

# Regulations of Methamphetamine Reward by Extracellular Signal-Regulated Kinase 1/2/ets-Like Gene-1 Signaling Pathway via the Activation of Dopamine Receptors

Hiroyuki Mizoguchi, Kiyofumi Yamada, Makoto Mizuno, Tomoko Mizuno, Atsumi Nitta, Yukihiro Noda, and Toshitaka Nabeshima

*Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya, Japan (H.M., K.Y., M.M., T.M., A.N., Y.N., T.N.); and Laboratory of Neuropsychopharmacology, Department of Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa, Japan (K.Y.)*

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## ABSTRACT

Little is known about molecular mechanisms for long-lasting neuroadaptation related to the rewarding effects of methamphetamine (MAP). In the present study, we examined the intracellular signaling that is associated with the expression of conditioned place preference (CPP) induced by MAP in rats. Rats were given MAP or saline (control group) for conditioning to the CPP test. MAP-treated and control animals were killed immediately after the CPP test [CPP<sup>+</sup>]. Some of the MAP-treated rats were killed without the CPP test [CPP<sup>-</sup>]. Hyperphosphorylation of mitogen-activated protein kinase (MAPK) ERK1/2, but not p38 and c-Jun N-terminal kinase/stress-activated protein kinase, was found in the nucleus accumbens (NAc) and striatum but not in other brain areas of MAP-treated CPP<sup>+</sup> animals. No such phosphorylation was seen in control and MAP-treated CPP<sup>-</sup> animals. Moreover, the transcription factor ets-like gene-1 (Elk-1), but not cAMP response element-binding pro-

tein, also showed a similar hyperphosphorylation in the same regions of MAP-treated CPP<sup>+</sup>. Tyrosine kinase receptors, including tyrosine kinase B, were not activated in any brain regions examined in all groups. Both the dopamine D1 receptor antagonist *R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SCH23390) and the D2 receptor antagonist raclopride inhibited the expression of CPP as well as the activation of ERK1/2 in MAP-treated CPP<sup>+</sup> animals, when they were injected before the CPP test. The microinjection of 2'-amino-3'-methoxyflavone (PD98059), a selective MAPK kinase inhibitor, into the NAc before the test, abolished the MAP-induced ERK1/2 activation and decreased the expression of MAP-induced CPP. These results suggest the importance of the ERK1/2 signaling pathway through activation of dopamine D1 and D2 receptors in the expression of CPP induced by MAP.

The mesolimbic dopaminergic projection to the nucleus accumbens (NAc) or striatum is thought to mediate the reinforcing effects of drugs of abuse through activation of dopamine receptors on NAc or striatal neurons (Koob, 1992; Self et al., 1998). Dopamine signals are mediated by two major classes of dopamine receptors, termed D1 and D2 re-

ceptors, that are distinguishable by their structural heterogeneity and action on the cAMP/protein kinase A (PKA) system (Silbley et al., 1993; Zanassi et al., 2001). Despite these opposing actions on cellular signaling via cAMP/PKA, previous studies have found that both dopamine D1 and D2 receptors can mediate reinforcing signals of abuse, because amphetamine-induced conditioned place preference (CPP) is blocked by either D1 or D2 receptor antagonists (Hiroi and White, 1991) and selective dopamine D1 and D2 receptor agonists were self-administered by rats (Self et al., 1996).

Psychostimulants act to enhance memory consolidation in general and facilitate the learning of specific behaviors un-

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**ABBREVIATIONS:** NAc, nucleus accumbens; MAP, methamphetamine; CPP, conditioned place preference; CREB, cAMP response element-binding protein; Elk-1, ets-like gene-1; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; NMDA, *N*-methyl-D-aspartate; PKA, protein kinase A; MEK, MAPK kinase; St, striatum; VTA, ventral tegmental area; Trk, tyrosine kinase; JNK, c-Jun N-terminal kinase/stress-activated protein kinase; BDNF, brain-derived neurotrophic factor; DARPP-32, dopamine and cAMP-regulated phosphoprotein of *M*<sub>r</sub> 32,000; PD98059, 2'-amino-3'-methoxyflavone; SCH23390, *R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; CGS19755, [(±)-2-carboxypiperidin-4-yl]methyl]-phosphonic acid; SL327, (Z)- & (E)-α-(Amino-((4-aminophenyl)thio)methyl)-2-(trifluoromethyl)benzeneacetonitrile.

related to drug intake. For example, systemic injections of amphetamine after training can enhance the learning of discrimination or avoidance takes (Berke and Hyman, 2000). Thus, the learning/memory mechanisms are considered to overlap with and be involved in the development of drug dependence that occurs on chronic administration of drugs of abuse (Berke and Hyman, 2000; Ammassari-Teule, 2001). Several lines of evidence suggest an important role for the intracellular signal transduction pathways in the mechanism of neural plasticity in response to drugs of abuse (Nestler, 2001). One of these signal transduction pathways is the extracellular signal-regulated kinase 1/2 (ERK1/2) cascade, a member of the mitogen-activated protein kinase (MAPK) family. ERK1/2 activation can phosphorylate tyrosine hydroxylase and stimulate dopamine synthesis in the brain (Lindgren et al., 2002). After activation, ERK1/2 proteins are translocated to the nucleus, resulting in phosphorylation and activation of transcription factors such as cAMP response element-binding protein (CREB) and Elk-1. These nuclear events would initiate cell-specific gene expression programs necessary for synaptic remodeling and long-term changes in synaptic efficacy. Recent evidence has demonstrated that the ERK signaling pathway is involved in the sensitization induced by cocaine (Valjent et al., 2000) and that ERK1 mutant mice have enhanced behavioral responses to the rewarding properties of morphine (Mazzucchelli et al., 2002), indicating that ERK may be involved in the response to drugs of abuse. However, still very little is known about the intracellular mechanisms leading to synaptic plasticity in reinforcing effects of drugs of abuse.

In the present study, we investigated intracellular signaling mechanisms that are associated with the expression of the MAP-induced CPP response in rats. The CPP response is a behavior that is developed by the association of reinforcing effects of drugs with the context in which animals have previously obtained positive reinforcing effects. Thus, it is considered that an understanding of the cellular signaling associated with the expression of MAP-induced CPP will provide insights into the mechanism of long-lasting neuroadaptation related to MAP dependence and drug-seeking behavior.

