[17] Franken IH, Kroon LY, Hendriks VM. Influence of individual differences in craving and obsessive cocaine thoughts on attentional processes in cocaine abuse patients. Addict Behav 2000;25:99–102.
[18] Fuchs RA, Evans KA, Ledford CC, Parker MP, Case JM, Mehta RH, et al. The Role of the dorsomedial prefrontal cortex, basolateral amygdala, and dorsal hippocampus in contextual reinstatement of cocaine seeking in rats. Neuropsychopharmacology 2005;30:296–309.
[19] Fuchs RA, Evans KA, Parker MP, See RE. Differential involvement of orbitofrontal cortex subregions in conditioned cue-induced and cocaine-primed reinstatement of cocaine seeking in rats. J Neurosci 2004;24:6600–10.
[20] Fuchs RA, See RE, Middaugh LD. Conditioned stimulus-induced reinstatement of extinguished cocaine seeking in C57BL/6 mice: a mouse model of drug relapse. Brain Res 2003;973:99–106.
[21] Gerber GJ, Stretch R. Drug-induced reinstatement of extinguished self-administration behavior in monkeys. Pharmacol Biochem Behav 1975;3:1055–61.
[22] Gottfried JA, O'Doherty J, Dolan RJ. Encoding predictive reward value in human amygdala and orbitofrontal cortex. Science 2003;301:1104–7.
[23] Grant S, London ED, Newlin DB, Villemagne VL, Liu X, Contoreggi C, et al. Activation of memory circuits during cue-elicited cocaine craving. Proc Natl Acad Sci USA 1996;93:12040–5.
[24] Hart CL, Ward AS, Haney M, Foltin RW, Fischman MW. Methamphetamine self-administration by humans. Psychopharmacology (Berl) 2001;157:75–81.
[25] Hester R, Garavan H. Executive dysfunction in cocaine addiction: evidence for discordant frontal, cingulate, and cerebellar activity. J Neurosci 2004;24:11017–22.
[26] Highfield DA, Mead AN, Grimm JW, Rocha BA, Shaham Y. Reinstatement of cocaine seeking in 129X1/SvJ mice: effects of cocaine priming, cocaine cues and food deprivation. Psychopharmacology (Berl) 2002;161:417–24.
[27] Kilts CD, Gross RE, Ely TD, Drexler KP. The neural correlates of cue-induced craving in cocaine-dependent women. Am J Psychiatry 2004;161:233–41.
[28] Morgan AD, Carroll ME, Loth AK, Stoffel M, Wickman K. Decreased cocaine self-administration in Kir3 potassium channel subunit knockout mice. Neuropsychopharmacology 2003;28:932–8.
[29] National Institute on Drug Abuse. Epidemiological trends in drug abuse: advance report. Washington, DC: U.S. Department of Health and Human Services; 2001.
[30] Panlilio LV, Yasar S, Nemeth-Coslett R, Katz JL, Henningfield JE, Solinas M, et al. Human cocaine-seeking behavior and its control by drug-associated stimuli in the laboratory. Neuropsychopharmacology 2005;179:374–82.
[31] Picciotto MR, Wickman K. Using knockout and transgenic mice to study neurophysiology and behavior. Physiol Rev 1998;78:1131–63.
[32] Picciotto MR, Zoli M, Rimondini R, Lena C, Marubio LM, Pich EM, et al. Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. Nature 1998;391:173–7.
[33] Roberts AJ, McDonald JS, Heyser CJ, Kieffer BL, Matthes HW, Koob GF, Gold LH. mu-Opioid receptor knockout mice do not self-administer alcohol. J Pharmacol Exp Ther 2000;293:1002–8.
[34] Rocha BA, Fumagalli F, Gainetdinov RR, Jones SR, Ator R, Giros B, et al. Cocaine self-administration in dopamine-transporter knockout mice. Nat Neurosci 1998;1:132–7.
[35] Rocha BA, Scearce-Levie K, Lucas JJ, Hiroi N, Castanon N, Crabbe JC, et al. Increased vulnerability to cocaine in mice lacking the serotonin-1B receptor. Nature 1998;393:175–8.
[36] See RE. Neural substrates of conditioned-cued relapse to drug-seeking behavior. Pharmacol Biochem Behav 2004;71:517–29.
[37] Shalev U, Grimm JW, Shaham Y. Neurobiology of relapse to heroin and cocaine: a review. Pharmacol Rev 2002;54:1–42.
[38] Sinha R, Fuse T, Aubin LR, O'Malley SS. Psychological stress, drug-related cues and cocaine craving. Psychopharmacology (Berl) 2000;152:140–8.
[39] Stewart J. Pathways to relapse: the neurobiology of drug- and stress-induced relapse to drug-taking. J Psychiatry Neurosci 2000;25:125–36.
[40] Soria G, Mendizabal V, Tourino C, Robledo P, Ledent C, Parmentier M, et al. Lack of CB1 cannabinoid receptor impairs cocaine self-administration. Neuropsychopharmacology 2005;30:1670–80.
[41] Szumilinski KK, Dehoff MH, Kang SH, Frys KA, Lominac KD, Klugmann M, et al. Homer proteins regulate sensitivity to cocaine. Neuron 2004;43:401–13.
[42] Uhl GR. Needed: mouse/human cross validation of reinstatement/relapse models (and drug reward models) to model human substance abuse vulnerability allelic variants. Psychopharmacology (Berl) 2003;168: 42–3.
[43] Ujike H, Sato M. Clinical features of sensitization to methamphetamine observed in patients with methamphetamine dependence and psychosis. Ann NY Acad Sci 2004;1025:279–87.
[44] Vanderschuren LJ, Everitt BJ. Drug seeking becomes compulsive after prolonged cocaine self-administration. Science 2004;305:1017–9.
[45] Volkow ND, Li TK. Drug addiction: the neurobiology of behaviour gone awry. Nat Rev Neurosci 2004;5:963–70.
[46] Weiss F, Maldonado-Vlaar CS, Parsons LH, Kerr TM, Smit DL, Ben-Shahar O. Control of cocaine-seeking behavior by drug-associated stimuli in rats: effects on recovery of extinguished operant-responding and extracellular dopamine levels in amygdala and nucleus accumbens. Proc Natl Acad Sci USA 2000;97:4321–6.

