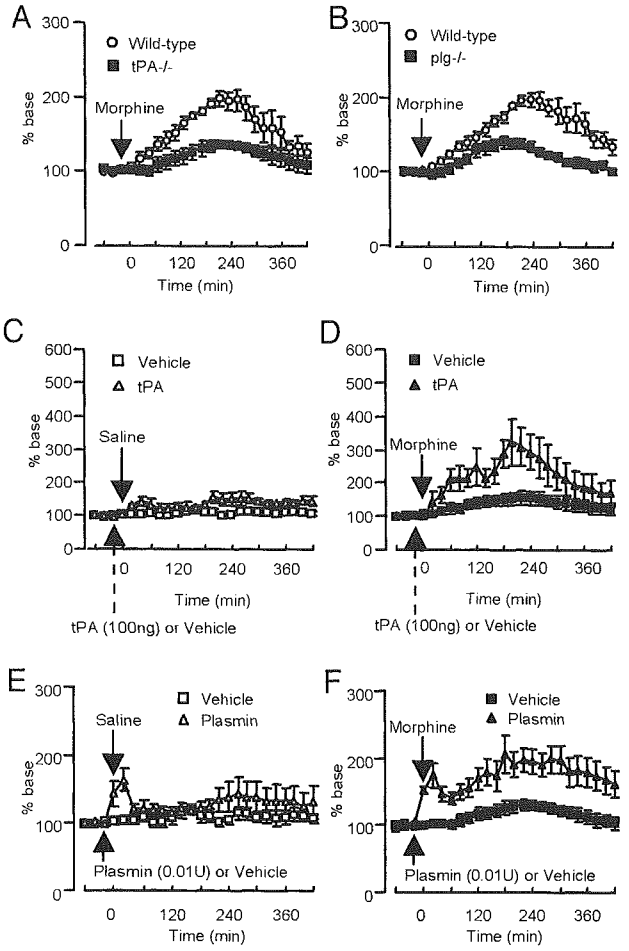

Fig. 4. Defect of morphine-induced dopamine release in $tPA^{-/-}$ mice and $plg^{-/-}$ mice. (*A*) Basal levels of dopamine in the NAcc did not differ between wild-type and $tPA^{-/-}$ mice (wild-type, 0.41 ± 0.13 nM, $n = 4$; $tPA^{-/-}$, 0.53 ± 0.01 nM, $n = 4$). Morphine (10 mg/kg s.c.)-induced dopamine release was markedly diminished in $tPA^{-/-}$ mice [$F_{(1, 6)} = 13.061$, $P < 0.05$]. (*B*) Basal levels of dopamine in the NAcc of $plg^{-/-}$ mice did not differ from those in wild-type mice (wild-type, 0.38 ± 0.10 nM, $n = 6$; $plg^{-/-}$, 0.38 ± 0.05 nM, $n = 6$). Morphine-induced dopamine release in the NAcc was significantly reduced in $plg^{-/-}$ mice compared to wild-type mice [$F_{(1, 10)} = 25.147$, $P < 0.01$]. (*C* and *D*) Effect of tPA on dopamine release in the NAcc of $tPA^{-/-}$ mice. Microinjection of tPA significantly increased basal levels of extracellular dopamine [$F_{(1, 8)} = 7.941$, $P < 0.05$] and morphine-induced dopamine release [$F_{(1, 8)} = 5.428$, $P < 0.05$) in $tPA^{-/-}$ mice]. (*E* and *F*) Effect of plasmin on dopamine release in the NAcc of $tPA^{-/-}$ mice. Microinjection of plasmin significantly increased basal levels of extracellular dopamine [$F_{(1, 8)} = 6.612$, $P < 0.05$] and morphine-induced dopamine release [$F_{(1, 8)} = 13.121$, $P < 0.01$] in $tPA^{-/-}$ mice. Values indicate means ± SE ($n = 4$ for *A*, $n = 6$ for *B*, and $n = 5$ for 4 *C–F*).

Single morphine treatment induced tPA mRNA expression in various regions of the rat brain. Although repeated morphine treatment reduced the ability of morphine to induce tPA mRNA expression in the brain, levels of tPA mRNA remained significantly higher in the frontal cortex, NAcc, striatum, and hippocampus. It is reported that the level of tPA is regulated by cAMP response element-binding protein (CREB) (8), and that CREB levels are reduced in the NAcc after chronic morphine treatment (38). Therefore, the reduced ability of morphine after repeated treatment to induce tPA mRNA expression in the brain may be due at least in part to the decrease in CREB levels.

Morphine-induced conditioned place preference was significantly attenuated in $tPA^{-/-}$ and $plg^{-/-}$ mice as compared with

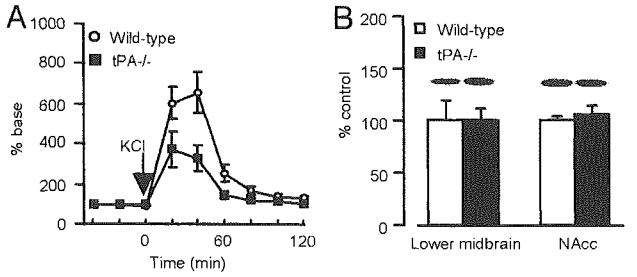

Fig. 5. Depolarization-evoked dopamine release and tyrosine hydroxylase content of $tPA^{-/-}$ mice. (*A*) High KCl (60 mM)-induced dopamine release in the NAcc of wild-type and $tPA^{-/-}$ mice. The lack of tPA significantly attenuated the depolarization-evoked dopamine release in the NAcc [$F_{(1, 10)} = 6.846$, $P < 0.05$]. (*B*) Immunoblot analysis of tyrosine hydroxylase of $tPA^{-/-}$ mice. There were no differences in the protein content of tyrosine hydroxylase in the lower midbrain and NAcc between $tPA^{-/-}$ and wild-type mice. Values indicate means ± SE ($n = 6$ for *A*, and $n = 4$ for *B*).

wild-type mice, suggesting that the tPA-plasmin system participates in the rewarding effects of morphine. However, because $tPA^{-/-}$ mice show impaired learning and memory in the context fear conditioning and two-way active avoidance test (18), we cannot exclude the possibility that $tPA^{-/-}$ mice failed to associate morphine-induced rewarding effects with the context during the conditioning. The mesolimbic dopaminergic pathway projecting from the VTA to the NAcc is thought to play a major role in mediating the rewarding effects of many stimuli, such as electrical brain stimulation and drugs of abuse (24). This dopamine system is important not only for rewarding effects but also locomotor-stimulating effects of morphine, the behavior being unaffected by the learning and memory function. The attenuation of both morphine-induced place preference and hyperlocomotion in $tPA^{-/-}$ and $plg^{-/-}$ mice suggests that the tPA-plasmin system plays a role in morphine-induced dopamine release in the NAcc.

Electrophysiological, biochemical, and behavioral data have suggested that tPA interacts extensively with dopamine D1 receptor-mediated responses in the brain. For instance, inactivation of the gene encoding tPA prevents the electrophysiological effects of D1 receptor agonists and mimics the effects of D1 receptor antagonists on the late phase of CA1 hippocampal long-term potentiation (39). The sensitivity of striatal cholinergic interneurons to dopamine D1 receptor stimulation is lost in $tPA^{-/-}$ mice (40). In contrast, we found that there were no differences in dopamine and morphine-induced increases in [^{35}S]GTPγS binding between wild-type and $tPA^{-/-}$ mice. No differences were evident in apomorphine-induced hyperlocomotion and tyrosine hydroxylase protein levels between the two types of mice. Accordingly, it is unlikely that the alterations of rewarding and locomotor-stimulating effects of morphine in $tPA^{-/-}$ mice are mainly due to the dysfunction of dopamine and opioid receptors in $tPA^{-/-}$ mice.

In vivo microdialysis and electrophysiological studies have provided evidence that the enhancement of dopamine release in the NAcc may be an essential process related to the morphine-induced rewarding effect (37, 41). In the present study, we demonstrated that morphine-induced dopamine release was attenuated in the NAcc of $tPA^{-/-}$ and $plg^{-/-}$ mice, and that microinjection of either exogenous tPA or plasmin into the NAcc, but not into the VTA, restored the morphine-evoked dopamine release in $tPA^{-/-}$ mice. The reduction of morphine-induced hyperlocomotion in $tPA^{-/-}$ mice was also reversed by microinjections of either exogenous tPA or plasmin into the NAcc. Western blotting of tyrosine hydroxylase contents of the brain revealed there are no differences be-

tween $tPA^{-/-}$ and wild-type mice, which is consistent with the immunohistochemical data (40). Therefore, plasmin that is converted from plg by tPA may have a role in regulating morphine-induced dopamine release in the NAcc.

The molecular mechanisms by which the tPA-plasmin system regulates morphine-induced dopamine release in the NAcc remain to be determined. However, it is known that the tPA-plasmin system degrades several extracellular matrix proteins (42), including laminin (43). Laminin in the synaptic cleft localizes calcium channels to the sites of active zones (44) and induces a small but significant increase in calcium levels in ciliary ganglion neurons when applied in soluble form to the culture medium (45). Accordingly, it is possible that a defect of the tPA-plasmin system may result in a malfunction of calcium channel activity, which leads to the reduction of depolarization-evoked dopamine release.

Conclusion

We have demonstrated that morphine increases tPA expression, and its enzyme activity in neuronal cells of the NAcc, by activating opioid receptors. The tPA-plasmin system plays a crucial role in regulating morphine-induced dopamine release in the NAcc and thereby is involved in the rewarding and locomotor-stimulating effects, without affecting the antinocipeptive effects.

We thank Eisai Co. Ltd. for providing us with human recombinant tPA. This study was supported in part by a Grant-in-Aid for Science Research (no. 14658249) and Special Coordination Funds for Promoting Science and Technology, Target-Oriented Brain Science Research Program, from the Ministry of Education, Culture, Sports, Science and Technology of Japan; a Grant-in-Aid for Health Sciences Research from the Ministry of Health, Labour and Welfare of Japan; and by the Fujisawa Foundation.