## Materials and Methods

**Animals.** Male Wistar rats (8 weeks old; Charles River Japan, Yokohama, Japan) weighing  $300 \pm 20$  g at the beginning of experiments were used in the study. They were housed three per cage with ad libitum access to food and water under controlled laboratory conditions (a 12-h light/dark cycle with lights on at 9:00 A.M.,  $23 \pm 0.5^\circ\text{C}$ ,  $50 \pm 0.5\%$  humidity). All experiments were performed in accordance with the Guidelines for Animal Experiments of the Nagoya University School of Medicine, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Drug Treatment.** A specific dopamine D1 receptor antagonist, *R*-(+)-SCH23390 (Sigma-Aldrich, St. Louis, MO), at 0.03 and 0.1 mg/kg; dopamine D2 receptor antagonist, *S*-(-)-raclopride (Sigma-Aldrich), at 0.3 and 1 mg/kg; and competitive NMDA receptor antagonist CGS19755 (Novartis, Basel, Switzerland), at 0.3 and 1 mg/kg, respectively, were intraperitoneally injected 30 min before the CPP test. For the microinjection of the specific MAPK kinase (MEK) inhibitor PD98059 (Sigma-Aldrich) into the NAc, rats were anesthetized with pentobarbital (50 mg/kg i.p.) and placed in a stereotaxic

apparatus. It is reported that PD98059 at 2  $\mu\text{g}$  specifically inhibits MAPK phosphorylation, but not the phosphorylation of stress-activated protein kinase isoforms, related MAPK family members, by inhibiting MEK, and the second-messenger activities of calcium/calmodulin-dependent protein kinase II, PKA, and protein kinase C are unchanged by the infusion of PD98059 at 2  $\mu\text{g}/\text{side}$  into the hippocampus, indicating that the dosage of drug used selectively inhibits the MAPK cascade (Blum et al., 1999). PD98059 has no significant effect on MAP kinase itself (Dudley et al., 1995). In addition, 2  $\mu\text{g}/\text{side}$  of PD98059 infused into the hippocampus resulted in an equilibrium concentration of  $\sim 37.5 \mu\text{M}$ , although the concentration was likely to be higher immediately surrounding the infusion site (Blum et al., 1999). A guide cannula ( $0.4 \times 0.5$  mm in diameter; Eicom, Kyoto, Japan) was implanted bilaterally into the NAc ( $+1.2$  mm anterior to bregma,  $\pm 1.9$  mm lateral, and  $-7.0$  mm for NAc ventral to dura), according to the atlas of Paxinos and Watson (1982). A dummy cannula (0.3 mm in diameter; Eicom) cut to extend 1.0 mm beyond the guide cannula was left in place throughout the experiment. PD98059 (2  $\mu\text{g}/\text{side}$ ) or vehicle (60% dimethyl sulfoxide-saline) was injected bilaterally through a 28 gauge injection cannula (Eicom) in a volume of 1.5  $\mu\text{l}/\text{side}$  over a 4-min period, 20 min before the CPP test in the NAc of the rats.

**Conditioned Place Preference (CPP).** The apparatus used for the place conditioning task consisted of two compartments: a black Plexiglas box and a transparent Plexiglas box (both  $27 \times 22 \times 26$  cm high) with a metal grid floor. To enable the rat to distinguish easily the transparent box from the black one, the floors of the transparent and black boxes were covered with white plastic mesh and with black frosting Plexiglas, respectively. Each box could be divided by a sliding door ( $10 \times 26$  cm high).

The place conditioning paradigm was performed according to the method of Kitaichi et al. (1996), with a minor modification. In the preconditioning test, the sliding door was opened and the rat was allowed to move freely between both boxes for 15 min once a day for 3 days. On the third day of the preconditioning test, we measured the time that the rat spent in the black and transparent boxes by using Scanet SV-10 LD (Melquest, Toyama, Japan). The box in which the rat spent the most time was referred to as the "preferred side," and the other box as the "nonpreferred side."

Conditioning was performed during 6 successive days. Rats were given drugs or vehicle in the apparatus with the sliding door closed. That is, a rat was subcutaneously given saline or MAP at 2 mg/kg and put in its nonpreferred side for 30 min. The next day, the rat was given saline and placed opposite the drug conditioning site for 30 min. These treatments were repeated for three cycles (6 days). In the postconditioning test, the sliding door was opened, and we measured the time that the rat spent in the black and transparent boxes for 15 min, using the Scanet SV-10 LD.

Place conditioning behaviors were expressed by Post-Pre, which was calculated as: [(postvalue) - (prevalue)], where post- and pre-values were the difference in time spent in the drug conditioning and the saline conditioning sites in the postconditioning and preconditioning tests, respectively.

Animals were killed by rapid decapitation as described previously (Berhow et al., 1996; Atkins et al., 1998; Cammarota et al., 2000). Saline-treated animals received saline during the 6 days of conditioning phase (control). MAP-treated animals were injected with MAP three times during the conditioning phase, and they were divided into CPP<sup>+</sup> and CPP<sup>-</sup> groups. CPP<sup>+</sup> and control animals were killed immediately after the CPP test, whereas CPP<sup>-</sup> rats were killed without the CPP test on the postconditioning day. Various brain regions including frontal cortex, NAc, striatum (St), hippocampus, ventral tegmental area (VTA), and amygdala were dissected out from control, CPP<sup>+</sup>, and CPP<sup>-</sup> animals and immediately frozen and stored at  $-80^\circ\text{C}$  until assayed.

**Western Blotting and Immunoprecipitation.** Brain tissues were homogenized in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM sodium orthovanadate, 2 mM EDTA, 50 mM NaF, 0.1% SDS, 1%

Nonidet P-40, 1% sodium deoxycholate, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml pepstatin, 10  $\mu$ g/ml aprotinin, and 10  $\mu$ g/ml leupeptin, pH 7.4), and microwaved for 15 s according to the protocol for immunoblotting with monoclonal antibodies. The homogenate was centrifuged at 10,000g for 10 min to pellet insoluble material. The protein concentration in the supernatant was determined using a Protein Assay Rapid Kit (Wako Pure Chemicals, Osaka, Japan). The sample was boiled in a sample buffer [0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol, and 20% 2 $\times$  TBE (90 mM Tris, 64.6 mM boric acid, and 2.5 mM EDTA, pH 8.4)] and electrophoresed by SDS-polyacrylamide gel electrophoresis on a 4.75% stacking gel and 10% separating gel, and then transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA). The same concentration (20 or 50  $\mu$ g) of protein per lane was located in all Western blotting. The membrane was incubated in the blocking solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for 2 h at room temperature and then incubated with primary antibodies. After washing, blots were incubated with the secondary antibodies. Immunoreactive materials on the membrane were detected using the ECL Western blotting detection reagents (Amersham Biosciences Inc., Piscataway, NJ) and exposed to X-ray film. The band intensities of the film were analyzed by densitometry. To calculate the amount of phosphorylated form versus total protein, the same membranes were stripped with a stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7) at 50°C for 20 min, incubated with primary antibodies for total protein, and detected as described above.

