Importance of ERK activation in behavioral and biochemical effects induced by MDMA in mice

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1   Little is known about the cellular effects induced by 3,4-methylenedioxymethamphetamine (MDMA, ecstasy), although changes in gene expression have been observed following treatments with other psychostimulants. Thus, the aim of this study was to investigate in mice, the relationships between the ras-dependent protein kinase ERK and MDMA-induced reinforcement using the conditioned place preference (CPP) and locomotor activity measurements. This was completed using real-time quantitative PCR method by a study of immediate early-genes (IEGs) transcription known to be involved in neuronal plasticity.

2   A significant CPP was observed after repeated MDMA treatment in CD-1 mice at a dose of $9 \, \mathrm{mg \, kg^{-1}}$ i.p. but not at 3 and $6 \, \mathrm{mg \, kg^{-1}}$. This rewarding effect was abolished by the selective inhibitor of ERK activation, SL327 ($50 \, \mathrm{mg \, kg^{-1}}$; i.p.). Similar results were obtained on MDMA-induced locomotor activity, clearly suggesting a role of ERK pathway in these behavioral responses.

3   Following acute i.p. injection, MDMA induced a strong *c-fos* transcription in brain structures, such as caudate putamen, nucleus accumbens and hippocampus, whereas *egr-1* and *egr-3* transcripts were only increased in the caudate putamen. MDMA-induced IEGs transcription was selectively suppressed by SL327 in the caudate putamen, suggesting a role for other signaling pathways in regulation of IEGs transcription in the other brain structures. In agreement with these results, MDMA-induced c-fos protein expression was blocked by SL327 in the caudate putamen.

4   This study confirms and extends to mice the reported role of ERK pathway in the development of addiction-like properties of MDMA. This could facilitate studies about the molecular mechanism of this process by using mutant mice.

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Abbreviations: CPP, conditioned place preference; IEG, immediate-early genes; MDMA, 3,4-methylenedioxymethamphetamine

Introduction

The rewarding properties of cocaine and various psycho-stimulants including 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) are related to their ability to enhance the release of dopamine (DA) in the nucleus accumbens (see review in Di Chiara, 1995). Moreover, MDMA was also shown to induce an increase in 5-hydroxytryptamine (5-HT) in the same structure. DA and serotonin (5-HT) interact with G protein-coupled receptors (GPCRs) expressed in different brain structures, including the mesolimbic pathway and hippocampus, which are involved in drug reinforcement and learning, respectively. Addiction is generally believed to result from molecular and cellular adaptations in specific brain neurons caused by repeated exposure to a drug of abuse (see review in Nestler, 2001). One of the present challenges is to identify and characterize the cellular targets accounting for the persistent aspects of addiction. This occurs by drug-induced stimulation of the receptors triggering alterations in intra-cellular biochemical processes and changes in gene expression. Thus, stimulation of D_1 DA receptor by psychostimulants led

to an increase in cAMP production through activation of the adenylyl cyclase by the α_s subunit of the G protein, with subsequent activation of the protein kinase A (Lovenberg *et al.*, 1991). This latter enzyme induces a phosphorylation of the transcription factor CREB, which under this activated form was shown to trigger the expression of early genes such as *c-fos* and *egr* family.

On the other hand, several studies have shown the existence of a link between the signaling pathway involving GPCR and the MAPK cascade, through $\beta\gamma$ subunits of the G-protein, leading to a ras-dependent cascade of phosphorylation (Lowes *et al.*, 2002). A key component of this cascade is the protein kinase ERK, which is activated by another protein kinase MEK. In the case of cocaine, inhibition of this phosphoryla-tion process in mice reversed both the rewarding effects and the enhanced c-fos expression evoked by the drug (Valjent *et al.*, 2000). The level of c-fos expression was also shown to be increased following acute MDMA administration in rats (Dragunow *et al.*, 1991; Erdtmann-Vourliotis *et al.*, 1999, 2000; Stephenson *et al.*, 1999), but no data are available in mice. Thus, owing to the usefulness of mice for gene manipulations and the recent description of the entire mouse

genome (Blake *et al.*, 2003), it was of interest to investigate whether MDMA is able to induce a conditioned place preference in mice, and if the behavioral effects of the drug are modified following ERK inhibition, using a specific inhibitor of ERK kinase (MEK), SL327 (Selcher *et al.*, 1999). Moreover, we analyzed the levels of *c-fos*, *egr-1* and *egr-3* transcription in discrete brain structures involved in drug dependence after injection of MDMA in mice, and we evaluated the role of ERK pathway in these effects.

The inhibition of ERK phosphorylation by SL327 completely abolished the place preference induced by MDMA showing the involvement of the ras-dependent signaling cascade in the observed reinforcement process. SL327 was also able to inhibit the acute effect of MDMA, by reversing the hyperlocomotor activity induced by this compound. Several early genes such as *c-fos*, *egr-1* and *egr-3* were found overexpressed by MDMA in particular structures, not necessarily identical. This overexpression was selectively inhibited by SL327 in the caudate putamen, suggesting that the neuronal plasticity induced by MDMA in this structure is probably controlled by ERK signaling pathway.