1. Sappino, A. P., Madani, R., Huarte, J., Belin, D., Kiss, J. Z., Wohlwend, A. & Vassalli, J. D. (1993) *J. Clin. Invest.* **92,** 679–685.
2. Chen, Z. L., Yoshida, S., Kato, K., Momota, Y., Suzuki, J., Tanaka, T., Ito, J., Nishino, H., Aimoto, S. & Kiyama, H. (1995) *J. Neurosci.* **15,** 5088–5097.
3. Backstrom, J. R., Lim, G. P., Cullen, M. J. & Tokes, Z. A. (1996) *J. Neurosci.* **16,** 7910–7919.
4. Hoffman, K. B., Larson, J., Bahr, B. A. & Lynch, G. (1998) *Brain. Res.* **811,** 152–155.
5. Gingrich, M. B., Junge, C. E., Lyuboslavsky, P. & Traynelis, S. F. (2000) *J. Neurosci.* **20,** 4582–4595.
6. Baranes, D., Lederfein, D., Huang, Y. Y., Chen, M., Bailey, C. H. & Kandel, E. R. (1998) *Neuron* **21,** 813–825.
7. Neuhoff, H., Roeper, J. & Schweizer, M. (1999) *Eur. J. Neurosci.* **11,** 4241–4250.
8. Qian, Z., Gilbert, M. E., Colicos, M. A., Kandel, E. R. & Kuhl, D. (1993) *Nature* **361,** 453–457.
9. Seeds, N. W., Williams, B. L. & Bickford, P. C. (1995) *Science* **270,** 1992–1994.
10. Ware, J. H., Dibenedetto, A. J. & Pittman, R. N. (1995) *Brain Res. Bull.* **37,** 275–281.
11. Gualandris, A., Jones, T. E., Strickland, S. & Tsirka, S. E. (1996) *J. Neurosci.* **16,** 2220–2225.
12. Parmer, R. J., Mahata, M., Mahata, S., Sebald, M. T, O'Connor, D. T. & Miles, L. A. (1997) *J. Biol. Chem.* **272,** 1976–1982.
13. Krystosek, A. & Seeds, N. W. (1981) *Science* **213,** 1532–1534.
14. Seeds, N. W., Basham, M. E. & Haffke, S. P. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 14118–14123.
15. Moonen, G., Grau-Wagemans, M. P. & Selak, I. (1982) *Nature* **298,** 753–755.
16. Frey, U., Muller, M. & Kuhl, D. A. (1996) *J. Neurosci.* **16,** 2057–2063.
17. Madani, R., Hulo, S., Toni, N., Madani, H., Steimer, T., Muller, D. & Vassalli, J. D. (1999) *EMBO J.* **18,** 3007–3012.
18. Calabresi, P., Napolitano, M., Centonze, D., Marfia, G. A., Gubellini, P., Teule, M. A., Berretta, N., Bernardi, G., Frati, L., Tolu, M., *et al.* (2000) *Eur. J. Neurosci.* **12,** 1002–1012.
19. Tsirka, S. E., Gualandris, A., Amaral, D. G. & Strickland, S. (1995) *Nature* **377,** 340–344.
20. Nicole, O., Docagne, F., Ali, C., Margaill, I., Carmeliet, P., MacKenzie, E. T., Vivien, D. & Buisson, A. (2001) *Nat. Med.* **7,** 59–64.
21. Siconolfi, L. B. & Seeds, N. W. (2001) *J. Neurosci.* **21,** 4336–4347.
22. Koob, G. F. (1992) *Trends. Pharmacol. Sci.* **13,** 177–184.
23. Wise, R. A. (1996) *Curr. Opin. Neurobiol.* **6,** 243–251.
24. Koob, G. F., Sanna, P. P. & Bloom, F. E. (1998) *Neuron* **21,** 467–476.
25. Johnson, S. W. & North, R. A. (1992) *J. Neurosci.* **12,** 483–488.
26. Bonci, A. & Williams, J. T. (1997) *J. Neurosci.* **17,** 796–803.
27. Nestler, E. J. (2001) *Nat. Rev. Neurosci.* **2,** 119–128.
28. Carmeliet, P., Schoonjans, L., Kieckens, L., Ream, B., Degen, J., Bronson, R., De Vos, R., van den Oord, J. J., Collen, D. & Mulligan, R. C. (1994) *Nature* **368,** 419–424.
29. Bugge, T. H., Flick, M. J., Daugherty, C. C. & Degen, J. L. (1995) *Genes Dev.* **9,** 794–807.
30. Heussen, C. & Dowdle, E. B. (1980) *Anal. Biochem.* **102,** 196–202.
31. Nitta, A., Ito, M., Fukumitsu, H., Ohmiya, M., Ito, H., Sometani, A., Nomoto, H., Furukawa, Y. & Furukawa, S. (1999) *J. Pharmacol. Exp. Ther.* **291,** 1276–1283.
32. Franklin, J. B. J. & Paxinos, G. T. (1997) *The Mouse Brain: In Stereotaxic Coordinates* (Academic, New York).
33. Miyamoto, Y., Yamada, K., Noda, Y., Mori, H., Mishina, M. & Nabeshima, T. (2002) *J. Neurosci.* **22,** 2335–2342.
34. Shintani, F., Kanba, S., Nakaki, T., Nibuya, M., Kinoshita, N., Suzuki, E., Yagi, G., Kato, R. & Asai, M. (1993) *J. Neurosci.* **13,** 3574–3581.
35. Noda, Y., Miyamoto, Y., Mamiya, T., Kamei, H., Furukawa, H. & Nabeshima, T. (1998) *J. Pharmacol. Exp. Ther.* **286,** 44–51.
36. Mamiya, T., Noda, Y., Nishi, M., Takeshima, H. & Nabeshima, T. (1998) *Brain Res.* **783,** 236–240.
37. Matthews, R. T. & German, D. C. (1984) *Neuroscience* **11,** 617–625.
38. Widnell, K. L., Self, D. W., Lane, S. B., Russell, D. S., Vaidya, V. A., Miserendino, M. J., Rubin, C. S., Duman, R. S. & Nestler, E. J. (1996) *J. Pharmacol. Exp. Ther.* **276,** 306–315.
39. Huang, Y. Y., Bach, M. E., Lipp, H. P., Zhuo, M., Wolfer, D. P., Hawkins, R. D., Schoonjans, L., Kandel, E. R., Godfraind, J. M., Mulligan, R., *et al.* (1996) *Proc. Natl. Acad. Sci. USA* **93,** 8699–8704.
40. Centonze, D., Napolitano, M., Saulle, E., Gubellini, P., Picconi, B., Martorana, A., Pisani, A., Gulino, A., Bernardi, G. & Calabresi, P. (2002) *Eur. J. Neurosci.* **16,** 713–721.
41. Narita, M., Funada, M. & Suzuki, T. (2001) *Pharmacol. Ther.* **89,** 1–15.
42. Schnaper, H. W. (1995) *Pediatr. Nephrol.* **9,** 104–111.
43. Goldfinger, L. E., Jiang, L., Hopkinson, S. B., Stack, M. S. & Jones, J. C. (2000) *J. Biol. Chem.* **275,** 34887–34893.
44. Sunderland, W. J., Son, Y. J., Miner, J. H., Sanes, J. R. & Carlson, S. S. (2000) *J. Neurosci.* **20,** 1009–1019.
45. Bixby, J. L., Grunwald, G. B. & Bookman, R. J. (1994) *J. Cell Biol.* **127,** 1461–1475.

0026-895X/04/6505-1293–1301$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2004 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 65:1293–1301, 2004

Vol. 65, No. 5
3028/1149379
Printed in U.S.A.

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.)

Received October 13, 2003; accepted February 17, 2004

This article is available online at http://molpharm.aspetjournals.org

0026-895X/04/6505-1293–1301$20.00
MOLECULAR PHARMACOLOGY

Mol Pharmacol 65:1293–1301, 2004

Vol. 65, No. 5
3028/1149379
Printed in U.S.A.

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.)

Received October 13, 2003; accepted February 17, 2004

This article is available online at http://molpharm.aspetjournals.org

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^+]. Some of the MAP-treated rats were killed without the CPP test [CPP^-]. 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^+ animals. No such phosphorylation was seen in control and MAP-treated CPP^- animals. Moreover, the transcription factor ets-like gene-1 (Elk-1), but not cAMP response element-binding protein, also showed a similar hyperphosphorylation in the same regions of MAP-treated CPP^+. 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^+ 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 receptors, 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-

This study was supported in part by grants-in aid for Health Sciences Research from the Ministry of Health, Labor and Welfare, Japan, Special Coordination Funds for Promoting Science and Technology, Target-Oriented Brain Science Research Program from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and an SRF grant for Biomedical Research.

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_r 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)methylene)-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 ± 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 ± 0.5°C, 50 ± 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 μg 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 μg/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 μg/side of PD98059 infused into the hippocampus resulted in an equilibrium concentration of ~37.5 μM, although the concentration was likely to be higher immediately surrounding the infusion site (Blum et al., 1999). A guide cannula (0.4 × 0.5 mm in diameter; Eicom, Kyoto, Japan) was implanted bilaterally into the NAc (+1.2 mm anterior to bregma, ±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 μg/side) or vehicle (60% dimethyl sulfoxide-saline) was injected bilaterally through a 28 gauge injection cannula (Eicom) in a volume of 1.5 μl/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 × 22 × 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 × 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 prevalues 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^+ and CPP^- groups. CPP^+ and control animals were killed immediately after the CPP test, whereas CPP^- 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^+, and CPP^- animals and immediately frozen and stored at −80°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 μg/ml pepstatin, 10 μg/ml aprotinin, and 10 μg/ml leupeptin, pH 7.4), and microwaved for 15 s according to the protocol for immunoblotting with monoclonal antibodies. The homogenate was centrifuged at 10,000*g* 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% bromphenol blue, 0.25% xylene cyanol, 30% glycerol, and 20% 2× 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 μg) 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 ± S.E. The significance of differences was determined by one-way analysis of variance, followed by the Student-Newman-Keuls test for multigroup 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$^+$ animals, compared with control and CPP$^-$ 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$^-$ 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$^+$ and

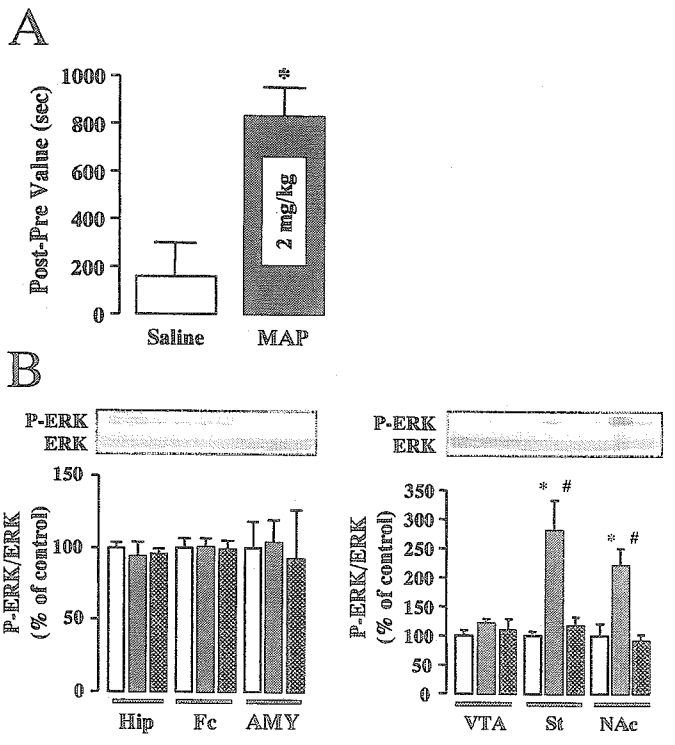

Fig. 1. A, MAP-induced CPP in rats; B, ERK1/2 activation associated with the expression of MAP-induced CPP. Rats were given MAP (2 mg/kg) or saline for the conditioning to the CPP test and were killed immediately after the test. Open column, saline-treated CPP$^-$ rats; closed column, MAP-treated CPP$^+$ rats; hatched column, MAP-treated CPP$^-$ rats. Data are presented as the mean ± S.E. ($n = 10$~12 for A; $n = 7$~8 for B). *, $P < 0.05$ vs. saline-treated CPP$^-$ rats; #, $P < 0.05$ vs. MAP-treated CPP$^-$ rats.