For phosphorylation analysis of TrkB, protein A Sepharose (Amersham Biosciences Inc.) was incubated with monoclonal anti-TrkB antibody for 6 h and then with each lysate (0.5 mg of protein) overnight. The immunoprecipitate was boiled in Laemmli sample buffer, separated on a 7.5% polyacrylamide gel, and subsequently transferred to a polyvinylidene difluoride membrane. The membranes were blocked and probed with anti-phosphotyrosine antibody (1:1000; Upstate Biotechnology, Lake Placid, NY), and detected as described above. To confirm equal loading of each protein, membranes were stripped with the stripping buffer, incubated with anti-TrkB antibody, and detected as described above. For the quantification of protein phosphorylation, the mean values in the control group were converted to 100%, and then individual data including those of control groups, were recalculated as percentages of the mean values. All the data in Western blotting are expressed as a percentage of the control.

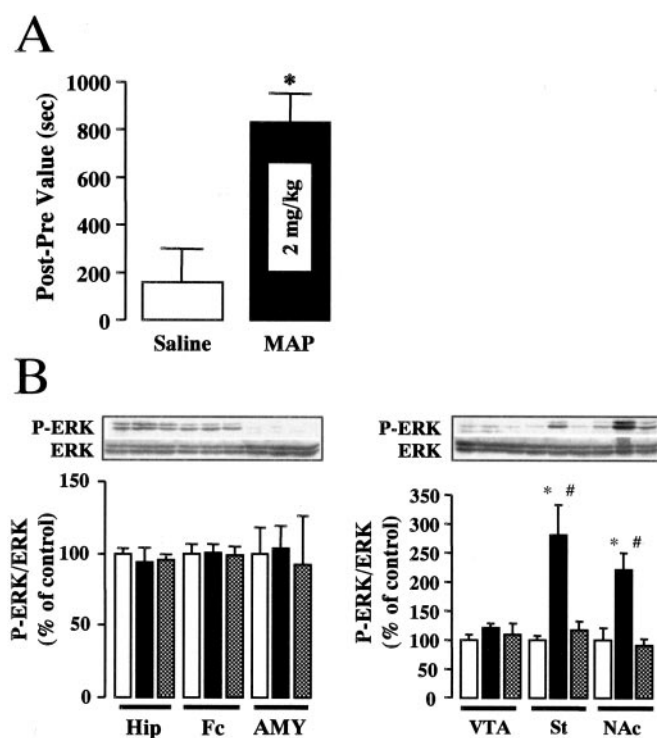
The primary monoclonal mouse antibodies used in the present study were anti-P-ERK (1:1000; Cell Signaling Technology Inc., Beverly, MA), P-Elk-1 (1:2000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), P-Pan-Trk (1:1000; Santa Cruz Biotechnology, Inc.), Pan-Trk (1:1000; Santa Cruz Biotechnology, Inc.), P-JNK (c-Jun N-terminal kinase/stress-activated protein kinase) (1:1000; Santa Cruz Biotechnology, Inc.), P-p38 (1:1000; Santa Cruz Biotechnology, Inc.), and CREB (1:1000; Santa Cruz Biotechnology, Inc.). The primary polyclonal mouse or rabbit antibodies were anti-Elk-1 (1:1000; Santa Cruz Biotechnology, Inc.), JNK (1:1000; Santa Cruz Biotechnology, Inc.), and P-CREB (1:1000; Santa Cruz Biotechnology, Inc.). The primary polyclonal rabbit antibodies were anti-p38 (1:1000; Santa Cruz Biotechnology, Inc.), TrkB (1:1000; Santa Cruz Biotechnology, Inc.), and ERK (1:2000; Upstate Biotechnology). The secondary antibodies, used at a 1:2000 or 1:5000 dilution, were horseradish peroxidase-linked anti-mouse or anti-rabbit IgG (Kirkegaard and Perry Laboratories).

**Statistical Analyses.** Results are expressed as the mean  $\pm$  S.E. The significance of differences was determined by one-way analysis of variance, followed by the Student-Newman-Keuls test for multi-group comparisons. Student's *t* test was used for two-group comparisons in Fig. 1A.

## Results

**Hyperphosphorylation of ERK1/2 Was Observed Specifically in the NAc and St after the Expression of MAP-Induced CPP.** MAP (2 mg/kg)-treated rats spent significantly more time in the drug-paired compartment than did control rats ( $P < 0.05$  by *t* test), indicating rewarding effects of MAP (Fig. 1A). To examine the intracellular signaling associated with the expression of MAP-induced CPP, MAP-treated rats were killed immediately after the CPP test, and the phosphorylation of various signaling molecules was examined by Western blotting. Hyperphosphorylation of ERK1/2 was found specifically in the NAc [ $F(2,18) = 7.33$ ;  $P < 0.005$ , and  $P < 0.05$  by post hoc] and St [ $F(2,21) = 11.5$ ;  $P < 0.001$ , and  $P < 0.05$  by post hoc], but not in other brain areas, of MAP-treated CPP<sup>+</sup> animals, compared with control and CPP<sup>-</sup> animals ( $P < 0.0001$ , and  $P < 0.05$  by post hoc). Importantly, no such phosphorylation was seen in the control and the MAP-treated CPP<sup>-</sup> animals (Fig. 1B). Accordingly, it is suggested that activation of ERK1/2 in the NAc and St is associated with the exposure of MAP-treated rats to the environment in which they had previously received the drug treatment, but not with the drug treatment itself.

**No Changes in the Phosphorylation of Other MAPKs, JNK, and p38 on MAP-Induced CPP.** Three members of the MAP kinase family have been identified: ERK, JNK, and p38, which are activated by stress stimuli. In this study, no changes in phosphorylated levels of JNK and p38 MAPK were observed in any brain areas in MAP-treated CPP<sup>+</sup> and



**Fig. 1.** A, MAP-induced CPP in rats; B, ERK1/2 activation associated with the expression of MAP-induced CPP. Rats were given MAP (2 mg/kg) or saline for the conditioning to the CPP test and were killed immediately after the test. Open column, saline-treated CPP<sup>+</sup> rats; closed column, MAP-treated CPP<sup>+</sup> rats; hatched column, MAP-treated CPP<sup>-</sup> rats. Data are presented as the mean  $\pm$  S.E. ( $n = 10\sim12$  for A;  $n = 7\sim8$  for B). \*,  $P < 0.05$  vs. saline-treated CPP<sup>+</sup> rats; #,  $P < 0.05$  vs. MAP-treated CPP<sup>-</sup> rats.