Available online at www.sciencedirect.com
ScienceDirect
Behavioural Brain Research 173 (2006) 39–46
BEHAVIOURAL BRAIN RESEARCH
www.elsevier.com/locate/bbr

Research report

Discriminative-stimulus effects of methamphetamine and morphine in rats are attenuated by cAMP-related compounds

Yijin Yan [a], Atsumi Nitta [a], Tomoko Mizuno [a], Akira Nakajima [a], Kiyofumi Yamada [b], Toshitaka Nabeshima [a,*]

[a] *Department of Neuropsychopharmacology & Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya 466-8560, Japan*
[b] *Laboratory of Neuropsychopharmacology, Kanazawa University Graduate School of Natural Science & Technology, Kanazawa 920-1192, Japan*

Received 22 April 2006; received in revised form 29 May 2006; accepted 31 May 2006
Available online 20 July 2006

Abstract

Animal models of drug discrimination have been used to examine the subjective effects of addictive substances. The cAMP system is a crucial downstream signaling pathway implicated in the long-lasting neuroadaptations induced by addictive drugs. We examined effects of rolipram, nefiracetam, and dopamine D2-like receptor antagonists, all of which have been reported to modulate cAMP level in vivo, on the discriminative-stimulus effects of methamphetamine (METH) and morphine in rats. All these compounds inhibited the discriminative-stimulus effects of METH, while only rolipram and nefiracetam attenuated the discriminative-stimulus effects of morphine. In addition, neither nifedipine nor neomycin, two voltage-sensitive calcium channel blockers, was found to modulate the effect of nefiracetam on METH-associated discriminative stimuli, suggesting that the inhibitory effect of nefiracetam may not involve the activation of calcium channels. These findings suggest that the cAMP signaling cascade may play a key role in the discriminative-stimulus effects of METH and morphine and may be a potential target for the development of therapeutics to counter drugs of abuse.

Keywords: Drug discrimination; Methamphetamine; Morphine; cAMP signaling pathway; Rolipram; Nefiracetam; Dopamine D2-like receptor antagonists; Rats

1. Introduction

The characteristic subjective and reinforcing effects of addictive drugs such as methamphetamine (METH) and opiate contribute to their widespread abuse [11,29]. It is well established that the cAMP signaling cascade plays a pivotal role in the long-lasting plasticity induced by addictive drugs, thus contributing to the development of drug dependence and psychosis [6,9,16,26,33–35]. For example, it has been reported that the cAMP system is involved in psychostimulant- and morphine-induced locomotor sensitization, conditioned place preference, intravenous self-administration, even in opiate abuse in addicts [7,34,35,38].

Rolipram and nefiracetam (*N*-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl) acetamide) are among the compounds known to modulate cAMP level. Rolipram is an inhibitor of cAMP phosphodiesterase IV, and its administration has been demonstrated to upregulate the intracellular cAMP level [10,16,24]. Nefiracetam is a derivative of pyrrolidine, which has been reported to ameliorate the impairment of learning and memory in animal models of aging [40], Alzheimer's disease [42], and head trauma [5] through increasing intracellular cAMP levels and enhancing currents via calcium ion channels [27,28,36,39,41]. In addition, the activation of dopamine D2-like receptors, including D2, D3, and D4 receptors, has been shown to down-regulate cAMP activity by coupling with inhibitory G proteins [8,13]. Previous reports have shown that rolipram, nefiracetam, and dopamine D2-like receptor antagonists attenuated drug-induced locomotor sensitization, withdrawal symptoms, conditioned place preference, intravenous self-administration, and reinstatement of drug-seeking behavior [2,9,10,14,16,32]. Thus, it is reasonable to postulate that all these compounds may modulate the development of addictive drug-induced dependence and psychosis through their regulations on the intracellular cAMP level.

* Corresponding author. Tel.: +81 52 744 2674; fax: +81 52 744 2682.
E-mail address: tnabeshi@med.nagoya-u.ac.jp (T. Nabeshima).

0166-4328/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbr.2006.05.029

Drug discrimination procedures in nonhuman primates and rodents have proven useful for clarifying the pharmacological mechanisms underlying the subjective effects of addictive drugs including psychostimulants and opioid ligands and for identifying potential compounds for use in therapy [3,4,11,19–21,29,31,37], since the discriminative-stimulus effects of addictive drugs in experimental animals are linked with the subjective effects of these drugs in humans [11,12,29]. Previously, we have reported that in animals, rolipram and nefiracetam attenuate the development of morphine dependence and tolerance by increasing the cAMP level in the brain [9,16]. We have further hypothesized that the cAMP signaling cascade is a determinant of the subjective effects of addictive drugs, and investigated effects of three types of cAMP-related compounds, rolipram, nefiracetam, and dopamine receptor antagonists, on the subjective effects of METH and morphine in a rat model of drug discrimination.

2. Materials and methods

2.1. Drugs

The following compounds were used in this study: METH and morphine hydrochloride (Dainippon Pharmaceutical Ltd., Osaka, Japan); rolipram, L-745870 hydrochloride, and raclopride tartrate salt (Sigma Chemicals Co., St. Louis, MO); nefiracetam (Daiichi Pharmaceutical Ltd., Tokyo, Japan); flunarizine hydrochloride (RBI Research Biomedicals International, MA); and neomycin sulfate and nifedipine (ICN Biomedicals Co., OH). METH, morphine, nefiracetam, raclopride, L-745870, and neomycin were dissolved in saline immediately before use. Rolipram was dissolved in dimethyl sulphoxide (Sigma Chemicals Co.) and then diluted with saline immediately before use. Flunarizine and nifedipine were dissolved in polyethylene glycol and then diluted with saline immediately before use. METH or morphine was subcutaneously administered 10 min before the test sessions. Rplipram (i.p.), nefiracetam (p.o.), and raclopride (i.p.) were administered 30 min before the METH or morphine treatment. The dopamine D4 antagonist (L-745870) and voltage-sensitive calcium channel blockers (flunarizine, neomycin, and nifedipine) were administered i.p. 40 min before the administration of METH. All drugs were administered in a volume of 1.0 ml/kg.

2.2. Animals

Thirty male Sprague–Dawley rats (Nihon SLC Co. Ltd., Shizuoka, Japan), initially 8 weeks old and weighing 260 ± 10 g, were used in this study. Body weights were gradually reduced to approximately 85% of free-feeding weights by limiting daily access to food. Water was available ad libitum. The rats were housed 3–4 per cage under controlled laboratory conditions (a 12 h reversed light/dark cycle with room lights on at 9:00 a.m., 23 ± 1 °C, 50 ± 5% humidity). All animal care and use was in accordance with the Principles of Laboratory Animal Care (National Institutes of Health Publication 85-123, 1983) and was approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine. Animals were treated according to the Guidelines of Experimental Animal Care issued from the Office of the Prime Minister of Japan.