CPP^- 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^+ and CPP^- 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^+ animals without any changes in the St [$F(2,12) = 8.37$; $P < 0.01$ compared with saline-treated animals or MAP-treated CPP^-, and $P < 0.05$ by post hoc comparison]. No such phosphorylation of CREB was seen in either the NAc or St of MAP-treated CPP^- 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-

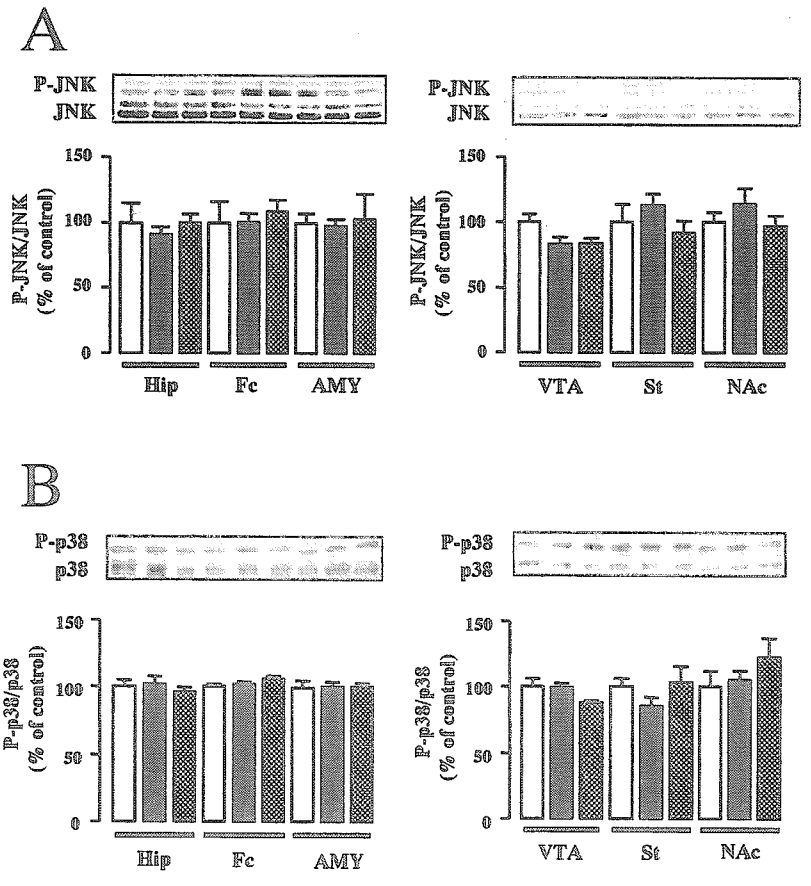

Fig. 2. No changes in JNK (A) and p38 (B) phosphorylation associated with the expression of MAP-induced CPP. Rats were given MAP (2 mg/kg) or saline for conditioning and were killed immediately after the test. Open column, saline-treated CPP^+ rats; closed column, MAP-treated CPP^+ rats; hatched column, MAP-treated CPP^- rats. Data are presented as the mean ± 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 μg/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

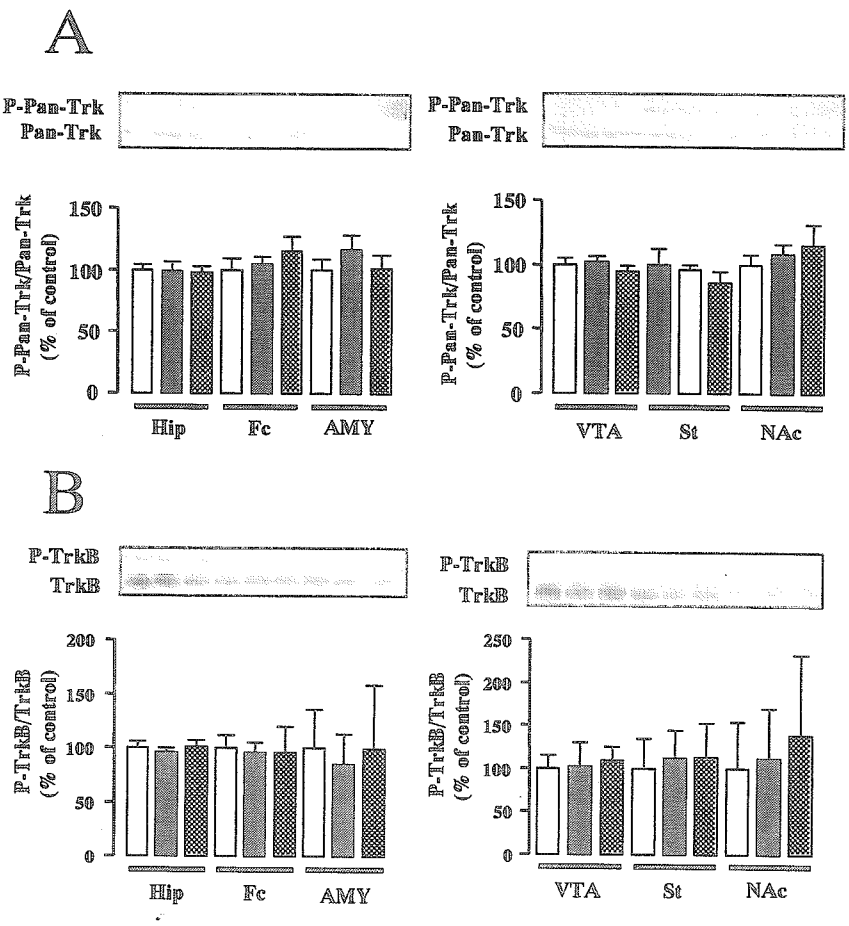

Fig. 3. No changes in Pan-Trk (A) and TrkB (B) phosphorylation on MAP-induced CPP. Rats were given MAP (2 mg/kg) or saline for the conditioning and were killed immediately after the CPP test. Open column, saline-treated CPP^+ rats; closed column, MAP-treated CPP^- rats; hatched column, MAP-treated CPP^- rats. Data are presented as the mean ± 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.

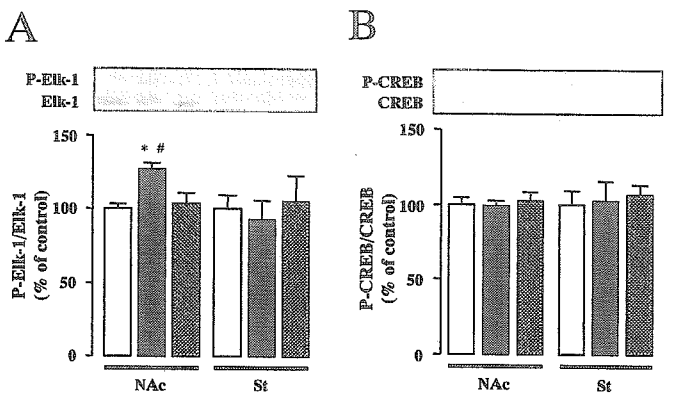

Fig. 4. A, Elk-1 activation associated with the expression of MAP-induced CPP. B, no changes in CREB phosphorylation on MAP-induced CPP. Rats were given MAP (2 mg/kg) or saline for the conditioning to the CPP test and were killed immediately after the test. Open column, saline-treated CPP^+ rats; closed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

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^+ animals, whereas no such phosphorylation was seen in the control and the MAP-treated CPP^- 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

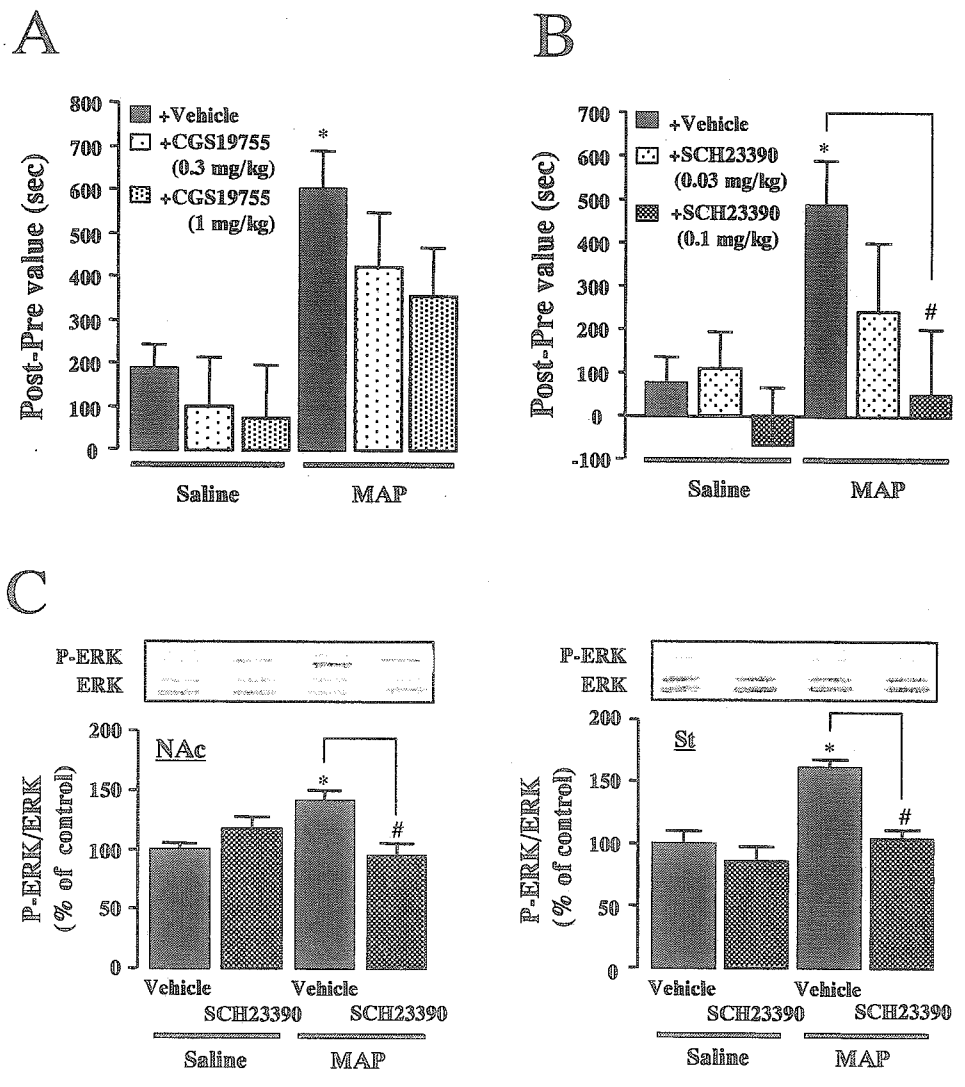

Fig. 5. Effect of NMDA receptor antagonist (A) and dopamine D1 receptor antagonist (B) on the expression of MAP-induced CPP; and effect of SCH23390 (0.1 mg/kg) on the hyperphosphorylation of ERK1/2 evoked by MAP (C). Rats were given MAP (2 mg/kg) or saline for conditioning to the test and were killed 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,