Methods

Animals and drugs

Male CD-1 mice (Iffa Credo, France) weighing 22–24 g were housed in a room with 12 h alternating ligth/dark cycle and controlled temperature ($21 \pm 1^\circ$C). Food and water were available *ad libitum*. All drugs were injected intraperitoneally (i.p.). D,L-MDMA (Lipomed, Switzerland) was dissolved in saline 0.9%. The MEK inhibitor SL327, a generous gift of Bristol-Myers Squibb (Wilmington), was dissolved in 100% DMSO, as previously described in behavioral studies (Selcher *et al.*, 1999), and injected 1 h before MDMA treatment. Volumes of injection were 0.1 and 0.02 ml per 10 g of body weight for MDMA and SL327, respectively. In preliminary studies, it has been verified that injection of DMSO 1 h before MDMA did not modify any effect as compared to MDMA alone in behavioral and biochemical studies.

The animals were treated in accordance with the *NIH Guidelines for the Care and Use of Laboratory Animals* (1985) and in agreement with the local ethical committee.

Place preference paradigm

A previously described unbiased place conditioning method was used (Valverde *et al.*, 2001). The place preference apparatus consisted of two conditioning compartments ($15 \times 15 \times 15$ cm^3) that were distinguished by different patterns on floors and walls, separated from each other by a neutral area. The movement and location of mice were recorded by computerized monitoring software (Videotrack, Viewpoint, Lyon, France). Briefly, the protocol consisted of three phases.

(1) *Preconditioning phase*. Drug-naive mice had free access to both compartments for 20 min, and the time spent in each compartment was recorded.

(2) *Conditioning phase*. This phase consisted of 6 days where each conditioning chamber was closed. In experiment A (MDMA induced place-preference), on the first conditioning day, mice ($n = 9$–10/group) were treated with MDMA (1, 3 or 9 mg kg^{-1}; i.p.), and were placed 20 min after injection in one of the conditioning environments individually for 20 min. The following conditioning day, mice were given saline in the opposite compartment and this sequence alternated during the next 6 days. Control animals received saline every day. In experiment B (effect of SL327 pretreatment on MDMA induced place-preference), on days 1, 3 and 5, according to their group, animals ($n = 7$–8/group) received SL327 (50 mg kg^{-1}; i.p.) or DMSO 1 h before MDMA (9 mg kg^{-1}; i.p.) or saline injection, and 20 min after, were placed in one of the conditioning environments individually for 20 min. All mice received DMSO/saline on days 2, 4 and 6.

(3) *Postconditioning phase*. This phase took place 24 h after the final conditioning session and was carried out exactly as the preconditioning phase.

Results are expressed in scores (mean $\pm$ s.e.m.) calculated as the difference between postconditioning and preconditioning time spent in the drug-paired compartment.

Locomotor activity

Locomotion was evaluated in transparent activity boxes ($10 \times 18 \times 14$ cm^3; Imetronic, Bordeaux, France). Displacements were measured by photocell beams located across the long axis, 20 mm above the floor (horizontal activity). Naive mice, not prehabituated to the activity boxes ($n = 10$–12/group), received MDMA (9 mg kg^{-1}; i.p.) or saline injection 1 h after SL327 (50 mg kg^{-1}; i.p.) or DMSO, and their locomotor activity was immediately recorded for 120 min. Locomotor activity was expressed in scores (mean $\pm$ s.e.m.) as the total number of interruption of the photocell beams, recorded every 15 min.

Drug treatment for RT–PCR analysis

Mice were injected with SL327 (50 mg kg^{-1}; i.p.) or DMSO, followed 1 h later by MDMA treatment (9 mg kg^{-1}; i.p.) or saline. At 1 h after the last injection, mice were killed by cervical dislocation, brain was removed and the following structures were dissected: caudate putamen, nucleus accumbens and hippocampus. Structures were frozen in isopentane at -50°C, and stored at -80°C until RNA extraction.

RNA extraction and real-time quantitative RT–PCR

Total RNA were extracted from the different brain structures by a modified acid-phenol guanidinum method (RNABle$^\circledR$, Eurobio). Each RNA sample was prepared with tissues pooled from five mice, which received the same treatment. The quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide. Reverse transcription of RNA was performed in first-strand buffer (Invitrogen, France), 500 μM of each dNTP, 20 U of Rnasin ribonuclease inhibitor (Promega, France), 10 mM dithiothreitol, 100 U Superscript II Rnase H$^-$ reverse transcriptase (Invitrogen, France), 0.15 μg μl^{-1} random hexanucleotide primers (Amersham Biosciences, France) and 1 μg of total RNA. The samples were incubated at 25°C for 10 min,

42°C for 30 min and 99°C for 5 min. PCR reactions were performed on a Light-Cycler® instrument (Roche Diagnostics) using the LC-FastStart DNA Master SYBR Green I kit (Roche Diagnostics). The PCR reactions were performed with 3–4 samples/drug treatment, in duplicate for each sample. Quantification was made on the basis of a calibration curve using total RNA from mouse brain. In addition to *c-fos*, *egr-1* and *egr-3* transcripts, the *TBP* mRNA (a component of the DNA binding complex TFIID) was quantified and each sample was normalized on the basis of its *TBP* content. The results are expressed as gene of interest transcript/*TBP* transcript.