2.3. Drug discrimination procedures

Experiments were performed in standard two-lever operant-conditioning chambers (Neuroscience Co., Tokyo, Japan) set in ventilated and sound-attenuated cubicles. The animal model of drug discrimination used here was established as described previously [25]. Briefly, rats ($n = 30$) were initially trained to press each of the two levers under a fixed ratio (FR) 1 schedule of food presentation. Once the rats acquired a stable rate of lever-presses, the FR was increased from FR1 to FR2, FR5, FR10, and then FR20. After the rats acquired a stable rate of lever-presses under a FR20 schedule, the animals were then assigned into two groups. One group of rats ($n = 14$) was trained to discriminate METH from saline. The other group ($n = 16$) was trained to discriminate morphine from saline. During the period of drug discrimination training, rats were injected with METH (0.5 mg/kg, s.c.), morphine (5.0 mg/kg, s.c.), or saline (1 ml/kg, s.c.) 10 min before the start of the session. Twenty consecutive responses on one lever resulted in the delivery of a food pellet after the drug injection (under an FR20 schedule), whereas 20 consecutive responses on the other lever resulted in the delivery of food pellet after injection of saline (under an FR20 schedule). Responding on the incorrect lever reset the FR requirement for the correct one. For half the rats, the right lever was the drug-paired lever and, for the other half, the left lever was the drug-paired lever. Each session ended after 20 food pellets were delivered or after 30 min elapsed, whichever came first. The criteria for the acquirement of drug discrimination were five consecutive sessions with: (1) more than 85% correct-lever responding before the first reinforcement and (2) more than 90% correct-lever responding over the session. Discrimination training sessions were conducted 6 days per week under a double alternation schedule (i.e., DDSSDDSS, etc., D = drug; S = saline).

The testing sessions were identical to the training sessions except that 20 consecutive responses on either lever resulted in the delivery of a food pellet. Each rat was tested the compounds according to a within-subjects design. After each testing session, rats were returned to daily drug-training sessions and then performed the next testing session until the criteria were met once again. Lever selection was examined after the administration of METH (0.2 mg/kg) or morphine (3.0 mg/kg), or the co-administration of the test compounds and METH or morphine.

2.4. Statistical analysis

Data were expressed as the mean ± SEM. The significance of differences was determined by a one-way analysis of variance (ANOVA), followed by Bonferroni's Multiple Comparison Test. p-Values of less than 0.05 were regarded as statistically significant.

3. Results

3.1. Establishment of METH and morphine-associated discrimination in rats

One group of rats ($n = 14$) was able to discriminate METH from saline and acquire a stable rate of METH-appropriate lever-presses under an FR20 schedule of food presentation (the training dose of METH, 0.5 mg/kg) after 60 ± 10 training sessions (Fig. 1A and B). Similarly, a separate group of rats ($n = 16$) were able to discriminate morphine from saline and acquired a stable rate of morphine-appropriate lever-presses under an FR20 schedule of food presentation (the training dose of morphine, 5.0 mg/kg) after 60 ± 10 training sessions (Fig. 1C and D). After the rats acquired the ability to discriminate the drugs from saline, the dose–response functions of METH- and morphine-induced discrimination were examined in the rats of METH and morphine-administered groups, respectively. METH-paired lever responses in the dose range of 0.2–0.5 mg/kg reached 90% while the rate of lever responses maintained stable (Fig. 2A and B). In the rats of morphine group, morphine-paired lever responses in the dose range of 2.0–5.0 mg/kg reached 90% (Fig. 2C). The rate of lever responses showed a tendency to decrease as the dose of morphine increased (Fig. 2D). Considering the selection of the drug-lever and the rate of lever-responses in rats, 0.2 mg/kg of METH and 3.0 mg/kg of morphine were chosen to examine the pharmacological effects of

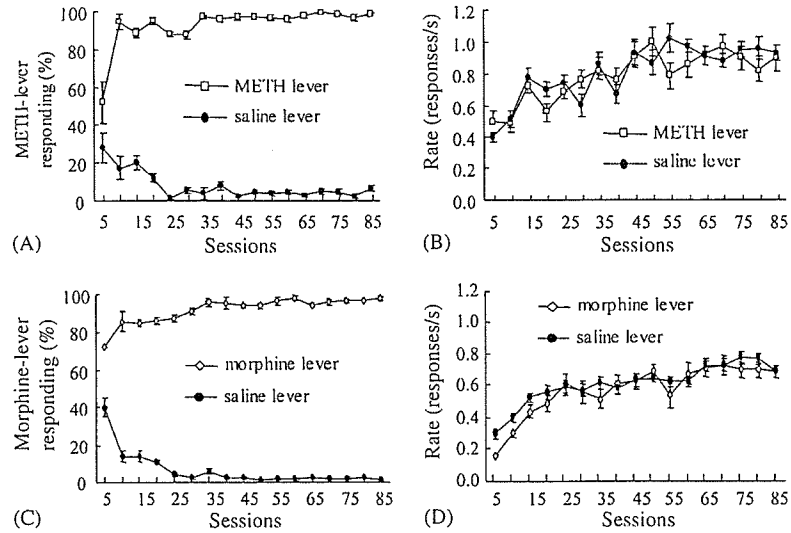

Fig. 1. Acquisition of METH and morphine-induced discrimination in rats. (A) The acquisition of METH (0.5 mg/kg, s.c.)-induced discriminative lever responses in rats ($n = 14$). (B) The rate of lever responses in rats during the period of training for METH-induced discrimination. (C) The acquisition of morphine (5.0 mg/kg, s.c.)-induced discriminative lever responses in rats ($n = 16$). (D) The rate of lever responses in rats during the period of training for morphine-induced discrimination. Data are presented as mean ± SEM.

all the compounds, whereas 0.5 mg/kg of METH and 5.0 mg/kg of morphine were used during all the training sessions of drug discrimination.

3.2. *Effects of rolipram on the discriminative stimuli induced by METH and morphine in rats*

It has been well documented that the cAMP signaling pathway plays a crucial role in the development of drug dependence [6,9,16,26,33–35]. We have previously reported that rolipram attenuates the development of morphine dependence through cAMP system [16]. To further evidence the role of cAMP in modulating drug-induced discrimination, we tested the effects of rolipram, a selective phosphodiesterase IV inhibitor, on METH- and morphine-associated discriminative lever responses. Rolipram at the dose of 0.05 mg/kg significantly inhibited the METH-associated discrimination (Fig. 3A, $p < 0.05$, ANOVA) but had no significant influence on the rate of lever responses in rats. However, rolipram significantly inhibited both METH-associated discrimination and the rate of lever responses at higher doses (0.1 and 0.3 mg/kg; Fig. 3A and B, $p < 0.01$, ANOVA). Similarly, rolipram (0.1 and 0.3 mg/kg) significantly attenuated the discriminative-stimulus effects of morphine in rats (Fig. 3C, $p < 0.05$ and 0.01, respectively, ANOVA), but not the rate of morphine-induced lever responses at any of the doses examined (Fig. 3D). Taken together, these findings have