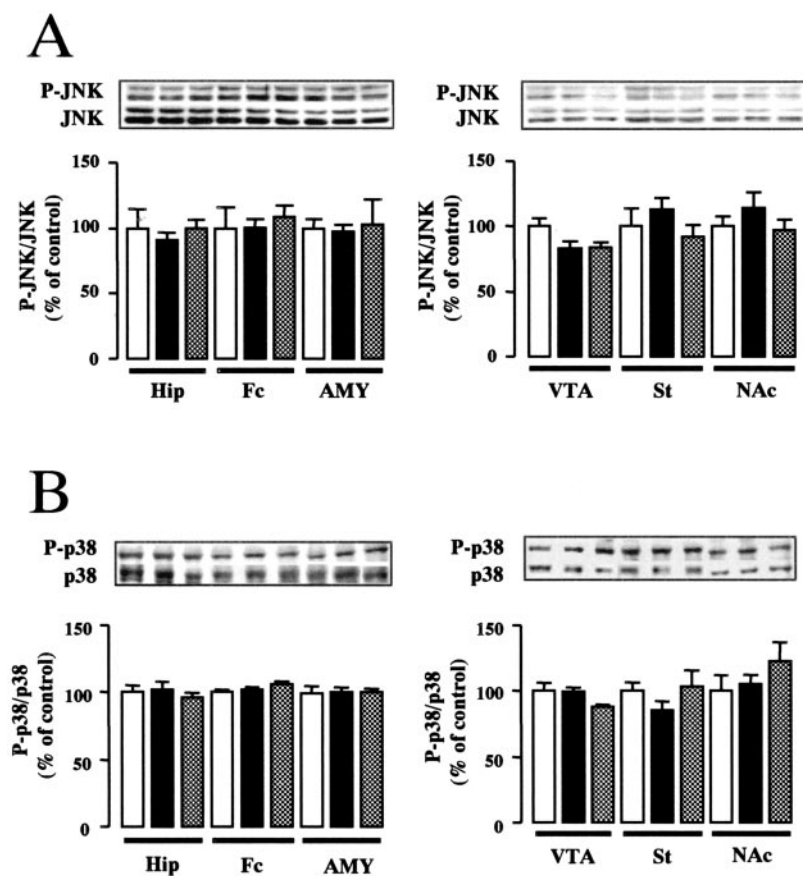
CPP<sup>-</sup> animals (Fig. 2). These results suggest that the three MAPKs are specifically and differentially activated by exposure to the context associated with the rewarding effects of MAP, and that the ERK1/2, but not JNK and p38 MAPK, phosphorylation may be related to drug-seeking/relapse behavior.

**No Changes in the Phosphorylation of Tyrosine Kinase Receptors, Including TrkB, on MAP-Induced CPP.** To examine whether Trk receptors are upstream of the hyperphosphorylation of ERK1/2 evoked by MAP-induced CPP, we investigated Trk receptor phosphorylation. Phosphorylated Trk receptors were detected with P-Pan-Trk antibodies, which recognize the phosphorylated form of TrkA, TrkB, and TrkC. Phosphorylated TrkB levels were also measured by immunoprecipitation with TrkB antibodies followed by Western blotting with anti-phosphotyrosine antibodies. There were no changes in phosphorylated levels of Pan-Trk and TrkB in any brain areas of MAP-treated CPP<sup>+</sup> and CPP<sup>-</sup> animals (Fig. 3).

**Hyperphosphorylation of Elk-1 Is Evoked after the Expression of MAP-Induced CPP.** Transcription factors such as CREB and Elk-1 are the nuclear targets of ERK1/2, and their activation by ERK1/2 is observed in various model systems (Davis et al., 2000). For instance, activation of ERK1/2 and Elk-1 has been reported in cocaine responses (Valjent et al., 2000). As illustrated in Fig. 4A, hyperphosphorylation of Elk-1 was specifically found in the NAc of MAP-treated CPP<sup>+</sup> animals without any changes in the St [ $F(2,12) = 8.37$ ;  $P < 0.01$  compared with saline-treated animals or MAP-treated CPP<sup>-</sup>, and  $P < 0.05$  by post hoc com-

parison]. No such phosphorylation of CREB was seen in either the NAc or St of MAP-treated CPP<sup>-</sup> animals (Fig. 4B).

**Involvement of Dopamine Receptors in MAP-Induced CPP and Activation of ERK1/2.** We then evaluated the involvement of dopamine receptors in the expression of MAP-induced CPP and the ERK activation evoked by MAP-induced CPP in the NAc and striatum. Both SCH23390 (dopamine D1 receptor antagonist) and raclopride (dopamine D2 receptor antagonist) dose dependently abolished the expression of MAP-induced CPP without affecting the behavior of control animals [Fig. 5B,  $F(3,51) = 4.68$ ,  $P < 0.05$ , and  $P < 0.05$  by post hoc comparison; Fig. 6A,  $F(3,42) = 3.99$ ,  $P < 0.05$ , and  $P < 0.05$  by post hoc comparison]. We also examined the involvement of NMDA receptors in the expression of MAP-induced CPP. CGS19755 at 1 mg/kg, which inhibits NMDA receptor function (Mori et al., 2001), failed to affect the expression of MAP-induced CPP (Fig. 5A), suggesting that the activation of NMDA receptors may not be critical to the expression of MAP-induced CPP. To further analyze the role of ERK1/2 activation in the expression of CPP, the effects of dopamine receptor antagonists on ERK1/2 phosphorylation were measured. The blockade of dopamine D1 receptors by administration of SCH23390 (0.1 mg/kg) resulted in the inhibition of ERK1/2 activation evoked by MAP-induced CPP in both NAc and St [Fig. 5C;  $F(3,19) = 4.60$ ,  $P < 0.05$ , and  $P < 0.05$  by post hoc comparison in the NAc, and  $F(3,21) = 12.5$ ,  $P < 0.0001$ , and  $P < 0.05$  by post hoc comparison in the St), whereas the treatment had no effect on the phosphorylation of ERK1/2 in control animals. These results suggest that dopamine D1 receptors are activated when MAP-treated an-



**Fig. 2.** No changes in JNK (A) and p38 (B) phosphorylation associated with the expression of MAP-induced CPP. Rats were given MAP (2 mg/kg) or saline for conditioning and were killed immediately after the test. Open column, saline-treated CPP<sup>+</sup> rats; closed column, MAP-treated CPP<sup>+</sup> rats; hatched column, MAP-treated CPP<sup>-</sup> rats. Data are presented as the mean  $\pm$  S.E. ( $n = 5$ ).

imals are exposed to the context in which they had previously received MAP, and that the activation is crucial for the expression of CPP and activation of ERK1/2 in the NAc and St.