Immunohistochemistry

Mice ($n = 5$–6/group) were injected with SL327 (50 mg kg^{-1}; i.p.) or DMSO, followed 1 h later by MDMA treatment (9 mg kg^{-1}; i.p.) or saline. At 2 h after the last injection, mice were deeply anesthetized by intraperitoneal injection of pentobarbital (Sanofi, France) prior to intracardiac perfusion of 4% paraformaldehyde in 0.1 M Na$_2$HPO$_4$/NaH$_2$PO$_4$ buffer, pH 7.5 (PB), delivered with a peristaltic pump at 20 ml min^{-1} over 5 min. The brains were then removed, postfixed for 4 h in the same fixative solution, and cryoprotected overnight in 30% sucrose in PB. Sections (60 μm) were cut with a microtome and kept in a solution containing 30% ethylene glycol, 30% glycerol in PBS, pH 7.4 at -20°C until they were processed for immunochemistry. Free-floating sections were rinsed in PB, incubated for 30 min at room temperature in a blocking solution of 3% normal goat serum in PB with 0.3% Triton X-100, and were then incubated overnight at 4°C in the primary antiserum directed against the c-fos protein (Tebu, sc-52, diluted at 1 : 6000). The sections were washed three times in 1% normal goat serum in PB with 0.3% Triton X-100 (NGST) and incubated in biotinylated goat anti-rabbit immunoglobulin G for 1 h at room temperature, then washed twice in NGST and incubated for 1 h in avidin–biotin–peroxidase complex (Vectastain, Vector Laboratories, Burlingame). Finally, the sections were washed three times in PB and developed using VIP chromogen (Vector Laboratories, Burlingame). After processing, the tissue sections were mounted on gelatin-coated slides, air-dried, dehydrated through a graded alcohol series, xylene treated and cover-slipped for light-microscopic examination. c-fos-positive neurons were counted at ×63 magnification using a computerized image analyser (NIH image software). Cell counts were performed for each mouse in the caudate putamen from bregma $+0.26$ to $+1.54$ mm (four to five sections per mouse), according to The Mouse Brain Paxinos and Franklin Atlas (Academic Press, 2nd edn, 2001). These counts were used to calculate an average number of c-fos-positive cells per section in the caudate putamen region.

Statistical analysis

Data were analyzed using one-way ANOVA between subjects for score values of conditioned place preference, locomotor activity, quantitative RT–PCR and immunohistochemistry. *Post hoc* comparisons were made using the Fisher–PLSD test. Effects of treatments on locomotor activity counts were analyzed by two-way ANOVA for repeated measures. The level of significance was set at $P < 0.05$.

Results

Effect of MDMA in the place preference paradigm in mice

Comparison of preconditioning times spent in the drug-paired compartment did not show any significant difference between the groups ($F_{3,33} = 1.301$; $P = 0.2905$), indicating the unbiased characteristics of the experimental design (Table 1a). One-way ANOVA ($F_{3,33} = 2.921$; $P = 0.0485$) revealed that repeated administration of MDMA induced a conditioned place preference (CPP). *Post hoc* comparisons ($P = 0.0072$) showed a significant effect of MDMA at the highest dose used (9 mg kg^{-1}), while no significant effect was observed with the doses of 1 and 3 mg kg^{-1} (Figure 1a).

Role of ERK activity on rewarding properties of MDMA

The selective MEK inhibitor SL327 (50 mg kg^{-1}; i.p.) was administered 1 h before MDMA (9 mg kg^{-1}; i.p.). Comparison of preconditioning times spent in the drug-paired compartment did not show any significant difference between the groups ($F_{3,26} = 0.009$; $P = 0.9989$), indicating the unbiased nature of the protocol (Table 1b) One-way ANOVA ($F_{3,26} = 3.188$; $P = 0.0403$) revealed a significant treatment effect between the four groups of animals treated during the conditioning phase with DMSO + Sal, DMSO + MDMA, SL327 + Sal or SL327 + MDMA. *Post hoc* comparisons showed a significant effect of MDMA vs control group; and interestingly, pretreatment with SL327 during the conditioning phase completely abolished the place preference induced by MDMA ($P = 0.0089$). No effect was observed after administration of SL327 alone (Figure 1b).

Role of ERK activity on MDMA-induced locomotor activity

Two-way ANOVA for repeated measures showed a significant treatment effect ($F_{3,39} = 11.901$; $P < 0.0001$), time effect

Table 1 Time spent in drug-associated compartment during the preconditioning and the testing phase

	Preconditioning (s)	Testing (s)
(a) *MDMA-induced place preference*[a]		
Saline	369 ± 16	367 ± 32
MDMA 1 mg kg^{-1}	365 ± 10	386 ± 21
MDMA 3 mg kg^{-1}	360 ± 15	388 ± 20
MDMA 9 mg kg^{-1}	335 ± 5	416 ± 17[##]
(b) *Effect of SL327 pretreatment on MDMA-induced place preference*[b]		
DMSO/saline	331 ± 21	347 ± 25
SL327/saline	330 ± 14	369 ± 28
DMSO/MDMA	331 ± 13	418 ± 20[#]
SL327/MDMA	327 ± 12	331 ± 14[**]