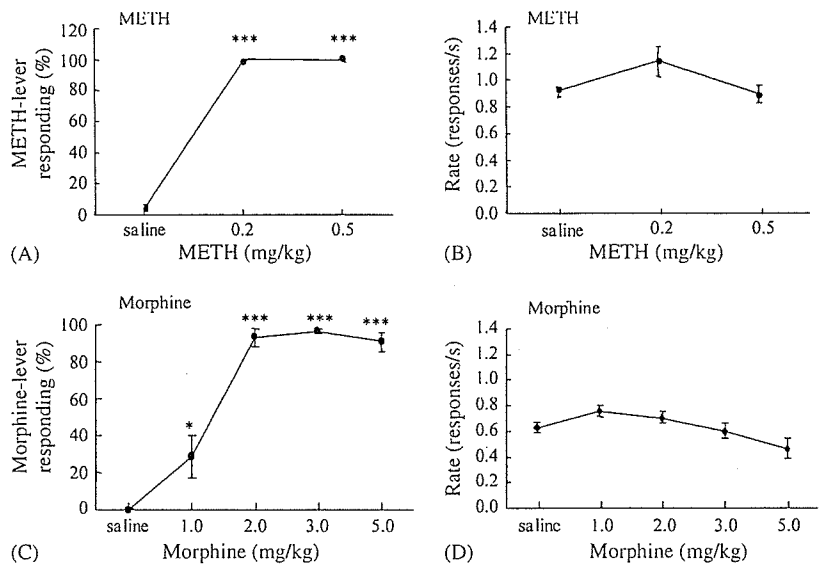

Fig. 2. Dose–response function of discriminative-stimulus effects of METH and morphine in rats. (A) The dose–response function for METH-associated discriminative lever responses in rats ($n = 14$). (B) The rate of lever responses in rats over the dose–response test sessions. (C) The dose–response function for morphine-associated discriminative lever responses in rats ($n = 16$). (D) The rate of lever responses in rats over the dose–response test sessions. Data are presented as mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. vehicle-associated lever responses (one-way ANOVA).

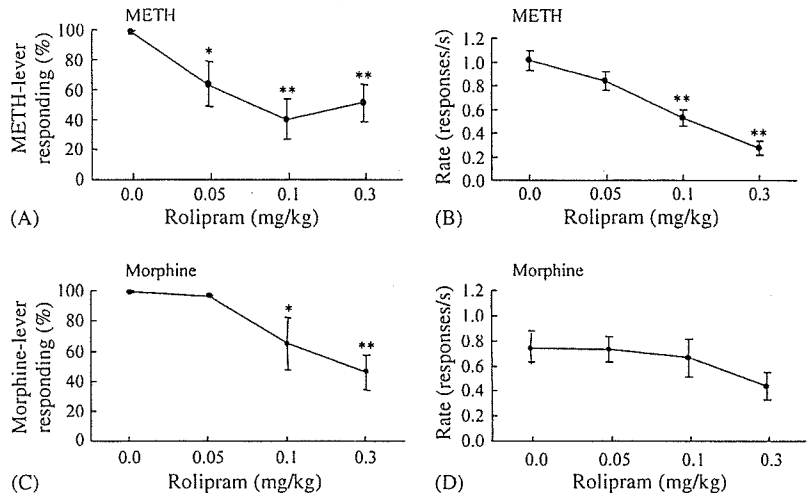

Fig. 3. Rolipram inhibits the discriminative-stimulus effects of METH and morphine in rats. (A) Effects of rolipram on METH (0.2 mg/kg)-induced discriminative lever responses in rats ($n = 14$). (B) The rate of lever responses in rats during the test sessions. (C) Effects of rolipram on morphine (3.0 mg/kg)-induced discriminative lever responses in rats ($n = 16$). (D) The rate of lever responses in rats during the test sessions. Data are presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. vehicle treatment (one-way ANOVA).

clearly implicated cAMP system in the discriminative-stimulus effects of METH and morphine in rats.

3.3. *Effects of nefiracetam on the discriminative stimuli induced by METH and morphine*

Previously, we have reported that nefiracetam attenuates the development of morphine dependence via the cAMP system [9]. In the present study, the effects of nefiracetam (5–50 mg/kg) on METH- and morphine-induced discrimination were examined. Although at lower doses (5–20 mg/kg), nefiracetam did not affect the discriminative-stimulus effects of METH, at 50 mg/kg, it significantly suppressed the effects of METH (Fig. 4A, $p < 0.01$, ANOVA). During the period of testing, the rate of lever responses remained stable (Fig. 4B), suggesting that the inhibitory effects of nefiracetam on METH-induced discriminative stimuli did not result from a general disruption of muscle function or motor activity. Similarly, nefiracetam attenuated the discriminative-stimulus effects of morphine at the dose of 10 mg/kg (Fig. 4C, $p < 0.01$, ANOVA). There was no significant effect of nefiracetam on the rate of morphine-induced lever responses observed during the period of testing (Fig. 4D). Together, these findings suggest that nefiracetam inhibits the discriminative-stimulus effects of both METH and morphine, which are not due to general disruption of muscle function and motor activity.

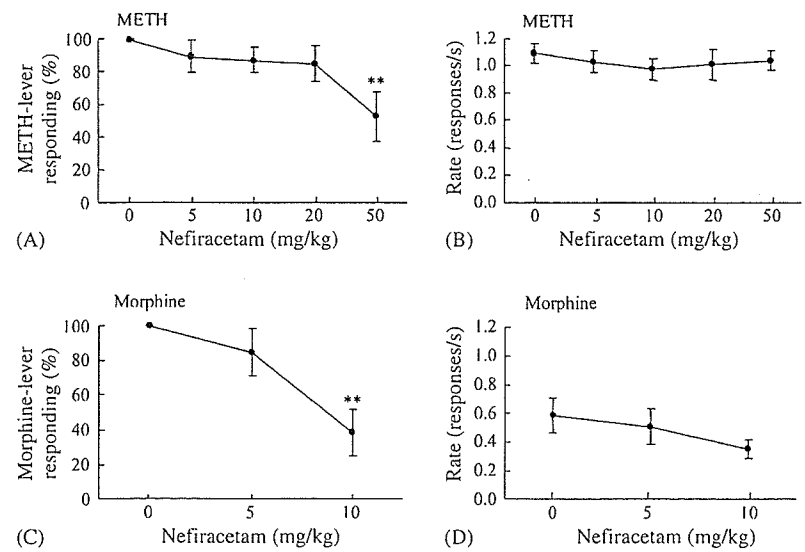

Fig. 4. Nefiracetam inhibits the discriminative-stimulus effects of METH and morphine in rats. (A) Effects of nefiracetam on METH (0.2 mg/kg)-induced discriminative lever responses in rats ($n = 14$). (B) The METH-associated rate of lever responses in rats during the test sessions. (C) Effects of nefiracetam on morphine (3.0 mg/kg)-induced discriminative lever responses in rats ($n = 16$). (D) The rate of lever responses in rats during the test sessions. Data are presented as mean ± SEM. $^{**}p < 0.01$ vs. vehicle treatment (one-way ANOVA).