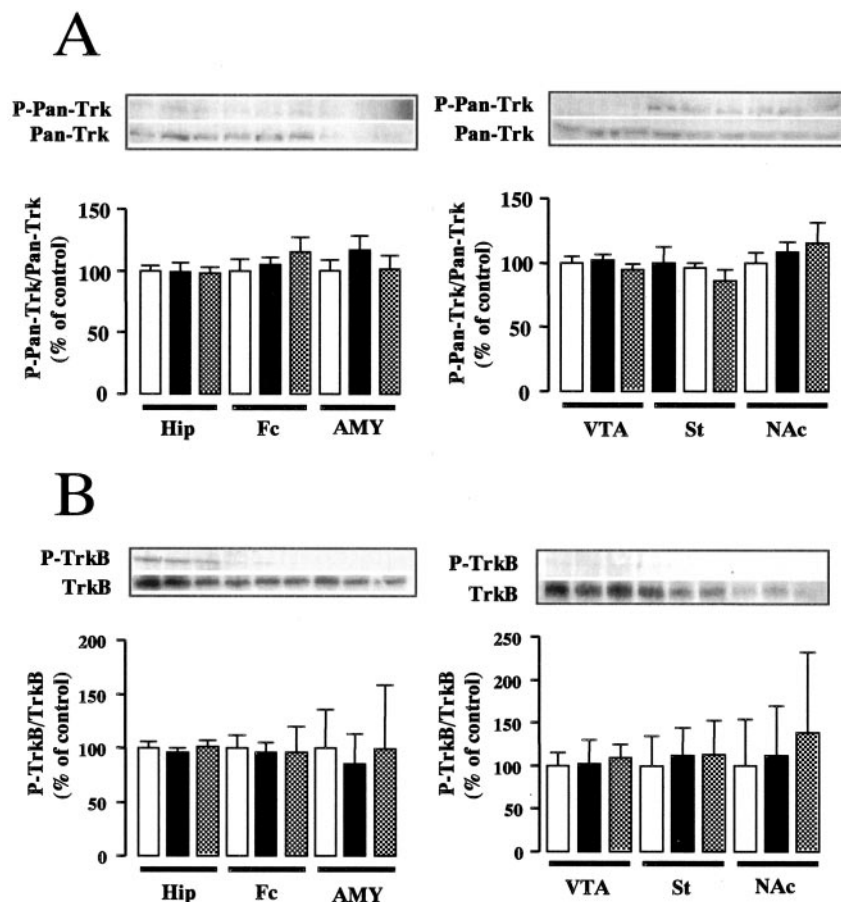
The possible involvement of the dopamine D2 receptor subtype in ERK activation evoked by MAP-induced CPP was also analyzed. Raclopride (1 mg/kg) failed to block the ERK activation evoked by MAP-induced CPP in the NAc but significantly decreased it in the St [Fig. 6B;  $F(3,17) = 12.3$ ,  $P < 0.001$ , and  $P < 0.05$  by post hoc comparison]. Taken together, these results show that dopamine D1 receptor-mediated ERK activation in the NAc and St is attributable to the expression of MAP-induced CPP, whereas the contribution of dopamine D2 receptors seems to be restricted to the St.

**Effect of MEK Inhibitor PD98059 on MAP-Induced CPP Expression.** Because both the MAP-induced CPP expression and ERK activation in the NAc were completely prevented by the dopamine D1 antagonist, we then tested the causal relation between MAP-induced CPP expression and ERK activation in the NAc. For this purpose, we assessed the effect of microinjection of PD98059, a selective MEK inhibitor, into the NAc. Bilateral microinjection of PD98059 into the NAc (2  $\mu$ g/side) significantly inhibited the expression of MAP-induced CPP [Fig. 7A;  $F(3,39) = 4.28$ ,  $P < 0.05$ , and  $P < 0.05$  by post hoc comparison]. The ERK activation evoked by MAP-induced CPP in the NAc was significantly abolished by PD98059 treatment [Fig. 7B;  $F(3,20) = 4.65$ ,  $P < 0.05$ , and  $P < 0.05$  by post hoc comparison]. These results suggest a critical role for the ERK1/2 signaling cascade in the expression of CPP in MAP-treated animals.

## Discussion

In the present study, we found that the hyperphosphorylation of ERK1/2, but not JNK and p38, in the NAc and St was associated with the expression of MAP-induced CPP. The transcription factor Elk-1, a nuclear target of ERK1/2, was also activated in the NAc by the expression of MAP-induced CPP. Moreover, the blockade of both D1 and D2 receptors by administration of SCH23390 and raclopride inhibited the expression of CPP induced by MAP. Administration of SCH23390 resulted in the inhibition of ERK1/2 hyperphosphorylation evoked by MAP-induced CPP in both NAc and St, whereas raclopride inhibited the hyperphosphorylation of ERK1/2 in the St but not the NAc. These findings suggest a role for the ERK1/2/Elk-1 signaling pathway via the activation of dopamine receptors in events underlying the expression of CPP induced by MAP. We suggest that the signaling pathway is crucial at least in part to the long-lasting neuroadaptation induced by MAP, which may be related to its abuse properties.