[a]Values are mean $\pm$ s.e.m. from nine or 10 independent mice per group. [##]$P < 0.01$ as compared to saline-treated group (Fisher–PLSD). [b]Values are mean $\pm$ s.e.m. from seven or eight independent mice per group. [#]$P < 0.05$ as compared to control group; [**]$P < 0.01$ as compared to MDMA-treated group (Fisher–PLSD). Statistical analysis was performed on the scores data, calculated as the difference between testing and preconditioning times spent in drug-associated compartment.
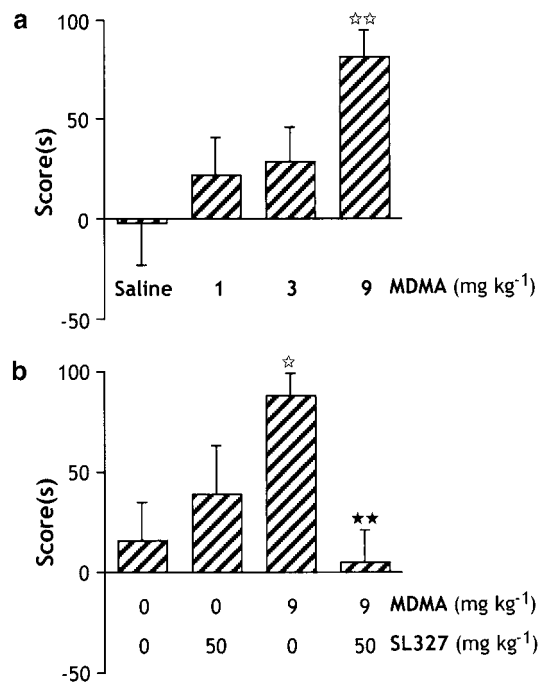
Figure 1 (a) Effects of MDMA in the place preference paradigm. The mice received MDMA (1, 3 and 9 mg kg^{-1}; i.p.) or saline (i.p.) 20 min before being confined in the associated compartment (see methods for details). Data are expressed in scores calculated as the difference between postconditioning and preconditioning time spent in the compartment associated with the drug (means $\pm$ s.e.m.). $\star\star P < 0.01$ as compared to saline-treated group (Fisher–PLSD). (b) Effect of pretreatment with the MEK inhibitor, SL 327 (50 mg kg^{-1}; i.p.) on MDMA (9 mg kg^{-1}; i.p.)-induced CPP. Data are expressed in scores calculated as the difference between postconditioning and preconditioning time spent in the compartment associated with the drug (means $\pm$ s.e.m.). $\star P < 0.05$ as compared to control group; $\star\star P < 0.01$ as compared to MDMA-treated group (Fisher–PLSD).

Figure 2 Effects of SL327 on spontaneous motor activity and MDMA-induced hyperlocomotion. Mice were i.p. injected with SL 327 (50 mg kg^{-1}; 0,02 ml 10 g^{-1} of body weight) or vehicle (DMSO, 0,02 ml 10 g^{-1} of body weight) followed 1 h later by MDMA (9 mg kg^{-1}; i.p.) or saline. The motor activity was monitored each 15 min for 120 min and started immediately after MDMA administration. Data represent means $\pm$ s.e.m. of cumulative photobeam disruptions ($n = 10–12$ mice). $\star P < 0.05$ and $\star\star P < 0.01$ vs DMSO/saline group. $\ast\ast P < 0.01$ and $\ast\ast\ast P < 0.001$ vs DMSO/MDMA group. $^{\#}P < 0.05$ vs DMSO/saline group (Fisher–PLSD).

($F_{7,273} = 132.78$; $P < 0.0001$) and time $\times$ treatment interaction ($F_{2,273} = 13.030$; $P < 0.0001$). Administration of MDMA (9 mg kg^{-1}) increased locomotor activity compared to saline-treated animals. This hyperactivity was antagonized by preinjection of SL327 (50 mg kg^{-1}) 1 h before MDMA treatment (after 120 min: $P = 0.0002$). Interestingly, SL327 pretreatment at 50 mg kg^{-1} significantly decreased spontaneous locomotor activity of mice at the beginning of the test (Figure 2).