3.4. Effects of voltage-sensitive calcium channel blockers alone or co-administration with nefiracetam on METH discrimination

There are two proposed intracellular mechanisms underlying nefiracetam's effects, cAMP system and calcium channel system [28,41]. To investigate how nefiracetam inhibits METH-induced discriminative stimuli, the pharmacological effects of voltage-sensitive calcium channel blockers were examined in the present study. We found that the co-administration of nifedipine (0.25–1.0 mg/kg), an L-type calcium channel blocker, with nefiracetam (50 mg/kg) failed to show modulation on METH-induced lever presses, although nifedipine, administered alone, inhibited METH-induced discriminative lever responses in a dose-dependent manner (Fig. 5A). The stable rate of lever responses in rats during all the test sessions indicated that the inhibitory effects of nifedipine on METH discrimination were not resulted from general disruption of muscle function and motor activity (Fig. 5B). Similarly, no modulation of the N-type calcium channel blocker neomycin (2–10 mg/kg) was observed on the regulatory effect of nefiracetam (50 mg/kg) on METH-induced discrimination in rats (Fig. 5C). In addition, neomycin when administered alone significantly attenuated METH-paired discriminative lever responses at the doses of 2 and 10 mg/kg (Fig. 5C, $p < 0.05$, ANOVA), whereas the rate of lever responses in rats maintained stable during all test sessions (Fig. 5D).

We also tested the effect of flunarizine, a non-selective voltage-sensitive calcium channel blocker. This compound, either administered alone (0.2–2.0 mg/kg) or co-administered with nefiracetam (50 mg/kg), failed to show any effect on METH-induced discrimination (Fig. 5E).

Taken together, these findings suggest that voltage-sensitive calcium channels may play no or little role in the inhibitory effects of nefiracetam on METH-induced discrimination.

3.5. Effects of dopamine D2-like receptor antagonists on METH-associated discrimination

We examined the effects of dopamine D2-like receptor antagonists on METH-induced discrimination, since dopamine D2-like receptors modulate the cAMP signaling pathway through inhibitory G proteins [8,13,34]. We found that the dopamine D2 receptor antagonist raclopride (0.3–1.0 mg/kg) significantly inhibited METH-induced discriminative lever responses (Fig. 6A, $p < 0.001$, ANOVA), and the rate of METH-induced lever responses in rats was also reduced by higher doses of raclopride (Fig. 6B, 0.6 and 1.0 mg/kg, $p < 0.05$ and 0.001, respectively, ANOVA). Raclopride at the dose of 0.3 mg/kg significantly prevented METH-induced discrimination but had no significant influence on the rate of lever responses, suggesting that this inhibitory effect of raclopride on METH-associated discrimination did not result from a disruption of motor activity. Finally, we found that L-745870, a selective dopamine D4 recep-

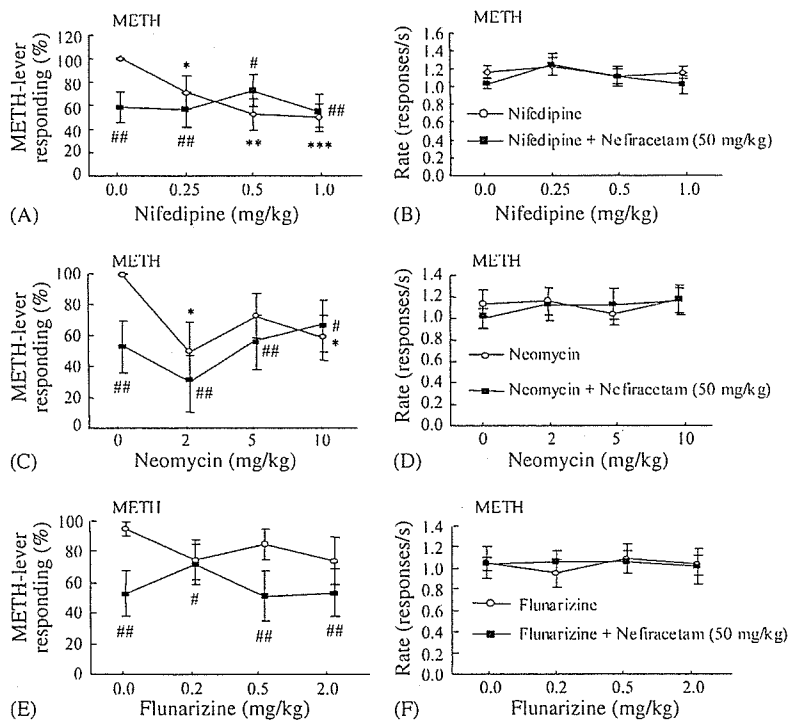

Fig. 5. Effects of voltage-sensitive calcium channel blockers alone or in combination with nefiracetam on METH-induced discriminative lever responses in rats. (A) Effects of nifedipine alone or together with nefiracetam (50 mg/kg) on METH (0.2 mg/kg)-induced discriminative lever responses in rats ($n = 14$). (B) The rate of lever responses in rats during the test sessions. (C) Effects of neomycin alone or in combination with nefiracetam (50 mg/kg) on METH (0.2 mg/kg)-induced discriminative lever responses in rats ($n = 14$). (D) The rate of lever responses in rats during the test sessions. (E) Effects of flunarizine alone or together with nefiracetam (50 mg/kg) on METH (0.2 mg/kg)-induced discriminative lever responses in rats ($n = 14$). (F) The rate of lever responses in rats during the test sessions. Data are presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. treatment with the vehicle for voltage-sensitive calcium channel blockers alone (one-way ANOVA); $^{\#\#}p < 0.01$ vs. treatment with the vehicle for nefiracetam alone (one-way ANOVA).

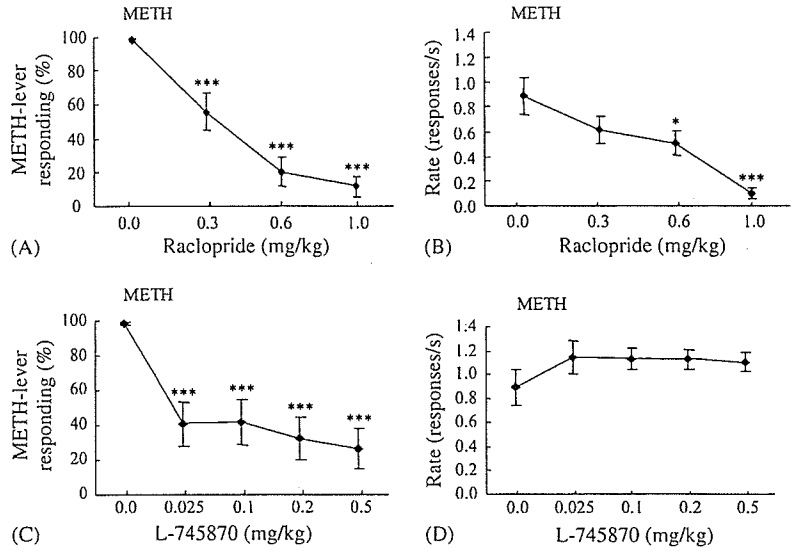

Fig. 6. Dopamine D2-like receptor antagonists inhibit the discriminative-stimulus effects of METH in rats. (A) Effects of raclopride on METH (0.2 mg/kg)-induced discrimination in rats ($n = 14$). (B) The rate of lever responses in rats during the test sessions. (C) Effects of L-745870 on METH (0.2 mg/kg)-induced discriminative lever responses in rats ($n = 14$). (D) The rate of lever responses in rats during the test sessions. Data are presented as mean ± SEM $^{***}p < 0.001$ vs. vehicle treatment (one-way ANOVA).

tor antagonist, disrupted the METH-induced discrimination at all doses examined (Fig. 6C, $p < 0.001$, ANOVA), and there was no change in the rate of lever responses in rats during the test sessions (Fig. 6D).