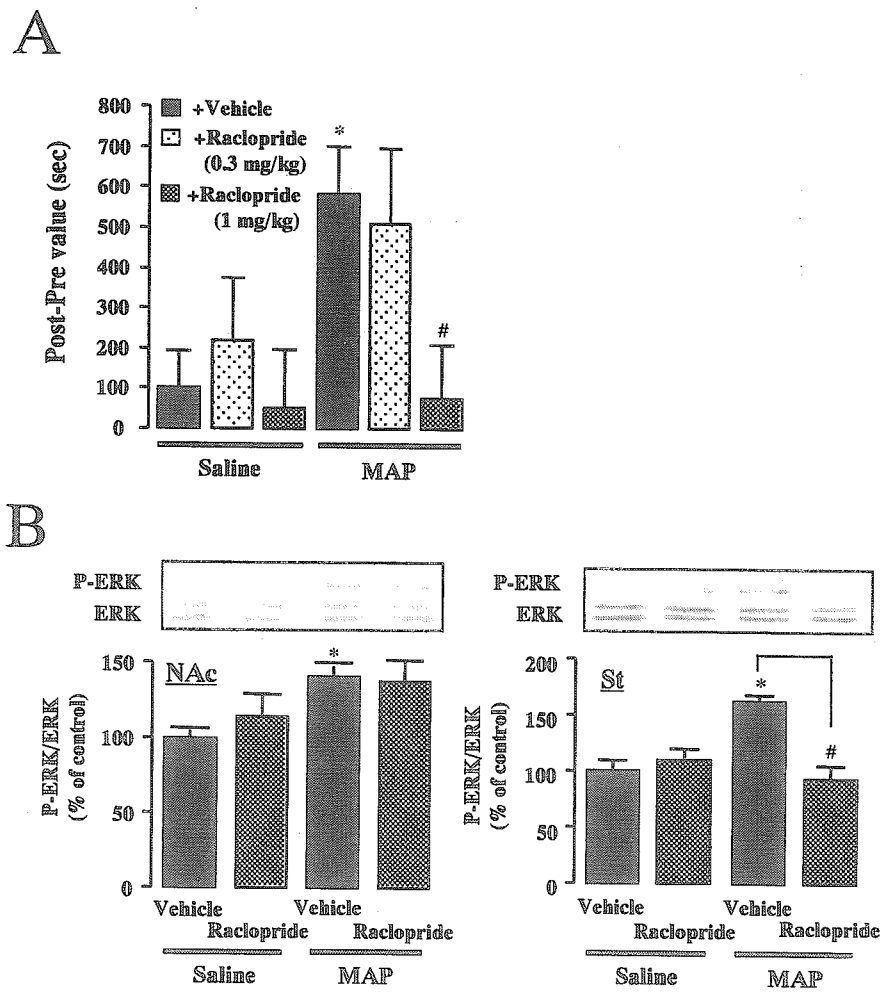

Fig. 6. Effect of dopamine D2 receptor antagonist on the expression of CPP (A) and hyperphosphorylation of ERK1/2 (B) evoked by MAP. Rats were given MAP (2 mg/kg) or saline for conditioning to the test and were killed immediately after the test. Raclopride was administered i.p. 30 min before the CPP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

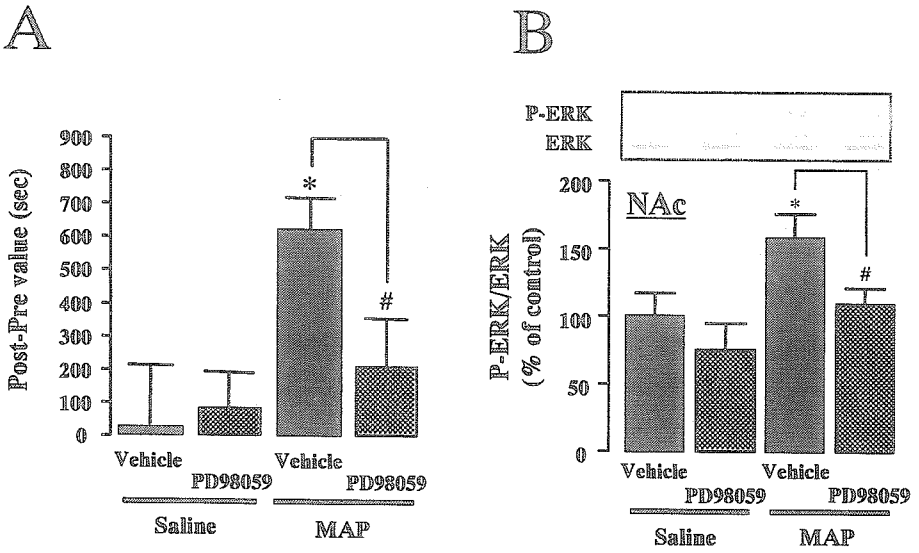

Fig. 7. Effects of microinjection of PD98059 into the NAc on the expression of CPP (A) and hyperphosphorylation of ERK1/2 (B) evoked by MAP. Rats were given MAP (2 mg/kg) or saline for conditioning to the CPP test. PD98059 (2 μg/side) or vehicle (60% dimethyl sulfoxide) was injected bilaterally into the NAc in a volume of 1.5 μl, 20 min before the CPP test. Data are presented as the mean ± 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^+ 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.

References

Ammassari-Teule M (2001) Drug addiction and memory systems: how neutral stimuli can gain control of behaviour. *Funct Neurol* **16**:227–235.

Atkins CM, Selcher JC, Petraitis JJ, Trzaskos JM, and Sweatt JD (1998) The MAPK cascade is required for mammalian associative learning. *Nat Neurosci* **1**:602–609.

Berhow MT, Hiroi N, and Nestler EJ (1996) Regulation of ERK (extracellular signal regulated kinase), part of the neurotrophine signal transduction cascade, in the rat mesolimbic dopamine system by chronic exposure to morphine or cocaine. *J Neurosci* **16**:4707–4715.

Berke JD and Hyman ST (2000) Addiction, dopamine and the molecular mechanisms of memory. *Neuron* **25**:515–532.

Blum S, Moore AN, Adams F, and Dash PK (1999) A mitogen-activated protein kinase cascade in the CA1/CA2 subfield of the dorsal hippocampus is essential for long-term spatial memory. *J Neurosci* **19**:3535–3544.

Cammarota M, Bevilaqua LR, Ardenghi P, Paratcha G, Levi de Stein M, Izquierdo I, and Medina JH (2000) Learning-associated activation of nuclear MAPK, CREB and Elk-1, along with Fos production, in the rat hippocampus after a one-trial avoidance learning: abolition by NMDA receptor blockade. *Brain Res Mol Brain Res* **76**:36–46.

Capper-Loup C, Canales JJ, Kadaba N, and Graybiel AM (2002) Concurrent activation of dopamine D1 and D2 receptors is required to evoke neural and behavioral phenotypes of cocaine sensitization. *J Neurosci* **22**:6218–6227.

Davies AM (1994) The role of neurotrophins in the developing nervous system. *J Neurobiol* **25**:1334–1348.

Davis S, Vanhoutte P, Pages C, Caboche J, and Laroche S (2000) The MAPK/ERK cascade targets both Elk-1 and cAMP response element-binding protein to control

long-term potentiation-dependent gene expression in the dentate gyrus in vivo. *J Neurosci* **20:**4563–4572.

Dudley DT, Pang L, Decker SJ, Bridges AJ, and Saltiel AR (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc Natl Acad Sci USA* **92:**7686–7689.

Hiroi N and White NM (1991) The amphetamine conditioned place preference: differential involvement of dopamine receptor subtypes and two dopaminergic terminal areas. *Brain Res* **552:**141–152.

Jovanovic JN, Czernik AJ, Fienberg AA, Greengard P, and Sihra TS (2000) Synapsins as mediators of BDNF-enhanced neurotransmitter release. *Nat Neurosci* **3:**323–329.

Keefe KA and Gerfen CR (1995) D1–D2 dopamine receptor synergy in striatum: effects of intrastriatal infusions of dopamine agonists and antagonists on immediate early gene expression. *Neuroscience* **66:**903–913.

Kitaichi K, Noda Y, Hasegawa T, Frukawa H, and Nabeshima T (1996) Acute phencyclidine induces aversion, but repeated phencyclidine induces preference in the place conditioning test in rats. *Eur J Pharmacol* **318:**7–9.

Koob GF (1992) Drugs of abuse: anatomy, pharmacology and function of reward pathways. *Trends Pharmacol Sci* **13:**177–184.

Kyosseva SV, Owens SM, Elbein AD, and Karson CN (2001) Differential and region-specific activation of mitogen-activated protein kinases following chronic administration of phencyclidine in rat brain. *Neuropsychopharmacology* **24:**267–277.

Lev S, Moreno H, Martinez R, Canoll P, Pleles E, Musacchio JM, Plowman GD, Rudy B, and Schlessinger J (1995) Protein tyrosine kinase PYK2 involved in Ca^{2+}-induced regulation of ion channel and MAP kinase functions. *Nature (Lond)* **376:**737–745.

Lezcano N, Mrzljak L, Eubanks S, Levenson R, Goldman-Rakic P, and Bergson C (2000) Dual signaling regulated by calcyon, a D1 dopamine receptor interacting protein. *Science (Wash DC)* **287:**1660–1664.

Lindgren N, Goiny M, Herrera-Marschitz M, Haycock JW, Hokfelt T, and Fisone G (2002) Activation of extracellular signal-regulated kinases 1 and 2 by depolarization stimulates tyrosine hydroxylase phosphorylation and dopamine synthesis in rat brain. *Eur J Neurosci* **15:**769–773.

Mazzucchelli C and Brambilla R (2000) Ras-related and MAPK signaling in neuronal plasticity and memory formation. *Cell Mol Life Sci* **57:**604–611.

Mazzucchelli C, Vantaggiato C, Ciamei A, Fasano S, Pakhotin P, Krezel W, Welzl H, Wolfer DP, Pages G, Valverde O, et al. (2002) Knockout of ERK1 MAP kinase enhances synaptic plasticity in the striatum and facilitates striatal-mediated learning and memory. *Neuron* **34:**807–820.

Mori A, Noda Y, Mamiya T, Miyamoto Y, Nakajima A, Furukawa H, and Nabeshima T (2001) Phencyclidine-induced discriminative stimulus is mediated via phencyclidine binding sites on the N-methyl-D-aspartate receptor-ion channel complex, not via $sigma_1$ receptors. *Behav Brain Res* **119:**33–40.

Nestler EJ (2001) Molecular basis of long-term plasticity underlying addiction. *Nat Rev Neurosci* **2:**119–128.

Orban PC, Chapman PF, and Brambilla R (1999) Is the Ras-MAPK signaling pathway necessary for long-term memory formation? *Trends Neurosci* **22:**38–44.

Paul ML, Graybiel AM, David JC, and Robertson HA (1992) D1-like and D2-like dopamine receptors synergistically activate rotation and c-fos expression in the dopamine-depleted striatum in a rat model of Parkinson's disease. *J Neurosci* **12:**3729–3742.

Paxinos G and Watson C (1982) *The Rat Brain in Stereotaxic Coordinates*. Academic Press, New York.

Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, and Cobb MH (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* **22:**153–183.

Pierce RC, Pierce-Bancroft AF, and Prasad BM (1999) Neurotrophin-3 contributes to the initiation of behavioral sensitization to cocaine by activating the Ras/mitogen-activated protein kinase signal transduction cascade. *J Neurosci* **19:**8685–8695.

Schwarzschild MA, Col RL, and Hyman SE (1997) Glutamate, but not dopamine, stimulates stress-activated protein kinase and AP-1-mediated transcription in striatal neurons. *J Neurosci* **17:**3455–3466.

Segal RA and Greenberg ME (1996) Intracellular signaling pathways activated by neurotrophic factors. *Annu Rev Neurosci* **19:**463–489.

Self DW, Barnhart WJ, Lehman DA, and Nestler EJ (1996) Opposite modulation of cocaine-seeking behavior by D1- and D2-like dopamine receptor agonists. *Science (Wash DC)* **271:**1586–1589.