Role of ERK activation on MDMA-induced IEG transcription

The effects of SL327 pretreatment on MDMA-induced *c-fos* transcription were analyzed by real-time quantitative RT–PCR (Figure 3). One-way ANOVA revealed significant differences of *c-fos* transcript level in the brain structures selected: caudate putamen ($F_{3,12} = 60.534$; $P < 0.0001$), nucleus accumbens ($F_{3,11} = 16.521$; $P = 0.0002$) and hippocampus ($F_{3,12} = 9.807$; $P = 0.0015$). *Post hoc* comparisons (Fisher–PLSD) showed a significant increase of *c-fos* transcription 1 h after MDMA administration (9 mg kg^{-1}) in these different regions: eight-fold ($P < 0.0001$), three-fold ($P = 0.0004$) and 2.5-fold ($P = 0.0013$). Moreover, as shown in Figure 3, SL327 (50 mg kg^{-1}) injected 1 h before MDMA partially decreased
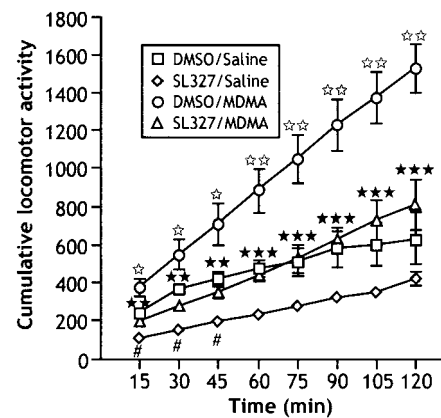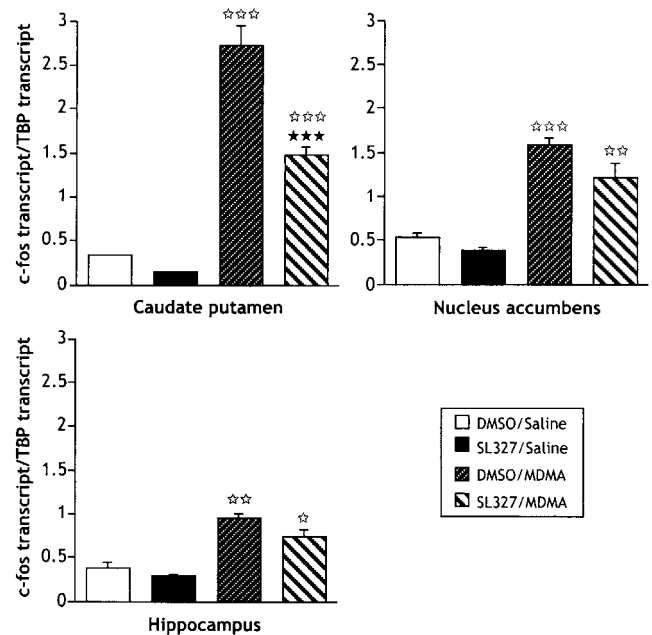

Figure 3 Real-time quantitative RT–PCR of *c-fos* transcript after acute treatment with MDMA (9 mg kg^{-1}; i.p.) $\pm$ SL327 (50 mg kg^{-1}; i.p.). SL327 was administered 1 h before MDMA, and brains were rapidly removed and the caudate putamen, nucleus accumbens and hippocampus were dissected 1 h after administration of MDMA (see Methods for details). Data represent means $\pm$ s.e.m. of *c-fos*/*TBP* fluorescence ratio ($n = 3–4$ samples/group; each sample is composed of structures pooled from five mice). $\star P < 0.05$, $\star\star P < 0.01$ and $\star\star\star P < 0.001$ vs DMSO/saline group. $\ast\ast\ast P < 0.001$ vs DMSO/MDMA group (Fisher–PLSD).

($P < 0.0001$), and did not modify MDMA-induced *c-fos* transcription in caudate putamen and hippocampus, respectively . Although not significant, the P-value (0.0761) indicates a trend to the decrease of MDMA-induced *c-fos* transcription

by SL327 administration in the nucleus accumbens. SL327 alone did not modify the levels of *c-fos* transcription as compared to control group in all the structures studied.

The effect of MDMA on *egr-1* transcription was also investigated by real-time quantitative RT–PCR (Figure 4). A significant increase (1.8-fold) was only observed in the caudate putamen, as revealed by one-way ANOVA ($F_{3,12} = 20.780$; $P < 0.0001$) and *post hoc* comparison ($P = 0.0006$), while no significant differences in *egr-1* transcription after MDMA injection were determined in the nucleus accumbens or hippocampus. In the caudate putamen, administration of SL327 1 h before MDMA completely abolished MDMA-induced *egr-1* transcription ($P < 0.0001$). However, SL327 pretreatment also reduced basal level of *egr-1* transcript ($P < 0.05$).

The profile of MDMA-induced *egr-3* transcription was very similar to *egr-1*, as shown in Figure 5. A slight but significant increase (1.2-fold) was only observed in the caudate putamen ($F_{3,12} = 4.386$; $P = 0.0265$ as revealed by one-way ANOVA; *post hoc* comparison: $P = 0.0221$), which was abolished by SL327 pretreatment ($P = 0.039$). MDMA did not modify the basal level of *egr-3* transcription in the nucleus accumbens and hippocampus.

Effect of SL327 on MDMA-induced c-fos protein expression in the caudate putamen

MDMA (9 mg kg^{-1}) produced a three-fold induction of c-fos-positive neurons in the caudate putamen 2 h after injection, as revealed by immunohistochemistry (Figure 6; $P = 0.0232$).
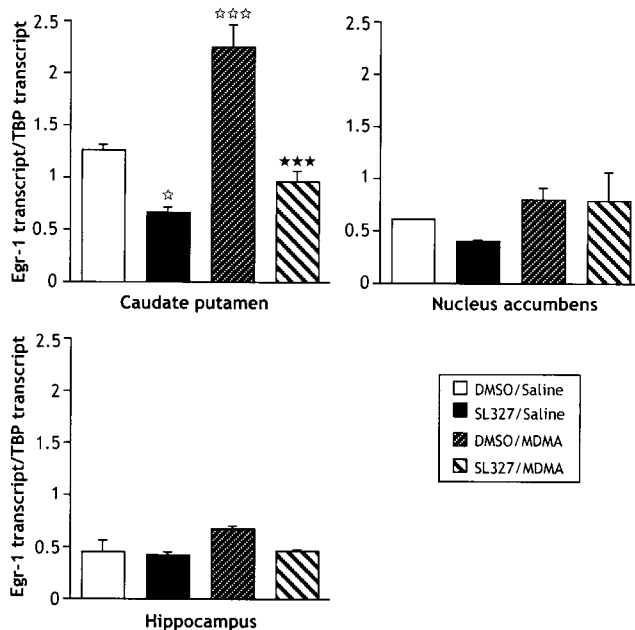