4. Discussion

In the present study, we tested the effects of several types of compounds including rolipram, nefiracetam, and dopamine D2-like receptor antagonists, and found that all these compounds were effective in inhibiting the discriminative-stimulus effects of METH and morphine in rats. Since all these compounds can regulate in vivo cAMP level, our findings suggest that cAMP signaling cascade plays a critical role in the subjective effects of addictive drugs.

cAMP signaling pathway has been implicated in the development of drug addiction (6, 9, 16, 26, 33, 34, 35). As a selective phophodiesterase IV inhibitor, rolipram (0.5–4.0 mg/kg, i.p.) has been documented to increase cAMP level or reverse a decreased cAMP level in the brain of rodents [10,16,24]. Accordingly, rolipram suppresses METH-induced behavioral sensitization, cocaine self-administration, and morphine-induced physical dependence via regulation of the cAMP system [10,14,16]. In present study, we further confirmed that rolipram attenuated discriminative-stimulus effects of both METH and morphine in rats, probably by maintaining a higher intracellular level of cAMP. Nefiracetam is another compound we have tested that might regulate cAMP system. It has been shown that nefiracetam ameliorates impairments of learning and memory by regulating cAMP activity and/or voltage-sensitive calcium channels in animal models [22,28,39,41]. Moreover, single or repeated administration of nefiracetam (5–10 mg/kg, p.o.) has been reported to increase cAMP level or reverse a decreased cAMP level in the brain of rodents [9,36]. Accordingly, we have previously reported that nefiracetam attenuates the development of morphine dependence via the cAMP system [9]. In the present study, we found that voltage-sensitive calcium channels play no or little role in the inhibitory effects of nefiracetam on METH-induced discriminative lever responses, suggesting that cAMP system may play an important role, at least in part, in the inhibitory effects of nefiracetam on discriminative-stimulus effects of METH and morphine. Thus, nefiracetam and rolipram may share common targets in the discriminative-stimulus effects of METH and morphine in rats by increasing the intracellular cAMP level. On the other hand, voltage-sensitive calcium channel blockers themselves showed inhibitory effects on METH-induced discriminative stimuli in our study. This observation is consistent with previous reports that voltage-sensitive calcium channel blockers inhibit psychostimulant-induced self-administration, conditioned place preference, and locomotor sensitization in a similar manner [17,18,30]. Given that co-administration of nefiracetam and the voltage-sensitive calcium channel blockers did not have any antagonistic effect, the most parsimonious explanation is that there are different neurotransmission circuits or brain areas for the inhibitory effects of nefiracetam and voltage-sensitive calcium channel blockers on METH-induced discriminative stimuli in rats [1,21,23,25,37,43].

It has been reported that the ventral tegmental area and nucleus accumbens are key brain areas involved in discriminative-stimulus effects of both METH and morphine, although the periaqueductal gray and parabranchial nucleus are also related to discriminative-stimulus effects of morphine in rats [15,25]. Dopaminergic transmission has been shown to play a key role in discriminative-stimulus effects of psychostimulants and opioids [21,29,31,37]. Once activated, dopamine D2-like receptors couple with inhibitory G-proteins and reduce cAMP activity [8,13,33,34]. Thus, we examined effects of both dopamine D2 and D4 receptor antagonists on discriminative-stimulus effects of METH. In our study, both dopamine D2 (raclopride) and D4 (L-745870) receptor

antagonists attenuated the discriminative-stimulus effects of METH in rats. These results are consistent with reports that dopaminergic transmission is involved in the discriminative-stimulus effects of METH [21,37]. Interestingly, the selective dopamine D4 receptor antagonist L-745870 showed strong antagonizing effects on METH-induced discrimination but had no influence on the rate of lever responses in rats, whereas the selective dopamine D2 receptor antagonist raclopride affected both METH-induced discriminative stimuli and the rate of lever responses in rats at higher doses. These results might suggest that, compared with dopamine D2 receptor, dopamine D4 receptor represents a better potential target in the treatment of the subjective effects of addictive drugs. The inhibitory effects of dopamine D2-like receptor antagonists may also support our hypothesis that the cAMP system plays an important role in the discriminative-stimulus effects of METH in rats.

In conclusion, we found that rolipram, nefiracetam, and dopamine D2-like receptor antagonists attenuated the discriminative-stimulus effects of METH and morphine in rats, suggesting an important role of the cAMP system in regulating drug-induced discriminative stimuli. Therefore, the cAMP signaling cascade may represent a potential target in the treatment of drug addiction.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Scientific Research 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; by a Grant-in-Aid for Health Science Research on Regulatory Science of Pharmaceuticals and Medical Devices, and Dementia and Fracture from the Ministry of Health, Labor and Welfare of Japan; by a Grant-in-Aid for Scientific Research (B) and Young Scientists (A); by the 21st Century Center of Excellence Program "Integrated Molecular Medicine for Neuronal and Neoplastic Disorders" from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and in part by Uehara Memorial Foundation Research Fellows.

References

[1] Abe E, Murai S, Saito H, Masuda Y, Itoh T. Effects of nefiracetam on deficits in active avoidance response and hippocampal cholinergic and monoaminergic dysfunctions induced by AF64A in mice. J Neural Transm 1994;95:179–93.

[2] Bari AA, Pierce RC. D1-like and D2 dopamine receptor antagonists administered into the shell subregion of the rat nucleus accumbens decrease cocaine, but not food, reinforcement. Neuroscience 2005;135:959–68.

[3] Czoty PW, Makriyannis A, Bergman J. Methamphetamine discrimination and in vivo microdialysis in squirrel monkeys. Psychopharmacology 2004;175:170–8.

[4] Czoty PW, Ramanathan CR, Mutschler A, Bergman J. Drug discrimination in methamphetamine-trained monkeys: effects of monoamine transporter inhibitors. J Pharmacol Exp Ther 2004;311:720–7.

[5] Deford SM, Wilson MS, Gibson CJ, Banarova A, Hamm RJ. Nefiracetam improves Morris water maze performance following traumatic brain injury in rats. Pharmacol Biochem Behav 2001;69:611–6.

[6] Ferrer-Alcon M, Garcia-Fuater J, La Harpe R, Garcia-Sevilla JA. Long-term regulation of signalling components of adenylyl cyclase and mitogen-activated protein kinase in the pre-frontal cortex of human opiate addicts. J Neurochem 2004;90:220–30.