The ERK pathway is a signaling cascade, controlled by the Ras family of small GTPases, which plays a vital role in a variety of cell-regulatory events (Orban et al., 1999; Pearson et al., 2001). The role of the Ras/ERK pathway in long-term synaptic changes and behavior is well established (Orban et al., 1999; Mazzucchelli and Brambilla, 2000). There is an increasing amount of evidence that indicates the participation of a Ras/MEK/ERK/Elk-1 signaling pathway during the formation of new memories (Atkins et al., 1998; Cammarota

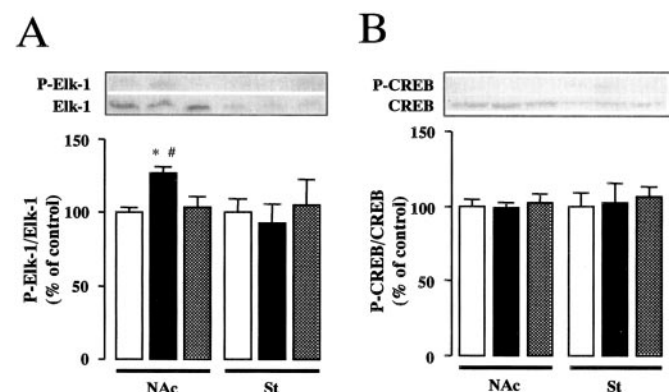


**Fig. 3.** No changes in Pan-Trk (A) and TrkB (B) phosphorylation on MAP-induced CPP. Rats were given MAP (2 mg/kg) or saline for the conditioning and were killed immediately after the CPP test. Open column, saline-treated CPP<sup>+</sup> rats; closed column, MAP-treated CPP<sup>+</sup> rats; hatched column, MAP-treated CPP<sup>-</sup> rats. Data are presented as the mean  $\pm$  S.E. (n = 5).

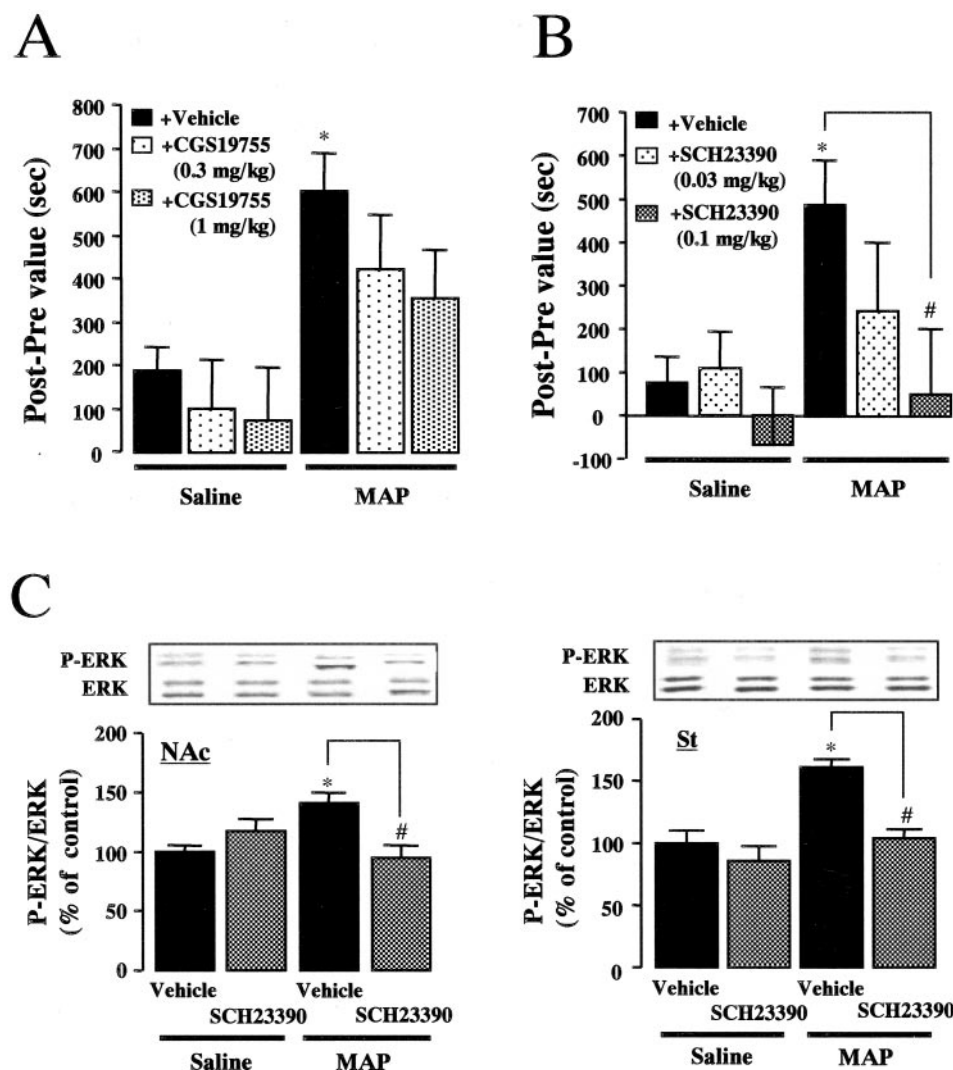
et al., 2000). As described in the introduction, learning/memory and the development of drug dependence share similar mechanisms. Kyosseva et al. (2001) have reported that

chronic administration of phencyclidine in rats produces a specific activation of ERK1/2, indicating a role for the ERK signaling pathway in phencyclidine abuse and perhaps in schizophrenia. Valjent et al. (2000) have reported that ERK activation was induced 10 min after acute administration of cocaine in the NAc and St of rat, and systemic administration of SL327, a selective MEK inhibitor, inhibits hyperlocomotion and acquisition of CPP induced by cocaine. Taken together, these reports strongly indicate that MAP and other psychostimulants activate the ERK1/2 signaling cascade in the brain.

In the present study, we found that ERK1/2 phosphorylation was activated in the NAc and St, but not in other brain areas of MAP-treated CPP<sup>+</sup> animals, whereas no such phosphorylation was seen in the control and the MAP-treated CPP<sup>-</sup> animals. Therefore, it is highly likely that ERK1/2 activation was induced by exposure to the context in which the animals had previously received MAP, not the MAP treatment itself. The microinjection of PD98059 into the NAc significantly inhibited the expression of MAP-induced CPP and abolished the ERK1/2 activation evoked by MAP-induced CPP, suggesting a critical involvement of the ERK signaling cascade in the expression of CPP induced by MAP. Our hypothesis is that the activation of ERK1/2 and Elk-1 in the



**Fig. 4.** A, Elk-1 activation associated with the expression of MAP-induced CPP. B, no changes in CREB phosphorylation on MAP-induced CPP. Rats were given MAP (2 mg/kg) or saline for the conditioning to the CPP test and were killed immediately after the test. Open column, saline-treated CPP<sup>-</sup> rats; closed column, MAP-treated CPP<sup>+</sup> rats; hatched column, MAP-treated CPP<sup>-</sup> rats. Data are presented as the mean ± S.E. (n = 5). \*, P < 0.05 vs. saline-treated CPP<sup>-</sup> rats; #, P < 0.05 vs. MAP-treated CPP<sup>-</sup> rats.