Figure 4 Real-time quantitative RT–PCR of *egr-1* transcript after acute treatment with MDMA (9 mg kg^{-1}; i.p.) $\pm$ SL327 (50 mg kg^{-1}; i.p.). SL327 was administered 1 h before MDMA, and brains were rapidly removed and the caudate putamen, nucleus accumbens and hippocampus were dissected 1 h after administration of MDMA (see Methods for details). Data represent means $\pm$ s.e.m. of *egr-1/TBP* fluorescence ratio ($n = 3-4$ samples/group; each sample is composed of structures pooled from five mice). ☆$P < 0.05$ and ☆☆☆$P < 0.001$ vs DMSO/saline group. ***$P < 0.001$ vs DMSO/MDMA group (Fisher–PLSD).
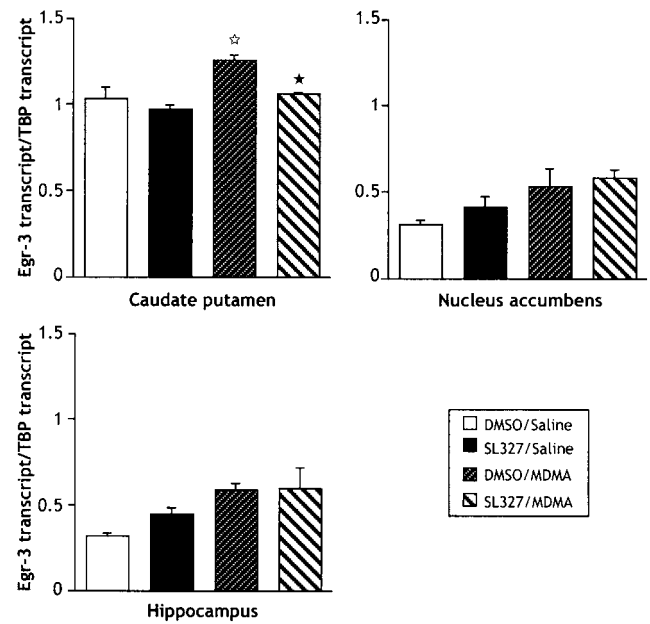

Figure 5 Real-time quantitative RT–PCR of *egr-3* transcript after acute treatment with MDMA (9 mg kg^{-1}; i.p.) $\pm$ SL327 (50 mg kg^{-1}; i.p.). SL 327 was administered 1 h before MDMA, and brains were rapidly removed and the caudate putamen, nucleus accumbens and hippocampus were dissected 1 h after administration of MDMA (see Methods for details). Data represent means $\pm$ s.e.m. of *egr-3/TBP* fluorescence ratio ($n = 3-4$ samples/group; each sample is composed of structures pooled from five mice). ☆$P < 0.05$ vs DMSO/saline group. *$P < 0.05$ vs DMSO/MDMA group (Fisher–PLSD).

This c-fos induction followed a specific pattern, with immunopositive cells predominantly in the dorso-medial region of the caudal caudate-putamen and in the rostral caudate putamen. Pretreatment with SL327 (50 mg kg^{-1}) 1 h before MDMA injection completely abolished this increase ($P = 0.0054$).

Discussion

MDMA has been studied in rats, but to date no data are available on rewarding effects of this substituted amphetamine in mice and on its mechanims of action. However, studies in these animals appear very interesting as spontaneous mutants or mice with genetic modifications, including null mutant mice (knockout) represent very useful animal models to investigate the molecular mechanisms involved in MDMA effects.