[7] Garcia-Sevilla JA, Ferrer-Alcon M, Martin M, Kieffer BL, Maldonado R. Neurofilament proteins and cAMP pathway in brains of mu-, delta- or kappa-opioid receptor gene knock-out mice: effects of chronic morphine administration. Neuropharmacology 2004;46:519–30.

[8] Hopf FW, Cascini MG, Gordon AS, Diamond I, Bonci A. Cooperative activation of dopamine D1 and D2 receptors increases spike firing of nucleus accumbens neurons via G-protein betagamma subunits. J Neurosci 2003;23:5079–87.

[9] 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.

[10] Iyo M, Bi Y, Hashimoto K, Inada T, Fukui S. Prevention of methamphetamine-induced behavioral sensitization in rats by a cyclic AMP phophodiesterase inhibitor, rolipram. Eur J Pharmacol 1996;312:163–70.

[11] Justinova Z, Ferre S, Segal PN, Antoniou K, Solinas M, Pappas LA, et al. Involvement of adenosine A1 and A2A receptors in the adenosinergic modulation of the discriminative-stimulus effects of cocaine and methamphetamine in rats. J Pharmacol Exp Ther 2003;307:977–86.

[12] Kamien JB, Bickel WK, Hughes JR, Higgins ST, Smith BJ. Drug discrimination by humans compared to nonhumans: current status and future directions. Psychopharmacology 1993;111:259–70.

[13] Kebabian J, Greengard P. Dopamine-sensitive adenyl cyclase: possible role in synaptic transmission. Science 1991;174:1346–9.

[14] Knapp CM, Foye MM, Ciraulo DA, Kornetsky C. The type IV phosphodiesterase inhibitors, Ro 20-1724 and rolipram, block the initiation of cocaine self-administration. Pharmacol Biochem Behav 1998;62:151–8.

[15] Krivsky JA, Stoffel EC, Sumner JE, Bryce C, Craft RM. Role of ventral tegmental area, periaqueductal gray and parabrachial nucleus in the discriminative stimulus effects of morphine in the rat. Behav Pharmacol 2006;17:259–70.

[16] Mamiya T, Noda Y, Ren X, Hamdy M, Furukawa S, Kameyama T, et al. Involvement of cyclic AMP systems in morphine physical dependence in mice: prevention of development of morphine dependence by rolipram, a phosphodiesterase 4 inhibitor. Br J Pharmacol 2001;132:1111–7.

[17] Martellotta MC, Kuzmin A, Muglia P, Gessa GL, Fratta W. Effects of the calcium antagonist isadipine on cocaine intravenous self-administration in rats. Psychopharmacology 1994;113:378–80.

[18] Martin-Iverson MT, Reimer AR. Effects of minodipine and/or haloperidol on the expression of conditioned locomotion and sensitization to cocaine in rats. Psychopharmacology 1994;114:315–20.

[19] McMahon LR, Sell SL, France CP. Cocaine and other indirect-acting monoamine agonists differentially attenuate a naltrexone discriminative stimulus in morphine-treated rhesus monkeys. J Pharmacol Exp Ther 2004;308:111–19.

[20] Mori A, Noda Y, Mamiya T, Miyamoto Y, Nakajima A, Furukawa H, et al. Phencyclindine-induced discriminative stimulus is mediated via phencyclidine binding sites on the *N*-methyl-D aspartate receptor-ion channel complex, not by sigma 1 receptors. Behav Brain Res 2001;119:33–40.

[21] Munzar P, Goldberg SR. Dopaminergic involvement in the discriminative-stimulus effects of methamphetamine in rats. Psychopharmacology 2000;148:209–16.

[22] Nabeshima T. Ameliorating effects of nefiracetam (DM-9384) on brain dysfunction. Drugs Today 1994;30:357–79.

[23] Nabeshima T, Noda Y, Tohyama K, Itoh J, Kameyama T. Effects of DM-9384 in a model of amnesia based on animals with GABAergic neuronal dysfunctions. Eur J Pharmacol 1990;178:143–9.

[24] Nagakura A, Niimura M, Takeo S. Effects of a phosphodiesterase IV inhibitor rolipram on microsphere embolism-induced defects in memory function and cerebral cyclic AMP signal transduction system in rats. Br J Pharmacol 2002;135:1783–93.

[25] Nakajima A, Yamada K, He J, Zeng N, Nitta A, Nabeshima T. Anatomical substrates for the discriminative stimulus effects of methamphetamine in rats. J Neurochem 2004;91:308–17.
[26] Nestler EJ. Molecular basis of long-term plasticity underlying addiction. Nat Rev Neurosci 2001;2:119–28.
[27] Nishizaki T, Matsuoka T, Nomura T, Mtsuyama S, Watabe S, Shiotani T, et al. A 'long-term-potentiation-like' facilitation of hippocampal synaptic transmission induced by the nootropic nefiracetam. Brain Res 1999;826:281–8.
[28] Nishizaki T, Matsuoka T, Nomura T, Sumikawa K, Shiotani T, Watabe S, et al. Nefiracetam modulates acetylcholine receptor currents via two different signal transduction pathways. Mol Pharmacol 1998;53:1–5.
[29] Platt DM, Rowlett JK, Spealman RD. Discriminative stimulus effects of intravenous heroin and its metabolites in rhesus monkeys: opioid and dopaminergic mechanisms. J Pharmacol Exp Ther 2001;299: 760–7.
[30] Rosenweig-Lipson S, Barrett JE. Modification of the behavioral effects of (±) BAY 8644, cocaine and D-amphetamine by L-type calcium channel blockers in squirrel monkeys. J Pharmacol Exp Ther 1995;274:842–51.
[31] Rowlett JK, Platt DM, Spealman RD. Cocaine-like discriminative stimulus effects of heroin: modulation by selective monoamine transport inhibitors. J Pharmacol Exp Ther 2004;310:342–8.
[32] Schmidt HD, Anderson SM, Pierce RC. Stimulation of D1-like or D2 dopamine receptors in the shell, but not the core, of the nucleus accumbens reinstates cocaine-seeking behaviour in the rat. Eur J Neurosci 2006;23:219–28.
[33] Self DW. Regulation of drug-taking and -seeking behaviors by neuroadaptations in the mesolimbic dopamine system. Neuropharmacology 2006;47:242–55.
[34] Self DW, Genova LM, Hope BT, Barnhart WJ, Spencer JJ, Nestler EJ. Involvement of cAMP-dependent protein kinase in the nucleus accombens in cocaine self-administration and relapse of cocaine-seeking behavior. J Neurosci 1998;18:1848–59.
[35] Shichinohe S, Ozawa H, Haahimoto E, Tatachner T, Riederer P, Saito T. Changes in the cAMP-related signal transduction mechanism in postmortem human brains of heroin addicts. J Neural Transm 2001;108:335–47.
[36] Takeo S, Niimura M, Miyake-Takagi K, Nagakura A, Fukatsu A, Ando T, et al. Persistent effects of delayed treatment with nefiracetam on the water maze task in rats with sustained cerebral ischemia. J Pharmacol Exp Ther 2003;304:513–23.
[37] Tidey JW, Bergman J. Drug discrimination in methamphetamine-trained monkeys: agonist and antagonist effects of dopaminergic drugs. J Pharmacol Exp Ther 1998;285:1163–74.
[38] Walters CL, Blendy JA. Different requirements for cAMP response element binding protein in positive and negative reinforcing properties of drugs of abuse. J Neurosci 2001;21:9438–44.
[39] Watabe S, Yoshii M. Ion channels and cognitive enhancers. Jpn J Neuropsychopharmacol 1993;15:249–57.
[40] Woodruff-Pak DS, Li YT, Hinchliffe RM, Port RL. Hippocampus in delay eyeblink classical conditioning: essential for nefiracetam amelioration of learning in older rabbits. Brain Res 1997;747:207–18.
[41] Yamada K, Nakayama S, Shiotani T, Hasegawa T, Nabeshima T. Possible involvement of the activation of voltage-sensitive calcium channels in the ameliorating effects of nefiracetam on scopolamine-induced impairment of performance in a passive avoidance task. J Pharmacol Exp Ther 1994;270:881–92.
[42] Yamada K, Tanaka T, Mamiya T, Shiotani T, Kameyama T, Nabeshima T. Improvement by nefiracetam of β-amyloid$_{1-42}$-induced learning and memory impairments in rats. Br J Pharmacol 1999;126:235–44.
[43] Zhao XL, Kuryatov A, Lindstrom JM, Yeh JZ, Narahashi T. Nootropic drug modulation of neuronal nicotinic acetylcholine receptors in rat cortical neurons. Mol Pharmacol 2001;59:674–83.