Self DW, Genova LM, Hope BT, Barnhart WJ, Spencer JJ, and Nestler EJ (1998) Involvement of cAMP-dependent protein kinase in the nucleus accumbens in cocaine self-administration and relapse of cocaine-seeking behavior. *J Neurosci* **18:**1848–1859.

Silbley DR, Monsma FJ Jr, and Shen Y (1993) Molecular neurobiology of dopaminergic receptors. *Int Rev Neurobiol* **35:**391–415.

Thoenen H (1995) Neurotrophins and neuronal plasticity. *Science (Wash DC)* **270:**593–598.

Valjent E, Corvol JC, Pages C, Besson MJ, Maldonado R, and Caboche J (2000) Involvement of the extracellular signal-regulated kinase cascade for cocaine-rewarding properties. *J Neurosci* **20:**8701–8709.

Vossler MR, Yao H, York RD, Pan M-G, Rim CS, and Stork PJS (1997) cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell* **89:**73–62.

Yamada K, Mizuno M, and Nabeshima T (2002) Role for brain-derived neurotrophic factor in learning and memory. *Life Sci* **70:**735–744.

Yan Z, Feng J, Fienberg AA, and Greengard P (1999) D2 dopamine receptors induce mitogen-activated protein kinase and cAMP response element-binding protein phosphorylation in neurons. *Proc Natl Acad Sci USA* **96:**11607–11612.

Zanassi P, Paolillo M, Feliciello A, Avvedimento EV, Gallo V, and Schinelli S (2001) cAMP-dependent protein kinase induces cAMP-response element-binding protein phosphorylation via an intracellular calcium release/ERK-dependent pathway in striatal neurons. *J Biol Chem* **276:**11487–11495.

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

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Behavioural Brain Research 152 (2004) 263–270
BEHAVIOURAL BRAIN RESEARCH
www.elsevier.com/locate/bbr

Research report

Molecular mechanisms in dizocilpine-induced attenuation of development of morphine dependence: an association with cortical Ca^{2+}/calmodulin-dependent signal cascade

Moustafa Mahmoud Hamdy [a,b], Yukihiro Noda [a], Masayuki Miyazaki [a], Takayoshi Mamiya [a,c], Ayumu Nozaki [a], Atsumi Nitta [a], Merfat Sayed [a], Abdel-Azim Assi [b], Adel Gomaa [b], Toshitaka Nabeshima [a,*]

[a] *Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[b] *Department of Pharmacology, Assiut University Graduate School of Medicine, Assiut, Egypt*
[c] *Department of Chemical Pharmacology, Faculty of Pharmacy, Meijo University, Nagoya, Japan*

Received 9 July 2003; received in revised form 6 October 2003; accepted 6 October 2003

Available online 15 December 2003

Abstract

We investigated how dizocilpine, a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, affects the development of morphine dependence in mice. Co-administration of dizocilpine (0.25 mg/kg) and morphine (10 mg/kg) for 5 days attenuated the development of tolerance to the antinociceptive effects of morphine. The withdrawal manifestation induced by the naloxone-challenge (5 mg/kg) was significantly reduced in mice that were treated with a combination of dizocilpine and morphine, compared to the mice treated with morphine and saline. The present study revealed a significant increase in c-Fos protein expression in the cortex and thalamus of mice showing naloxone-precipitated withdrawal syndrome. The combination of dizocilpine and morphine prevented the increase of c-Fos protein expression in the cortex and thalamus. Interestingly, repeated co-administration of dizocilpine and morphine prevented the withdrawal-induced phosphorylation of Ca^{2+}/calmodulin kinase II (p-CaMK II) in the cortex, but not in the thalamus. Acute dizocilpine treatment prior to the naloxone-challenge and repeated treatment with dizocilpine alone had no effect on analgesia, withdrawal manifestations, p-CaMK II levels or c-Fos protein levels. These results showed that co-administration of dizocilpine and morphine prevented the development of morphine tolerance and dependence and suggested that the preventive effect of dizocilpine results from the regulation of c-Fos protein expression, which is possibly involved in the activation of the Ca^{2+}/calmodulin-dependent signal cascade in the cortex.

Keywords: Morphine; Withdrawal manifestations; Dizocilpine; c-Fos protein; Ca^{2+}/calmodulin kinase

1. Introduction

Opioid analgesics such as morphine are widely used in the management of pain. Repeated use may lead to the development of tolerance and dependence. Tolerance is indicated by a decreased efficacy of the drug after chronic use leading to the requirement for a higher dose to get the desired analgesic effect. Dependence is a continued need for the drug to maintain a state of physiological equilibrium, following repeated administration, and is evidenced by withdrawal manifestations when drug administration is terminated [7]. The mechanisms underlying the development of morphine tolerance, dependence and withdrawal manifestations are not fully understood.

The *N*-methyl-D-aspartate (NMDA) receptor is one of the glutamate receptors that plays a key role in synaptic plasticity and neuronal development [19]. Recent studies proved that excitatory amino acids (EAA) and their receptors have a role in the chronic actions of opioids [13,15] and the NMDA receptor plays an important role in opiate tolerance [23] and dependence [8,33]. The activation of NMDA receptors leads to the opening of receptor-gated ion channels, which allow Ca^{2+} to enter the neuron, where it participates in numerous processes, including the activation of protein kinases [35]. Protein kinases such as Ca^{2+}/calmodulin kinase II (CaMK II) have been reported to play an important role in various neuronal adaptive processes such as long-term potentiation [21], drug addiction [24] and the induction of

* Corresponding author. Tel.: +81-52-744-2674; fax: +81-52-744-2979.
E-mail address: tnabeshi@med.nagoya-u.ac.jp (T. Nabeshima).

0166-4328/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbr.2003.10.014

immediate early gene expression such as c-*fos* and c-*jun*, etc. [18]. Chronic morphine treatment increases the expression of CaMK II [20], and causes adaptive increases in Ca^{2+} accumulation [35]. Further, opioid withdrawal has been demonstrated to lead to an induction of the c-*fos* mRNA and its c-Fos protein product in several regions of the rat brain [10,12,30]. Dizocilpine, a non-competitive NMDA receptor antagonist, has been shown to prevent the morphine dependence in rodents [8,34]. However, the pathway by which dizocilpine could modulate opioid dependence and tolerance, and the effects of dizocilpine on opiate-induced changes in neuronal activity remain unclear.

In the present study, we here investigated how the dizocilpine affects the development of morphine dependence and tolerance in mice: (1) whether dizocilpine could attenuate the development of opioid dependence and tolerance according to previous reports [8,30]. (2) The effects of dizocilpine on withdrawal-induced changes in neuronal activity by using expression of c-Fos protein as a marker in the cortex and thalamus. (3) The possible role of the Ca^{2+}/calmodulin-dependent signal cascade in the symptoms of morphine withdrawal [16,22,27] by measuring phosphorylation of CaMK II (p-CaMK II) in the cortex and thalamus. We have previously demonstrated that both brain regions are involved in the development morphine dependence, since ERK activation and/or the increased cAMP levels are observed at the cortex and/or thalamus of morphine-dependent mice showing naloxone-precipitated withdrawal syndrome [10,22,28]. Thus, we used these both regions in the present study.

2. Materials and methods

2.1. Animals

Male C57BL/6J mice, 7 weeks old, weighing 20–25 g were used. The animals were handled in accordance with the guidelines established by the Institute for Laboratory Animal Research of Nagoya University. They were housed in groups of five in a temperature and light controlled room (23±1 °C, a 12-h light/dark cycle, with lights on at 7.30 a.m.) with free access to food and water.

2.2. Drug treatment

Morphine hydrochloride (Shionogi Pharmaceutical, Co. Ltd., Osaka, Japan), dizocilpine hydrogen maleate (Sigma, St. Louis, MO, USA) and naloxone hydrochloride (Sigma) were used.

To develop morphine tolerance and dependence, mice received morphine (10 mg/kg s.c.) with or without dizocilpine twice daily for 5 days. Dizocilpine was administered 30 min before every morphine treatment. In our preliminary experiments, we found that the higher doses (0.5 and 1 mg/kg i.p.) of dizocilpine markedly induced motor dysfunction such as ataxic behavior and motor in-coordination in mice. A dose of 0.25 mg/kg i.p. of dizocilpine was chosen for subsequent experimental steps. This dose has a significant effect on the attenuation of withdrawal manifestations and avoids the toxic interaction between higher doses of dizocilpine and morphine during induction of tolerance and dependence.

2.3. Hot plate test

The temperature of the hot plate was kept at 52 ± 0.2 °C. The analgesic threshold was considered to be the latency between the moment an animal was placed on the plate and the time when it started licking its hind paws or jumping with all four feet. Prior to drug administration, all mice were tested on the hot plate for 4 days in order to obtain a stable control response level. The animals were removed from the hot plate if they did not respond within 30 s in order to avoid tissue damage. Any animal, which failed to respond within 30 s was excluded immediately and re-tested again after 30 min. The antinociceptive effect of morphine was determined 60 min after the first injection on the first, third and fifth day.

2.4. Induction of withdrawal syndrome

All groups received naloxone (5 mg/kg i.p.) 2 h after the last injection of morphine on the sixth day of treatment. Immediately after the naloxone injection, each animal was placed in a transparent acryl cylinder (20 cm in diameter, 35 cm in height) to observe withdrawal manifestations (jumping, rearing, teeth chatter, paw tremors and diarrhea) for 30 min. The withdrawal manifestations were manually evaluated by co-workers blind to the treatment protocol. Diarrhea was scored as 0: no, 1: mild, 2: moderate, and 3: severe diarrhea.

2.5. Immunocytochemistry

2.5.1. Brain fixation

One hour after naloxone treatment according to our previous report [10], the animal was anesthetized with sodium pentobarbital (50 mg/kg i.p.), and perfused transcardially with 60 ml of heparinized saline, followed by 60 ml of 10% formalin in 0.1 M phosphate-buffered saline (PBS) (pH 7.4). The brain was removed, post-fixed at 4 °C overnight in the same fixative and then cryoprotected in 30% sucrose in the same buffer for one night, and kept at −80 °C until the assay.

2.5.2. Staining

The brains were cut into 50 μm thick coronal sections by a cryostat, extensively rinsed with PBS, and processed for immunocytochemistry as described previously [11]. Briefly, the sections were incubated in 1.5% normal goat serum in PBS for 1 h at room temperature. Then, they were incubated with primary antibody diluted (1:1000) in PBS for 48 h at 4 °C, washed three times in PBS (for 10 min each) and incubated with biotylinated secondary antibody (1:200)

in PBS for 2 h at room temperature. Next, they were washed as above and then incubated with avidin–biotin-peroxidase complex (1:100) for 1 h at room temperature. After another wash the sections were incubated in 0.04% diaminobenzidine with 0.0075% hydrogen peroxide and 10 μl of imidazole in PBS for 4–5 min. The reaction was stopped in PBS, and the sections were mounted on gelatin-subbed slides. The slides were allowed to dry prior to being dehydrated in gradually concentrated ethanol, cleared in xylene and cover-slipped with mounting medium.

2.5.3. Counting of c-Fos protein-positive cells

Tissue sections were examined at low power (50×) by light microscopy, to determine the segmental level at the same coordinates according to the atlas of Franklin and Paxinos [6], as well as gray matter landmarks. Positive c-Fos cells were identified using light field microscopy at (200×) and detected by the brown color of their nuclei. Positive nuclei were counted only when structures of the appropriate size and shape demonstrated clear increases in immunoreactivity compared to the background level. Questionable structures were examined at higher power (500×) and were not counted if identification remained uncertain [2,10]. The total number of positive cells in five similar sections through each area was counted in every animal. The number (mean±S.E.) of positive cells in the area for one animal in each group ($n = 5$) was calculated.