This work reports for the first time that MDMA is able to generate a CPP in mice at a dose of 9 mg kg^{-1}. This is consistent with the dose used in rats to produce a reliable CPP (Bilsky & Reid, 1991). Moreover, the effect observed following repeated administration of MDMA in the CPP reflects the reinforcing properties of MDMA, which was reported to induce feeling of euphoria and well-being in humans (Teter & Guthrie, 2001). This effect is very likely due to the effects of MDMA on the release and reuptake of different monoamines in the mesolimbic pathway. Thus, administration of MDMA results in increased synaptic levels of both 5-HT and DA in the rat nucleus accumbens (White *et al.*, 1996; Kankaanpaa *et al.*, 1998). DA increase, mainly in the core of the nucleus accumbens, has been shown to play a critical role in reward
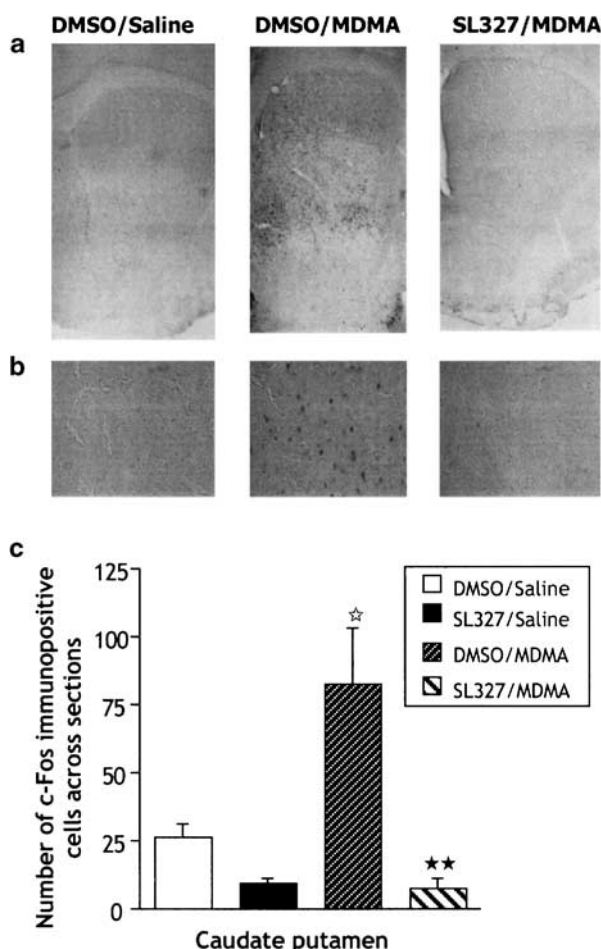
Figure 6 Immunohistochemistry of c-fos 2 h after acute treatment with MDMA (9 mg kg^{-1}; i.p.)$\pm$SL327 (50 mg kg^{-1}; i.p.). SL327 was administered 1 h before MDMA and mice were perfused 2 h after the last injection (see Methods for details). (a) Representative example showing c-fos immunoreactivity in the whole striatum. (b) High magnification of caudate putamen region ($\times$400) showing c-fos-positive nuclei. (c) Data represent means$\pm$s.e.m. of c-fos immunopositive neurons ($n=5-6$/group). $\dstar P<0.05$ vs DMSO/saline group. ** $P<0.01$ vs DMSO/MDMA group (Fisher–PLSD).

and drug dependence and is a common response generated by all drugs of abuse (see reviews in Koob, 1992; Di Chiara, 1995).

The initial event leading to addiction involves the action of the drug on its target(s) protein(s) and on neurons that express these proteins, followed by a repercussion on the intracellular signaling. The best established molecular adaptation to chronic drug exposure is an upregulation of the cAMP pathway (Terwilliger *et al.*, 1991; Ortiz *et al.*, 1995; Schoffelmeer *et al.*, 1996; Unterwald *et al.*, 1996). However, this phenomenon is just one of a large number of changes at the molecular and cellular levels that have been documented after chronic drug administration, involving, for instance, activation of MAP kinases or changes in protein kinase C activity (Narita *et al.*, 1994). We have focused our attention on the ERK pathway, as once activated ERK can phosphorylate and activate other protein kinases as well as an array of effector proteins, which include proteins and transcription factors (Seger & Krebs, 1995). Thus, ERK is an important regulator of neuronal functions, and has been shown to be implicated in

various neurobiological events such as synaptic plasticity and memory (see review in Sweatt, 2001). In this study, we used the selective MEK inhibitor SL327, which has no significant effect on a variety of other kinases such as PKC, CAMKII or PKA (Atkins *et al.*, 1998; Selcher *et al.*, 1999). This inhibitor totally abolished the expression of MDMA-induced CPP, demonstrating that the ERK pathway is involved in MDMA-induced rewarding effects. This suggests that ERK may be a common pathway to different drugs of abuse, leading to addictive state, as ERK has also been directly related to the development of THC- and cocaine-rewarding properties *in vivo* (Valjent *et al.*, 2000; 2001).

To evaluate the role of ERK pathway in MDMA-induced acute behavioral effect, we have also investigated the effect of SL327 on MDMA-induced hyperlocomotion. SL327 alone was able to induce a short-lasting significant decrease of locomotor activity in mice. At the moment, we have no clear explanation for this effect occurring more specifically during the exploration of the activity boxes. This behavior might be expected to engage dopaminergic systems, known to activate the ERK signaling pathway. In rats, a trend toward a decrease in locomotor activity measured in an open-field has already been observed following SL327 injection (Atkins *et al.*, 1998). The most important result obtained in this experiment is the demonstration that ERK pathway seems to be involved in the acute behavioral effect of MDMA, as SL327 completely reversed the hyperlocomotion induced by a single administration of this amphetamine derivative. This suggests that psychostimulant drugs, belonging to different chemical series, may have several common mechanisms of action as illustrated by the partial inhibition of cocaine-induced hyperlocomotor effect by SL327 (Valjent *et al.*, 2000). MDMA-induced hyperactivity is very likely mediated by both DA, acting on D_1 receptor, and by 5-HT, as this substituted amphetamine is known to act by increasing the extracellular levels of these neurotransmitters (see above). Moreover, it has been demonstrated that $5-HT_{1B}$ and $5-HT_{2A}$ receptors play a role in the MDMA-induced locomotor activity (Kehne *et al.*, 1996; Scearce-Levie *et al.*, 1999). Thus, activation of these receptors by MDMA-induced endogenous 5-HT release may lead to ERK activation, as this was associated to a coupling to different second messengers, including ERK pathway (Pullarkat *et al.*, 1998; Watts *et al.*, 2001). Other systems may also play a role in the effects observed following MDMA administration, as this compound can directly interact with relatively high affinities to α_2-adrenoreceptors, M-1 muscarinic and H-1 histamine receptors (Battaglia *et al.*, 1988), all possibly activating ERK pathways (Berkeley *et al.*, 2001).

