05, **p < 0.01, ***p < 0.001.
culture (Schatz et al. 1999). Due to the high sensitivity of our assay, this represents definitely more than 99% depletion of DA and indicates the completion of the degeneration of dopaminergic axons and terminals. At this point we investigated the effect of MDMA using this isolated slice model. It could be shown that MDMA induces expression of c-fos mRNA and a transient increase of c-fos-like protein in isolated striatal slices. Our data indicate that the MDMA-induced c-fos expression appears independently of the dopaminergic input of the nigrostriatal system and independently of the glutamatergic input of the corticostriatal system. Thus, MDMA may directly induce c-fos expression in the dorsal striatum without the extrinsic neuronal circuitry and the neurotransmitters DA or glutamate.

To investigate the types of striatal cells expressing c-fos after MDMA stimulation, we performed c-fos and substance P-colocalization experiments. Recently, it was reported that cocaine induced c-fos in striatal SP neurons (Kosofsky et al., 1995). Our study shows that about one-third of all SP neurons, which mainly are GABAergic nigrostriatal projections, exhibited c-fos immunoreactive nuclei after MDMA stimulation. This suggests that c-fos expression is induced in neuronal cells and that SP neurons are one of the cell subtypes within the striatum that show MDMA-induced c-fos expression. However, other types of neurons are likely to be involved and MDMA-induced c-fos expression in glial cells cannot be excluded.

Dragunow et al. (1991) showed that MDMA enhanced c-fos-like protein in the dorsomedial and ventromedial region of the rostral striatum and in the dorsomedial region of the caudal striatum in vivo after about 3 h, being in line with our data. This group has also demonstrated that the MDMA-induced c-fos expression was reversed by the NMDA antagonist MK 801 (Dragunow et al., 1991). However, in vivo studies are not appropriate for studying isolated effects without extrinsic inputs and are not appropriate for determining whether a specific receptor stimulation leads to direct
downstream induction of c-fos expression. The isolated organotypic single slice model allows to investigate such isolated effects and direct downstream actions. Our data showed that 100 µM MDMA interacted with various receptors (Battaglia et al., 1988), which seem to be responsible for a receptor-mediated induction of c-fos gene expression. In our investigations, the expression of c-fos-like protein was inhibited by the selective NMDA antagonist MK 801 and the selective DA D2 antagonist metopramide, indicating the involvement of dopaminergic D2 receptors and glutamatergic NMDA receptors. Since the dopaminergic D1 receptor antagonist SCH 23390 did not lead to an effect on the c-fos expression, direct D1 receptor activation does not seem to be involved.

MDMA has been shown to stimulate DA release in the rat striatum (Yamamoto and Spanos, 1988). Furthermore, it was demonstrated that cocaine induced c-fos-immunoreactive proteins via activation of D1 receptors (Young et al., 1991). Liu et al. (1995) showed that DA enhanced c-fos-like protein by activating D1 receptors. The exact mechanism of c-fos induction is unclear, but our data indicate that the MDMA-induced c-fos expression is not due to a D1 receptor activation in the isolated slice model. Since c-fos expression via potentiation of dopaminergic neurotransmission and D1 receptor activation seems to be an important mechanism for cocaine and amphetamine in vivo (Graybiel et al., 1990; Young et al., 1991), a similar indirect effect of MDMA in vivo in addition to our findings seems likely. However, in vivo effects independent of the presence of dopamine cannot be investigated. Since the isolated single slice model lacks DA (Humpel et al., 1996), the MDMA-induced c-fos expression is not due to DA release and our findings point to additional mechanisms of MDMA. Also, direct interactions within the striatum must be considered when explaining the interaction of MDMA with the dopamine/glutamate systems leading to a c-fos expression. It remains to be established if and to what extent DA or glutamate are involved in the MDMA-induced c-fos expression in vivo.

Besides DA D1 receptors, DA D2 receptors also seem to be involved in the cocaine-induced c-fos expression in the striatum (Graybiel et al., 1990). Activation of DA D2 receptors following inhibition of cAMP formation is generally involved in drug reinforcement behavior (Self and Nestler, 1995). The role of D2 receptors in the c-fos induction is unclear. Criswell et al. (1990) proposed a functional link between NMDA receptors and dopaminergic D1 receptors. Furthermore, Konradi et al. (1996) demonstrated the requirement of enhanced Ca2+ entry via NMDA receptors for DA-mediated induction of immediate early genes in dissociated striatal cultures. Ca2+ entry via NMDA seems to be a general crucial factor in the DA receptor-mediated c-fos expression. An interaction between NMDA receptors and dopaminergic D2 receptors seems possible. However, it is not clear if the activation of D2 and NMDA receptors are independent pathways or interdependent.

In conclusion, our results show that direct effects of MDMA within the striatum must be considered when looking for mechanisms of action. MDMA induces c-fos in isolated rat striatum via involvement of the activation of glutamatergic NMDA and dopaminergic D2 receptors. The presence of afferent inputs such as dopamine or glutamate does not seem to be necessary for the induction of c-fos. Our findings point to a direct receptor-mediated influence of MDMA in gene transcription in the striatum, which may underlie long-term neuronal plasticity and behavioral changes.

ACKNOWLEDGMENTS

We thank Iris Berger and Astrid Saria for excellent technical assistance.

REFERENCES


Gazzara RA, Takeda H, Cho AK, Howard SG. 1989. Inhibition of...