2.6. Western blot analysis

Fifteen minutes after naloxone injection, the animals were decapitated and different areas of the brain were dissected and kept at −80 °C. The dissected brain tissue was homogenized by sonication in an ice-cold lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 10 mM NaF, 10 mM EDTA, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, 10 mM sodium diphosphate decahydrate, 0.2 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin, 4 μg/ml leupeptin and 4 μg/ml pepstatin). The homogenate was centrifuged at 13,000 × g for 20 min and the supernatant was used for the measurement of phosphorylation of CaMK II. Samples (20 μg of protein) were boiled in Laemmli sample buffer, separated on a 10% polyacrylamide gel and subsequently transferred to PVDF membranes (Millipore). The membranes were blocked with a Detector Block Kit (KPL) for 2 h at room temperature and probed with anti-phospho-CaMK IIα/β subunit antibody (polyclonal anti-rabbit peptide antibodies at 0.5 μg/ml, Upstate, VA, USA) overnight at 4 °C. Membranes were washed with TBST buffer (10 mM Tris–HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.4) and subsequently incubated with a goat anti-rabbit horseradish peroxydase-conjugated secondary antibody for 1 h at room temperature. The immune complexes were detected by chemiluminescence (ECL, Amersham) and exposed to X-ray film. The band intensities of the film were analyzed by densitometry. To confirm equal loading of each protein, membranes were stripped with stripping buffer (62.5 mM Tris–HCl, 100 mM 2-mercaptoethanol, and 2% SDS, pH 6.7) at 50 °C for 30 min, and then incubated with anti-CaMK IIα subunit antibody (monoclonal anti-rabbit peptide antibodies at 0.2 μg/ml, Sigma) and detected as described above.

2.7. Statistical analysis

The results are expressed as means ± S.E.M. for each group. Statistical analysis of the difference between groups was done with the one-way ANOVA and Student's t test as a post hoc analysis. Differences were considered statistically significant at a level of $P < 0.05$.

3. Results

3.1. Effect of dizocilpine on morphine-induced analgesia

Acute morphine treatment (10 mg/kg s.c.) produced an antinociceptive effect in the hot plate test (Fig. 1A): the average analgesic latency in morphine-treated mice (25.5±1.0 s) was significantly increased compared to that in saline-treated mice (11.0 ± 1.3 s). When dizocilpine (0.25 mg/kg i.p.) was administered 30 min before the morphine, it had no effect on the morphine-induced analgesia (23.8 ± 1.0 s) or the analgesic latency itself (7.4 ± 1.1 s).

3.2. Effect of dizocilpine on the development of morphine tolerance to analgesia

Tolerance to morphine was developed with the same administration schedule as dependence. The effect of dizocilpine co-administered with morphine on the development of morphine tolerance is shown in Fig. 1B and C shows the time course of development of morphine tolerance and the effect of co-administration of dizocilpine and morphine on it. Chronic administration of morphine alone induces tolerance to its antinociceptive effect. The analgesic latency in chronic morphine-treated mice on day 5 (11.8 ± 1.2 s) was not significantly different from the latency in chronic saline-treated mice (7.8 ± 0.9 s). The development of tolerance was significantly inhibited by the co-administration of dizocilpine and morphine. The analgesic latency (22.7±1.8 s) in the chronic (dizocilpine+morphine)-treated group was significantly increased compared to the chronic (saline + morphine)-treated group or chronic saline-treated group. Chronic dizocilpine treatment alone had no effect on analgesic latency (5.7 ± 0.5 s) compared to chronic saline treatment.

3.3. Effects of dizocilpine on naloxone-precipitated withdrawal manifestations in morphine-dependent mice

Chronic morphine treatment produces physical dependence as assessed by a characteristic set of behavioral

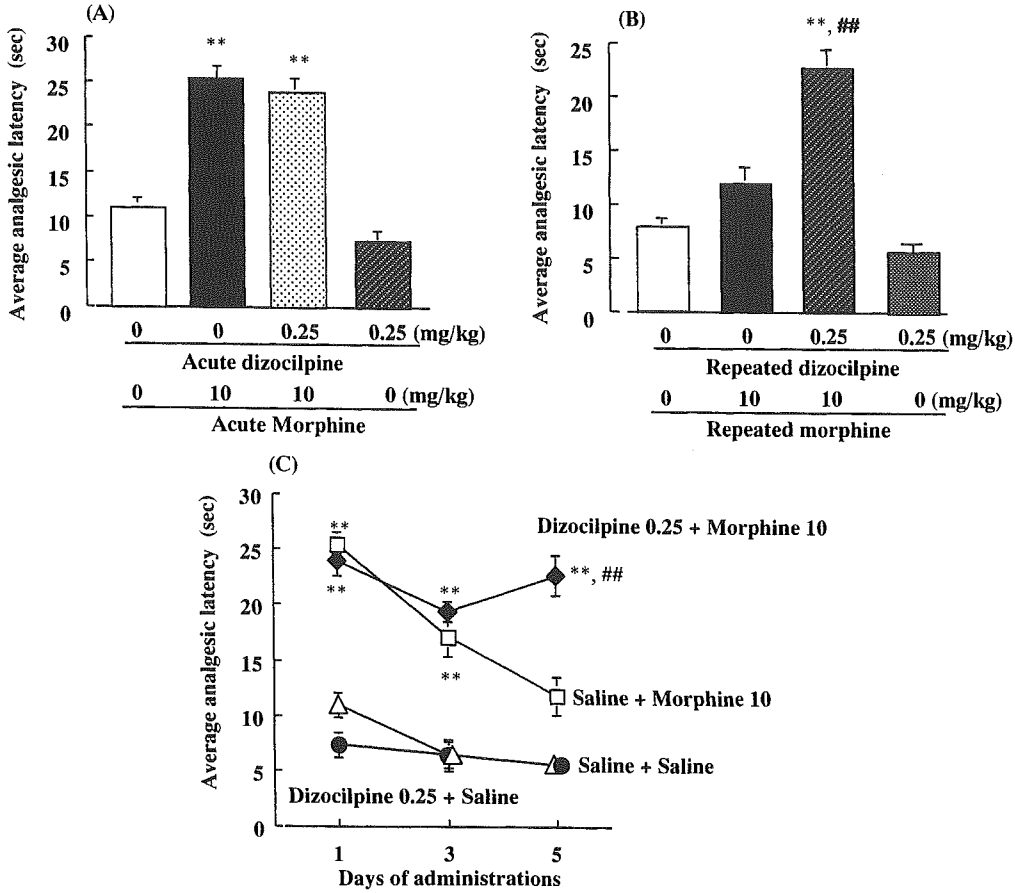

Fig. 1. Effects of dizocilpine on morphine-induced analgesia in mice. (A) Effect of acute dizocilpine treatment, (B) effect of repeated co-administration of dizocilpine and morphine, (C) the time course of tolerance to morphine-induced analgesia. The values are expressed as mean analgesic latency (hot plate) ± S.E.M. $^{**}P < 0.01$ vs. (saline + saline)-treated group. $^{\#\#}P < 0.01$ vs. (saline + morphine 10)-treated group ($n = 5$).

responses, including teeth chatter, erection, ptosis, chews, lacrimation, diarrhea, salivation, writhes, jumps, wet dog shakes and weight loss, following naltrexone challenge [26]. In agreement with previous reports, chronic morphine treatment produced a highly significant increase in withdrawal manifestations such as jumping, paw tremors, rearing, teeth chatter and diarrhea following the naloxone (5 mg/kg) challenge compared to the saline treatment (Fig. 2A–E). Co-administration of dizocilpine (0.25 mg/kg) and morphine (10 mg/kg) attenuated all withdrawal manifestations significantly (Fig. 2A–E). No significant difference could be detected between dependent mice and the dependent mice that received acute dizocilpine treatment (0.25 mg/kg) on the day of withdrawal. Further, chronic treatment with dizocilpine (0.25 mg/kg) alone also had no effect on withdrawal manifestations compared to the saline-treated group after the naloxone-challenge (Fig. 2A–E).

3.4. c-Fos protein expression

Naloxone-precipitated morphine withdrawal syndrome has been reported to associate with c-Fos protein expression in different areas of the brain [10,12]. The numbers of c-Fos-positive cells in the cingulate cortex (A) and cingulate thalamus (B) of morphine-dependent mice were significantly increased, compared to those in the saline-treated mice (Fig. 3). Chronic (dizocilpine + morphine)-treated mice showed a significant reduction of c-Fos protein expression in the above-mentioned areas compared to the chronic (saline + morphine)-treated mice or the dependent mice, which received acute dizocilpine treatment before the naloxone-challenge (Fig. 3). No significant difference was detected between the dependent mice and acute dizocilpine-treated dependent mice. Chronic dizocilpine treatment alone has no significant effect on the expression of c-Fos-positive cells (Fig. 3).

3.5. Changes in the phosphorylation of Ca^{2+}/calmodulin kinase II (p-CaMK II) levels

No difference in the total CaMK II level was detected between in the cingulate cortex and thalamus of each treatment following naloxone-challenge (data not shown). However, the mice with the naloxone-precipitated withdrawal syndrome showed a significant increase in p-CaMK II levels in the cingulate cortex, but not the thalamus. Although

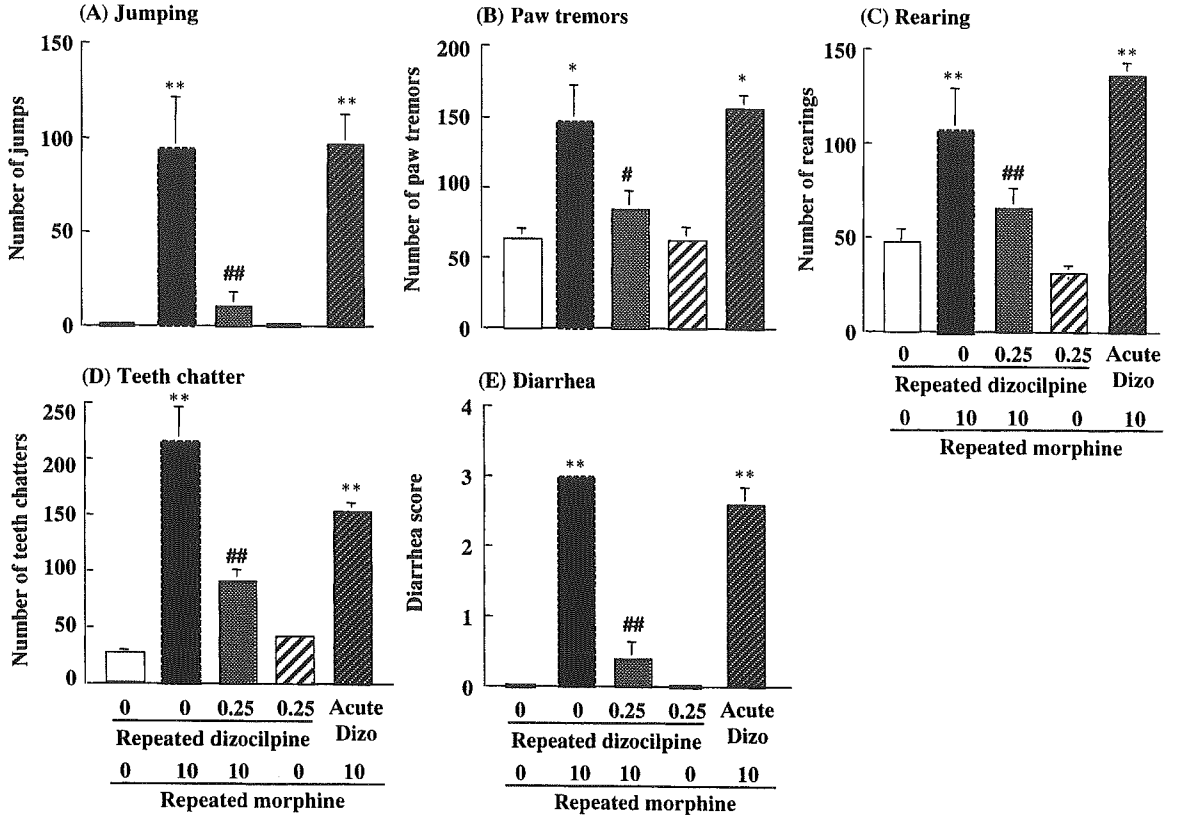

Fig. 2. Effects of co-administration of dizocilpine and morphine on the naloxone-induced withdrawal symptoms. (A) jumping, (B) paw tremor, (C) rearing, (D) teeth chatter, (E) diarrhea. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. chronic (saline + saline)-treated group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. chronic (saline + morphine 10)-treated group ($n = 5$). Dizo: dizocilpine.