This leads to the phosphorylation of important proteins, effectors and transcription factors, and results in a variety of responses. Thus, for instance, both Elk-1 and CREB transcription factors, critically involved in immediate-early genes (IEG) mRNA induction, may be phosphorylated by ERK (Seger & Krebs, 1995). IEG have certainly a key role for persistent drug-induced plasticity, as regulation of gene expression is one mechanism that should lead to relatively stable changes within neurons. As previously mentioned, a prime nuclear target of activated ERK is the ternary complex factor Elk-1, which acts as a transcriptional activator *via* the serum-response element (SRE) of various IEGs, including *c-fos*. In mice, using for the first time, the real-time quantitative PCR method, we observed a strong induction

by MDMA of *c-fos* transcription in three brain structures: nucleus accumbens, caudate putamen and hippocampus. This is in agreement with previous studies performed in rats, showing, by *in situ* hybridization or immunohistochemistry, an increase in *c-fos* transcription and expression in the medial prefrontal cortex, caudate putamen and nucleus accumbens following acute MDMA treatment (Erdtmann-Vourliotis *et al.*, 1999; Stephenson *et al.*, 1999). One of the most important result was the inhibition of MDMA-induced *c-fos* transcription when mice were pretreated with SL327, which clearly appears to be tissue-specific. Thus, the MEK inhibitor partially blocked MDMA-induced *c-fos* expression in the caudate putamen, while no inhibition was observed in the nucleus accumbens or the hippocampus. This suggests that MDMA-induced *c-fos* transcription can be mediated by different signaling pathways, one ERK-dependent and the other one -independent. Two DNA regulatory elements, at least, are crucial for the transcriptional regulation of c-fos: the cAMP/calcium-responsive element (CRE) and the SRE. This latter site is essentially controlled by phosphorylation of Elk-1 by ERK proteins, while activation of the CRE site is dependent on CREB phosphorylation, which may occur following activation of different kinases, PKA, CAMKs or ERK (Herdegen & Leah, 1998). However, as revealed by immunohistochemistry, c-fos increase was totally blocked by SL327 pretreatment, indicating that ERK inhibition is suffisant to suppress its induction at the protein level.

Other IEGs may be regulated by CRE and/or SRE sites, such as *egr-1* or *egr-3*, which code for transcription factors that have become popular neurobiological tools for imaging functional activity, and Egr-1 or Egr-3 proteins may be involved in neuronal plasticity (O'Donovan *et al.*, 1999). The present study showed that MDMA is also able to induce *egr-1* and *egr-3* transcription in the caudate putamen of mice, consistent with the data obtained in rat following acute administration of MDMA or cocaine, respectively (Shirayama *et al.*, 2000; Jouvert *et al.*, 2002). However, the changes noted in *egr-3* transcripts were small and may not be biologically significant at the protein level. In the caudate putamen, pretreatment with the MEK inhibitor abolished MDMA-induced *egr-1* or *egr-3* transcription. It is interesting to observe that ERK inhibition also reduced basal *egr-1* transcription. This could result from the fact that *egr-1* mRNA is constitutively expressed at high level in several brain structures (Herdegen *et al.*, 1995). However, the absence of significant difference between SL327/MDMA and SL327/saline groups clearly shows that MDMA-mediated *egr-1* transcription is dependent on ERK activation.

All these findings demonstrate that the abused drug MDMA induces a rapid and transient expression of several IEGs in mice. However, these genes can be activated by a wide range of stimuli (Ali *et al.*, 1999; Thiriet *et al.*, 2001; Nakao *et al.*, 2002). Thus, the challenge in the next few years will be to identify downstream effectors, which have their expression levels modulated by these IEGs, and are specifically related to MDMA's action. However, it is interesting to observe that activation of ERK through phosphorylation, and Egr-1 protein are implicated in numerous forms of neuronal plasticity, including long-term synaptic changes, such as those occurring during long-term potentiation (Atkins *et al.*, 1998; Davis *et al.*, 2000; Williams *et al.*, 2000), which appears to play a key role in the development of drug addiction (Davis *et al.*, 2000; Ungless *et al.*, 2001). Thus, the identification of the molecular events leading to ERK activation by MDMA will be of great interest.

In summary, these data show for the first time the presence of a rewarding effect and the induction of IEGs in specific brain structures by MDMA in mice. Moreover, this study clearly reveals the major role of ERK in the behavioral responses induced by this substituted amphetamine. Finally, the ERK pathway appears differentially implicated in MDMA-induced IEG transcription depending on the brain structure and the IEG studied, showing therefore the complex MDMA action at the intracellular level.

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