**Fig. 5.** Effect of NMDA receptor antagonist (A) and dopamine D1 receptor antagonist (B) on the expression of MAP-induced CPP; and effect of SCH23390 (0.1 mg/kg) on the hyperphosphorylation of ERK1/2 evoked by MAP (C). Rats were given MAP (2 mg/kg) or saline for conditioning to the test and were killed immediately after the CPP test. SCH23390 was administered i.p. 30 min before the CPP test. Data are presented as the mean ± S.E. (n = 7-16 for A; n = 7-16 for B; n = 5-7 for C). \*, P < 0.05 vs. vehicle-treated saline group; #, P < 0.05 vs. vehicle-treated MAP group.

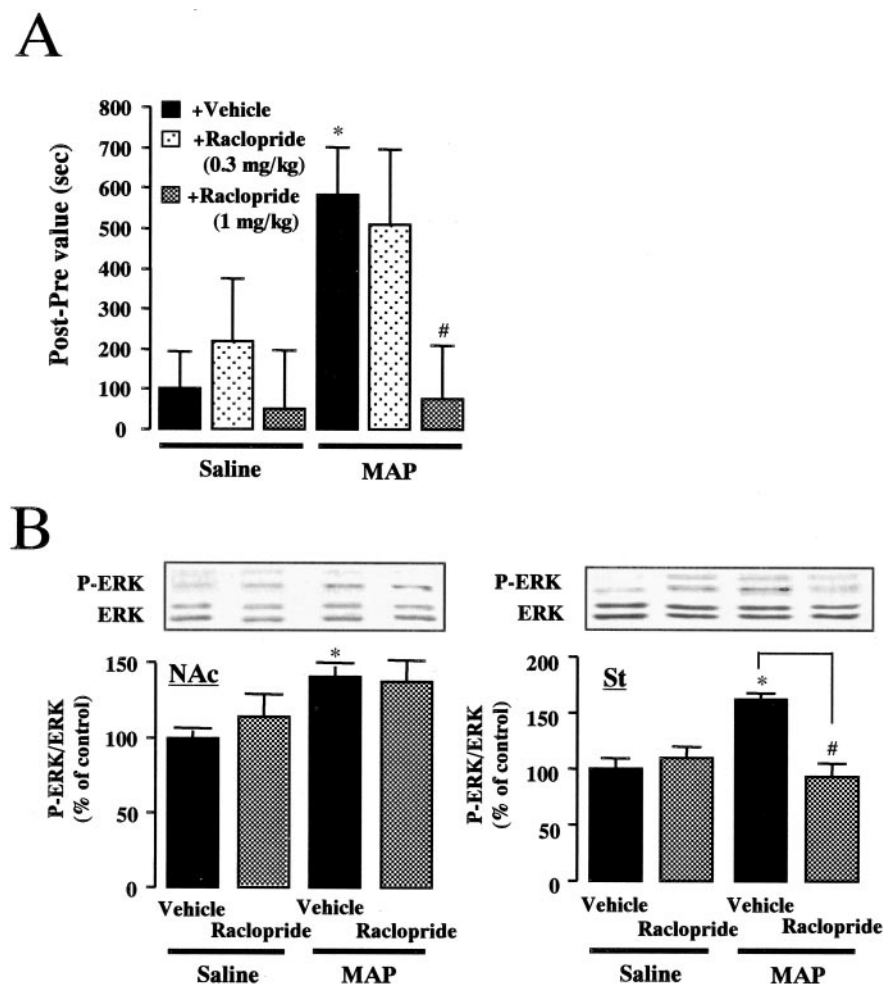


NAC represents the neuronal response related to the contextual memory of the rewarding effects of MAP, and is thereby associated with long-lasting neuroadaptation in MAP dependence.

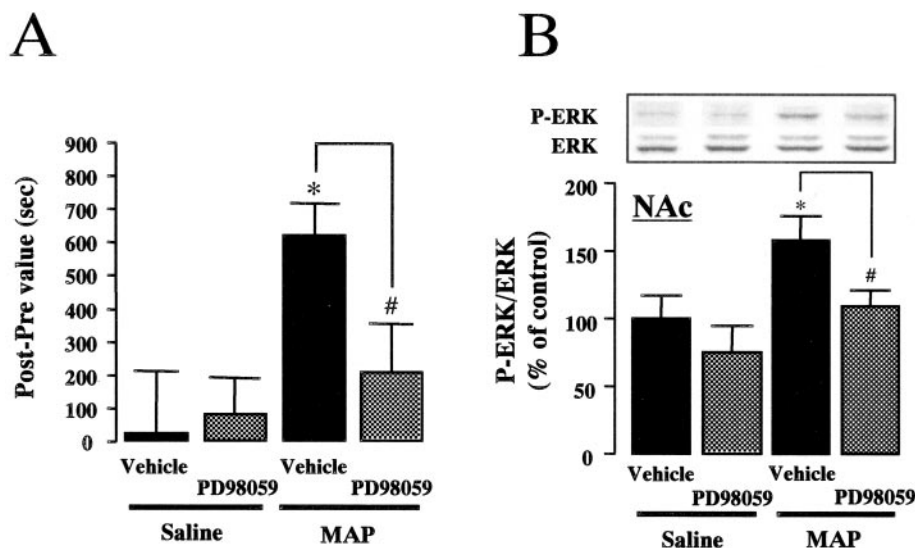
The dopamine D1 receptor antagonist SCH23390 at 0.1 mg/kg significantly reversed the hyperphosphorylation of ERK1/2 in the NAc and St evoked by MAP-induced CPP as well as the expression of CPP in MAP-treated animals. These results suggest that the expression of CPP induced by MAP may be related to the ERK1/2 activation via dopamine D1 receptors. It is well known that stimulation of dopamine D1 receptors results in the activation of ERK1/2 (Valjent et al., 2000; Zanassi et al., 2001). Several molecules could be responsible for the link between dopamine D1 receptors and ERK, such as the small Ras-related G protein Rap1, activated by PKA, and the subsequent activation of the B Raf isoform (Vossler et al., 1997; Zanassi et al., 2001). Another possible intermediate between the D1 receptor and ERK activation could be calcyon, a dopamine D1 receptor-interacting protein, expressed in the St (Lezcano et al., 2000). Calcyon stimulates intracellular calcium release, which is known to activate the Ras/ERK pathway (Lev et al., 1995). It should be investigated whether these intermediates are responsible or not for MAP-induced CPP in the near future. The dopamine D2 receptor antagonist raclopride significantly abolished the expression of MAP-induced CPP. However, ERK1/2 hyperphosphorylation evoked by MAP-induced