dizocilpine itself did not significantly affect the level of p-CaMK II in the cingulate cortex, co-administration of dizocilpine significantly inhibited activation of CaMK II in the cingulate cortex (Fig. 4).

4. Discussion

We found that the chronic co-administration of dizocilpine and morphine inhibited the development of tolerance to morphine analgesia, as evidenced by a significant increase in the latency of analgesia in comparison with the chronic (saline + morphine)-treated group, but acute administration of dizocilpine had no effect on the morphine-induced tolerance. In addition, chronic dizocilpine treatment alone had no effect on the analgesic latency. Our results are consistent with previous reports that pretreatment with a non-competitive NMDA receptor antagonist, dizocilpine [8,34], can block the development of antinociceptive tolerance to morphine without affecting its antinociceptive actions [31,32]. An important characteristic of dizocilpine is that it attenuates the development of morphine tolerance without antagonizing the analgesic effect of morphine. In this respect it differs from the opioid receptor antagonists, naloxone [3] and β-funaltrexamine [4], which attenuate morphine tolerance only at doses that block the analgesia.

Chronic co-administration of dizocilpine (0.25 mg/kg) and morphine also inhibited the development of morphine dependence, evidenced by a significant reduction of withdrawal manifestations consistent with previous findings that selective antagonists for the NMDA receptor attenuate the manifestations of naloxone-induced withdrawal in morphine-dependent animals [14,26]. It is unlikely that the effect of dizocilpine is due to acute interaction between dizocilpine and morphine or naloxone on the day of withdrawal, since acute injection of dizocilpine before morphine or naloxone does not modify withdrawal behavioral manifestations in chronic morphine-treated mice. These results suggest that chronic co-administration of dizocilpine and morphine attenuates the development of morphine tolerance and dependence without affecting the acute effects of morphine.

Gene expression is thought to play an important role in many forms of neuronal plasticity. The severity of morphine dependence is related to the extent of morphine use, and withdrawal symptoms persist long after elimination [5,17]. Such long lasting behavioral modifications hint of plastic changes within the nervous system, some of which may be partially mediated by the regulation of gene expression

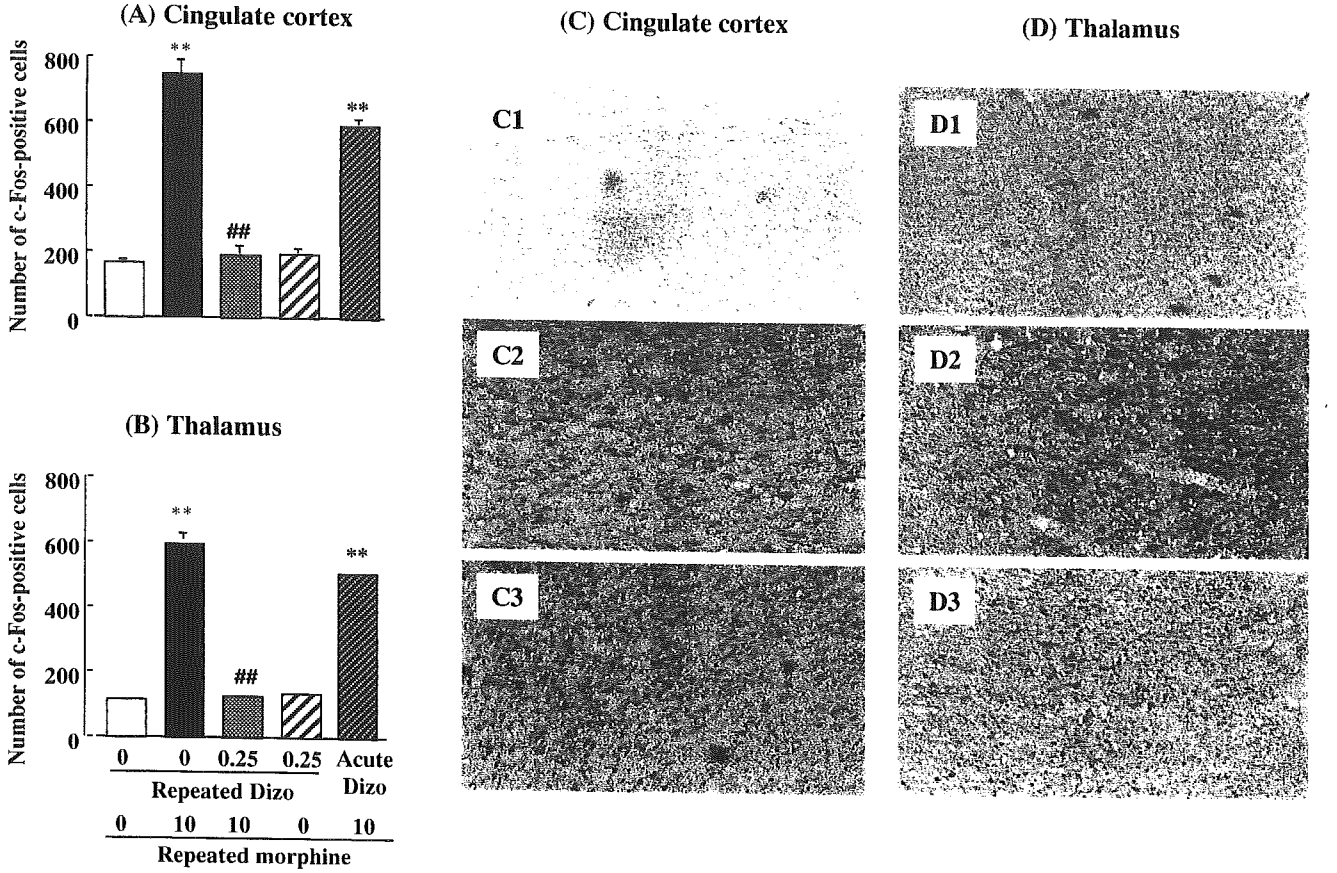

Fig. 3. Effects of repeated co-administration of dizocilpine and morphine on naloxone-induced c-Fos protein expression in the cingulate cortex (A) and cingulate thalamus (B). Representative photomicrographs of the cortex (C1–3) and thalamus (D1–3) for c-Fos protein histochemistry in different groups: (C1 and D1) = chronic (saline + saline)-treated group, (C2 and D2) = chronic (saline + morphine 10)-treated group, (C3 and D3) = chronic (dizocilpine 0.25 + morphine 10)-treated group. $^{**}P < 0.01$ vs. chronic (saline + saline)-treated group, $^{\#\#}P < 0.01$ vs. chronic (saline + morphine 10)-treated group ($n = 5$). Dizo: dizocilpine.

[9,24]. Acute and chronic morphine treatment decreases c-*fos* expression in the locus coeruleus [12]. Precipitated morphine withdrawal produced brain region-specific increases in the c-*fos* mRNA level. In the present study, the number of c-Fos protein-positive cells was significantly increased in the cingulate cortex and thalamus in morphine-dependent animals showing withdrawal manifestations, and the dependent mice injected with acute dizocilpine before the naloxone-challenge in comparison with chronic (saline + saline)-treated mice. In this study, interestingly, we observed a significant reduction in the number of c-Fos protein-positive cells in the group co-administered dizocilpine and morphine in the above-mentioned brain areas after naloxone-challenge. As regards c-Fos protein expression, our data are consistent with a previous report that levels of c-*fos* mRNA and protein are significantly increased in neuronal circuits mediating different functions during morphine withdrawal. Expression of c-Fos protein is significantly increased in several brain regions [10,30]. The frontal cortex and thamalus shows an increase of c-*fos* mRNA levels in morphine withdrawal [1,25]. Thus, this result supports our previous hypothesis that induction of c-Fos protein expression in different brain areas represents one feature of morphine withdrawal and may play a role in the expression of withdrawal manifestations [10].

The entry of Ca^{2+} has the ability to affect numerous intracellular processes, including the activation of protein kinases [35]. Protein kinases have been reported to play an important role in various neuronal adaptive processes such as drug addiction [24]. Chronic opioid treatment causes adaptive increases in Ca^{2+} accumulation and an increase in the expression of CaMK II [20]. The increase in Ca^{2+} activates CaMK II [29]. CaMK II has an important role in the phosphorylation of CREB (cAMP response element binding protein) and the phosphorylation of CREB produces an increase in its activity, which leads to an increase in c-*fos* expression [29]. We investigated whether the Ca^{2+}/calmodulin-dependent signal pathway is involved in the dizocilpine-mediated attenuation of withdrawal syndrome and c-Fos protein expression. We did find an increase in the level of p-CaMK II only in the cingulate cortex. It has been suggested that the cortex is the terminal/intermedial area for noradrenergic neurons associated with drug addiction and plays a key role in the naloxone-precipitated morphine withdrawal syndrome [31].

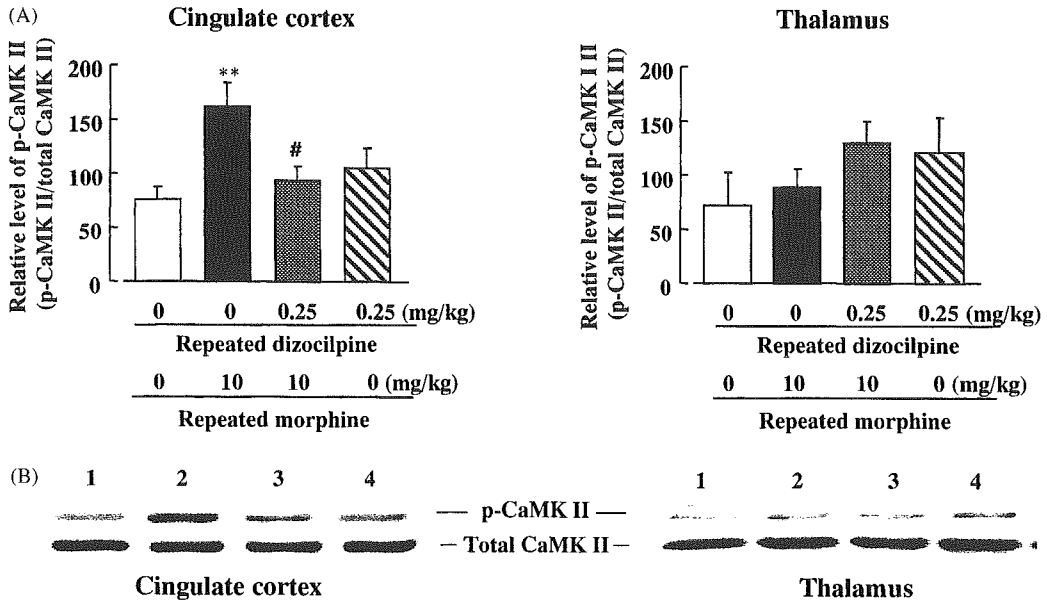

Fig. 4. The effects of dizocilpine on the p-CaMK II in the cingulated cortex and other brain areas of morphine-dependent mice following naloxone-precipitated withdrawal. (A) The ratio of p-CaMK II to total CaMK II in each brain region. $^{**}P < 0.01$ vs. chronic (saline + saline)-treated group, $^{\#}P < 0.05$ vs. chronic (saline + morphine 10)-treated group. (B) Lanes 1, 2, 3 and 4 represent chronic (saline + saline)-treated group, chronic (saline + morphine 10)-treated group, chronic (dizocilpine 0.25 + morphine 10) and chronic (dizocilpine 0.25 + saline)-treated group, respectively. $^{**}P < 0.01$ vs. chronic (saline + saline)-treated group, $^{\#}P < 0.05$ vs. chronic (saline + morphine 10)-treated group. Dizo: dizocilpine.

This activation of CaMK II after naloxone-precipitated withdrawal in the cingulate cortex was inhibited by repeated co-administration of dizocilpine and morphine. Activation of NMDA receptors leads to the opening of receptor-gated ion channels, which allow Ca^{2+} to enter the neuron, where it participates in numerous processes, including the activation of protein kinases [35]. Dizocilpine is a non-competitive receptor antagonist that acts by blocking the ion channel [34]. According to these findings, we speculate that, the chronic co-administration of dizocilpine and morphine inhibits Ca^{2+} participation in neurons. This inhibits the activation of CaMK II responsible for the phosphorylation of CREB. The lack of phosphorylation of CREB decreases its activity resulting in a reduced expression of c-Fos protein after the naloxone-challenge. These results support that chronic co-administration of dizocilpine and morphine, at least in part, through inhibition of CaMK II in the cingulate cortex, can attenuate the development of morphine tolerance, morphine dependence and c-Fos protein expression induced by naloxone-challenge. However, further study should be carried out to clarify other molecular mechanisms such as cyclic AMP and/or extracellular signal-regulated protein kinase signaling cascades, since there is a discrepancy between the changes of c-Fos protein and p-CaMK II in the thalamus.

In summary, we confirmed that dizocilpine attenuated the development of morphine dependence and tolerance, and found that naloxone-precipitated withdrawal-induced expression of c-Fos protein and activations of CaMK II in the cortex were prevented by repeated co-administration of dizocilpine and morphine. These results suggest that the preventive effect of dizocilpine results from the regulation of c-Fos protein expression, which is possibly involved in the activation of a Ca^{2+}/calmodulin-dependent signal cascade in the cortex.

Acknowledgements

This work was supported, in part, by Special Coordination Funds for Promoting Science and Technology, Target-oriented Brain Science Research Program, from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by Health Science Research Grants for Research on Pharmaceutical and Medical Safety from the Ministry of Health, Labour and Welfare of Japan.

References

[1] Beckmann AM, Matsumoto I, Wilce PA. Immediate early gene expression during morphine withdrawal. Neuropharmacology 1995;34:1183–9.
[2] Bullitt E. Expression of c-*fos*-like protein as a marker for neuronal activity following noxious stimulation in the rat. J Comp Neurol 1990;296:517–30.
[3] Cochin J, Mushlin BE. Effect of agonist–antagonist interaction on the development of tolerance and dependence. Ann NY Acad Sci 1976;281:244–51.
[4] DeLander GE, Portoghese PS, Takemori AE. Role of spinal mu opioid receptors in the development of morphine tolerance and dependence. J Pharmacol Exp Ther 1984;231:91–6.

[5] Eddy NB, Halbach H, Isbell H, Seevers MH. Drug dependence: its significance and characteristics. Bull WHO 1965;32:721–33.
[6] Franklin KBJ, Paxinos G. The mouse brain in stereotaxic coordinates. San Deigo: Academic Press; 1997.
[7] Fundytus ME, Ritchie J, Coderre TJ. Attenuation of morphine withdrawal symptoms by subtype-selective metabotropic glutamate receptor antagonists. Br J Pharmacol 1997;120:1015–20.
[8] Gonzalez P, Cabello P, Germany A, Norris B, Contreras E. Decrease of tolerance to, physical dependence on morphine by, glutamate receptor antagonist. Eur J Pharmacol 1997;332:257–62.
[9] Graybiel AM, Moratalla R, Robertson HA. Amphetamine and cocaine induce drug-specific activation of the c-*fos* gene in striosome-matrix compartments and limbic subdivisions of the striatum. Proc Natl Acad Sci USA 1990;87:6912–6.
[10] Hamdy MM, Mamiya T, Noda Y, Sayed M, Assi A, Gomaa A, et al. A selective phosphodiesterase IV inhibitor, rolipram blocks both withdrawal behavioral manifestations, and c-Fos protein expression in morphine dependent mice. Behav Brain Res 2000;118:85–93.
[11] Harlan RE, Garcia MM. Charting of Jun family member proteins in the rat forebrain and midbrain: immunocytochemical evidence for a new Jun-related antigen. Brain Res 1995;692:1–22.
[12] Hayward MD, Duman RS, Nestler EJ. Induction of the c-*fos* proto-oncogene during opiate withdrawal in the locus coeruleus and other regions of rat brain. Brain Res 1990;525:256–66.
[13] Herman BH, Vocci F, Bridge P. The effects of NMDA receptor antagonists and nitric oxide synthase inhibitors on opioid tolerance and withdrawal. Medication development issues for opiate addiction. Neuropsychopharmacology 1995;13:269–93.
[14] Higgens GA, Nguyen P, Sellers EM. The NMDA antagonist dizoclipine (MK-801) attenuates motivational as well as somatic aspects of naloxone-preciipitated opioid withdrawal. Life Sci 1992;50:PL67–PL172.
[15] Inturrisi CE. Pre-clinical evidence for a role of glutamatergic systems in opioid tolerance and dependence. Semin Neurosci 1997;9:110–9.
[16] Itoh A, Shiotani T, Nakayama S, Mamiya T, Hasegawa T, Noda Y, et al. Attenuation of the development of morphine dependence/tolerance by nefiracetam: involvement of adenosine 3′:5′-cyclic monophosphate system. Behav Brain Res 2000;115:65–74.
[17] Jaffe JH. Drug addiction of drug abuse. In: Gilman AG, Rall TW, Nies AS, Taylor P, editors. Eighth Goodman and Gilman's the pharmacological basis of therapeutics. New York: Pergamon Press; 1990. p. 22–573.
[18] Kaczmarek L. Glutamate receptor-driven gene expression in learning. Acta Neurobiol Exp (Warsz) 1993;53:187–96.
[19] Linden DJ, Wong KL, Sheu FS, Routtenberg A. NMDA receptor blockade prevents the increase in protein kinase C substrate (protein F1) phosphorylation produced by long term potentiation. Brain Res 1988;458:142–6.
[20] Lou L, Zhou T, Wang P, Pei G. Modulation of Ca^{2+}/calmodulin dependent protein kinase II activity by acute and chronic morphine administration in rat hippocampus: Differential regulation of α and β isoforms. Mol Pharmacol 1999;55:557–63.
[21] Malenka J, Kauer JA, Perkel DJ, Mauk MD, Kelly PT, Nicoll RA, et al. An essential role for postsynaptic calmodulin and protein kinase activity in long term potentiation. Nature 1989;340:554–7.
[22] Mamiya T, Noda Y, Ren X, Yamada K, Furukawa S, Kameyama T, et al. Involvement of cyclic AMP system in morphine physical dependence in mice: prevention of development of morphine dependence by rolipram, a cyclic AMP specific phosphodiesterase inhibitor. Br J Pharmacol 2000;132:1111–7.
[23] Marek P, Ben-Eliyahu S, Vaccarino AL, Liebeskind JC. Delayed application of MK-801 attenuates development of morphine tolerance in rats. Brain Res 1991;558:163–5.
[24] Nestler EJ, Hope BT, Widnell KL. Drug addiction a model for the molecular basis of neural plasticity. Neuron 1993;11:995–1006.
[25] Rasmussen K. The role of the locus coeruleus and *N*-methyl-D-aspartic acid (NMDA) and AMPA receptors in opiate withdrawal. Neuropsychopharmacology 1995;13:295–300.
[26] Rasmussen K, Fuller RW, Stockton ME, Perry KW, Swinford RM, Ornstein PL. NMDA receptor antagonists suppress behaviors but not norepinephrine turnover or locus coeruleus unit activity induced by opiate withdrawal. Eur J Pharmacol 1991;117:9–16.
[27] Ren X, Mamiya T, Noda Y, Yamada K, Oike Y, Yamamura K, et al. The role of cyclic AMP (cAMP) response element-binding protein (CREB)-binding protein (CBP) in mediating morphine withdrawal syndrome and the regulations of related genes: an investigation by using CBP knockout mice. Soc Neurosci Abs 1999;25: 1322.
[28] Ren X, Noda Y, Mamiya T, Nagai T, Nabeshima T. A neuroactive steroid, dehydroepiandrosterone sulfate, prevents the development of morphine dependence and tolerance via c-*fos* expression linked to the extracellular signal-regulated protein kinase. Behav Brain Res 2003, in press.
[29] Sheng M, Thompson MA, Greenberg ME. CREB: a Ca^{2+}-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science 1991;252:1427–30.
[30] Stornetta RL, Norton FE, Guyenet PG. Autonomic areas of rat brain exhibit increased Fos-like immunoreactivity during opiate withdrawal in rats. Brain Res 1993;624:19–28.
[31] Terwilliger RZ, Beiter-Johnson D, Sevarion KA, Crain SM, Nestler EJ. A general role for adaptations in G-proteins and the cyclic AMP systems in mediating the chronic actions of morphine and cocaine on neuronal function. Brain Res 1991;548:100–10.
[32] Tiseo PJ, Inturrisi CE. Attenuation of morphine tolerance by the competitive *N*-methyl-D-aspartate receptor antagonist, LY274614. J Pharmacol Exp Ther 1994;264:1090–6.
[33] Trujillo S, Akil H. Inhibition of morphine tolerance and dependence by the NMDA antagonist MK-801. Science 1991;252:85–7.
[34] Wong EHF, Kemp JA, Priestley T, Knight AR, Woodruff GN, Iversen LL. The anticonvulsant MK-801 is a potent *N*-methyl-D-aspartate antagonist. Proc Natl Acad Sci USA 1986;83:7104–8.
[35] Wroblewski JT, Danysz W. Modulation of glutamate receptors: molecular mechanisms and functional implications. Ann Rev Pharmacol Toxicol 1989;29:441–7.