CPP was blocked in the St but not the NAc. Dopamine D1 and D2 receptors are coupled with  $G_s$  and  $G_{i/o}$  protein, respectively, and thus they have opposite effects on intracellular signaling, such as ERK1/2 activation via the cAMP/PKA pathway (Zanassi et al., 2001). However, a recent study demonstrated that the dopamine D2 receptor agonist quinpirole induces ERK1/2 and CREB phosphorylation in neurons via protein kinase C/Ras/Raf/MEK and DARPP-32, a dopamine and cAMP-regulated phosphoprotein (Yan et al., 1999). Furthermore, it is shown that dopamine D1 and D2 receptors synergistically activate immediate early gene expression and locomotion in dopamine-depleted rats (Paul et al., 1992; Keefe and Gerfen, 1995) and are required to evoke neural and behavioral phenotypes of cocaine sensitization (Capper-Loup et al., 2002). Thus, it is possible that raclopride inhibits the expression of MAP-induced CPP by inhibiting the ERK1/2 hyperphosphorylation through the blockade of dopamine D2 receptors in the St.

Activation of tyrosine kinase receptors or NMDA receptors results in an activation of ERK1/2. The neurotrophins, which play an important role in several forms of synaptic plasticity, such as learning and memory (Thoenen, 1995; Yamada et al., 2002), are expressed by dopamine neurons in the ventral midbrain (Davies, 1994). The members of the nerve growth factor family of neurotrophins that are active in the brain include nerve growth factor, brain-derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin-4/5 (Davies,



**Fig. 6.** Effect of dopamine D2 receptor antagonist on the expression of CPP (A) and hyperphosphorylation of ERK1/2 (B) evoked by MAP. Rats were given MAP (2 mg/kg) or saline for conditioning to the test and were killed immediately after the test. Raclopride was administered i.p. 30 min before the CPP test. Data are presented as the mean  $\pm$  S.E. ( $n = 8\sim 14$  for A;  $n = 4\sim 7$  for B). \*,  $P < 0.05$  vs. vehicle-treated saline group; #,  $P < 0.05$  vs. vehicle-treated MAP group.



**Fig. 7.** Effects of microinjection of PD98059 into the NAc on the expression of CPP (A) and hyperphosphorylation of ERK1/2 (B) evoked by MAP. Rats were given MAP (2 mg/kg) or saline for conditioning to the CPP test. PD98059 (2  $\mu$ g/side) or vehicle (60% dimethyl sulfoxide) was injected bilaterally into the NAc in a volume of 1.5  $\mu$ l, 20 min before the CPP test. Data are presented as the mean  $\pm$  S.E. ( $n = 9\sim 13$  for A;  $n = 4\sim 8$  for B). \*,  $P < 0.05$  vs. vehicle-treated saline group; #,  $P < 0.05$  vs. vehicle-treated MAP group.

1994; Pierce et al., 1999). The effects of neurotrophin are initiated by binding to their receptor tyrosine kinase, TrkA, TrkB, and TrkC, respectively (Segal and Greenberg, 1996). The MAP kinase pathway is one of the major signaling cascades activated downstream of neurotrophin stimulation of Trk receptors (Segal and Greenberg, 1996). For instance, chronic morphine treatment or chronic infusion of BDNF induces ERK activation in the VTA, and the morphine-induced increase in ERK activity is blocked by local infusion of NMDA receptor antagonist into the VTA (Berhow et al., 1996). Moreover, neurotrophin-3 contributes to the initiation of behavioral sensitization to cocaine by activating the Ras/ERK kinase signal transduction cascade through TrkC (Pierce et al., 1999). However, no phosphorylation of Trk receptors was seen in any brain regions of MAP-treated CPP<sup>+</sup> animals. Moreover, CGS19755 did not inhibit the expression of CPP evoked by MAP. Therefore, it is unlikely that tyrosine kinase receptors and NMDA receptors play an important role in the activation of ERK1/2 for the expression of MAP-induced CPP. However, in the present study, Trk phosphorylation was analyzed at the same time point as ERK1/2 immediately after the CPP. Because this is an early event in the Ras/ERK1/2 cascade, it remains possible that Trk phosphorylation occurs before this event.

Neither JNK nor p38 was phosphorylated by the expression of CPP evoked by MAP. This result is consistent with a report that neither dopamine nor forskolin activates JNK in striatal primary neuronal cells (Schwarzschild et al., 1997). Therefore, it seems likely that among MAP kinases, ERK1/2 is related and important to the expression of CPP induced by MAP.

How does ERK1/2 activation contribute to the expression of CPP induced by MAP? ERK1/2 is localized to both pre- and postsynaptic neurons in the hippocampus and cerebral cortices (Atkins et al., 1998; Jovanovic et al., 2000) and regulates synaptic vesicle proteins such as synapsin, voltage-gated ion channels, and transcription factors, including CREB and Elk-1. In postsynaptic neurons, ERK1/2 activation through both dopamine D1 and D2 receptors results in its translocation to the nucleus, resulting in phosphorylation of Elk-1. These signalings to the nucleus would initiate cell-specific gene expression programs necessary for synaptic remodeling

in the expression of CPP induced by MAP. Moreover, BDNF-induced glutamate and GABA release is linked to the phosphorylation of synapsin via the activation of TrkB and ERK1/2 (Jovanovic et al., 2000). Depolarization-induced activation of ERK1/2 stimulates tyrosine hydroxylase phosphorylation and dopamine synthesis in rat striatal slices (Lindgren et al., 2002), suggesting that ERK1/2, which has been extensively studied in relation to postsynaptic changes such as gene expression, is also able to regulate presynaptic function transiently in the brain. Therefore, it is possible that activated ERK1/2 in presynaptic neurons plays a role in increasing dopamine biosynthesis and release for the expression of CPP induced by MAP. These events in pre- and postsynaptic neurons in the NAc and St might be concerned with the expression of MAP-induced CPP when MAP-treated rats are exposed to the environment in which they had previously received drug treatment.

In conclusion, our study indicates that ERK1/2 activation can lead to the expression of CPP induced by MAP through both dopamine D1 and D2 receptors. ERK1/2 activation observed in both NAc and St may play a role, at least in part, in the learning/memory mechanisms of drug dependence induced by MAP.

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**Address correspondence to:** Dr. Toshitaka Nabeshima, Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya 466-8560, Japan. E-mail: tnabeshi@med.nagoya-u.ac.jp