Available online at www.sciencedirect.com
ScienceDirect
Behavioural Brain Research 177 (2007) 261–268
BEHAVIOURAL BRAIN RESEARCH
www.elsevier.com/locate/bbr

Research report

Transient drug-primed but persistent cue-induced reinstatement of extinguished methamphetamine-seeking behavior in mice

Yijin Yan[a,b], Kiyofumi Yamada[c], Atsumi Nitta[a], Toshitaka Nabeshima[a,d,*]

[a] *Department of Neuropsychopharmacology & Hospital Pharmacy, Nagoya University, Graduate School of Medicine, Nagoya 466-8560, Japan*
[b] *College of Bioengineering, Henan University of Technology, Zhengzhou 450052, China*
[c] *Laboratory of Neuropsychoparmacology, Graduate School of Natural Science & Technology, Kanazawa University, Kanazawa 920-1192, Japan*
[d] *Japanese Drug Organization of Appropriate Use and Research, Nagoya 468-0069, Japan*

Received 19 September 2006; received in revised form 13 November 2006; accepted 15 November 2006
Available online 19 December 2006

Abstract

It is essential to develop animal models to study the role of genetic factors in the relapse of drug-seeking behavior in genetically engineered mutant mice. This paper reports a typical model of drug-primed and cue-induced reinstatement of extinguished methamphetamine (METH)-seeking behavior in mice. C57BL/6J mice were trained to self-administer METH (0.1 mg/kg/infusion) by poking their nose into an active hole under a fixed ratio schedule in daily 3-h sessions. After acquiring stable METH self-administration behavior, the mice were subjected to extinction training in the absence of both METH and METH-associated cues. Once the active nose-poking responses were extinguished, drug-primed and cue-induced reinstatement were investigated according to a within-subjects design. A priming injection of METH reliably reinstated the extinguished drug-seeking behavior in the absence of both METH and METH-associated cues. Interestingly, the drug-primed METH-seeking behavior disappeared within 2 months after withdrawal from METH, while cue-induced reinstatement of extinguished METH-seeking behavior lasted for at least 5 months after the withdrawal. A correlation study revealed that drug-primed, but not cue-induced, reinstatement behavior was positively correlated with the total amount of METH taken by individuals during METH self-administration. In conclusion, our findings suggest that the present reinstatement procedure for mouse model of relapse is useful and reliable, and different neural mechanisms may be involved in drug-primed and cue-induced METH-seeking behavior.

Keywords: Drug-primed reinstatement; Cue-induced reinstatement; Intravenous self-administration; Methamphetamine; Mice

1. Introduction

Epidemiological reports suggest that genetic factors contribute to 30–60% of the variability in drug dependence/addiction and alcoholism including vulnerability to relapse of taking drugs of abuse [15,16,21,23,32,33,35]. In 1991, a mouse model of i.v. self-administration was reported [4], and since then several specific genes/proteins have been identified as neural substrates of addiction, which are involved in the acquisition and maintenance of drug dependence, including the dopamine D2 receptor [3,7,11], the serotonin 1B receptor [26], an acetylcholine receptor containing beta 2 [22], the mu-opioid receptor [24], metabotropic glutamate receptor 5 [5], cannabinoid receptor 1 [8,29], the Kir3 potassium channel subunit [20], and the homer 2 [30]. In contrast, no direct evidence has been obtained of genetic factors involved in susceptibility to relapse. Since most genetically modified model animal strains are inbred mice, there is an impetus to develop reinstatement procedures in mice. More recently, efforts have been made to establish a mouse model of relapse [12,14,37]. Unfortunately, in mice, neither a widely acceptable drug-primed reinstatement procedure nor information on the characterization of reinstatement behavior is available. Importantly, failure of drug-primed reinstatement behavior in mice may cast doubt on the validity of previously published data on drugs of abuse from other mouse models.

Over the past several years, abuse of methamphetamine (METH) has spread world-wide, resulting in serious health

* Corresponding author at: Department of Neuropsychopharmacology & Hospital Pharmacy, Nagoya University, Graduate School of Medicine, Showa-ku, Nagoya 466-8560, Japan. Tel.: +81 52 744 2674; fax: +81 52 744 2682.
E-mail address: tnabeshi@med.nagoya-u.ac.jp (T. Nabeshima).

0166-4328/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbr.2006.11.033

and social issues. A major clinical concern in the treatment of METH addiction is relapse even after long-term abstinence [2,6,13,34,35]. In the present study, we developed a typical animal model of METH-primed reinstatement behavior and found that C57BL/6J mice showed a short-term drug-primed, but a long-lasting cue-induced, reinstatement of extinguished METH-seeking behavior.

2. Materials and methods

2.1. Subjects and drugs

Male C57BL/6J mice (SLC, Tokyo, Japan) were 8 weeks old and weighed 25–30 g at the beginning of the experiments. All mice were kept in a regulated environment (23 ± 0.5 °C; 50 ± 0.5% humidity) with a reversed 12-h light/dark cycle (lights on at 9:00 a.m.). After the surgery to implant catheter into jugular vein, mice were housed singly until the end of all the experiments. Water and food were available *ad libitum* throughout the experiments. Animal care and use was in accordance with the Principles of Laboratory Animal Care (National Institutes of Health Publication 85-123, 1983) and was approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine. Animals were treated according to the Guidelines of Experimental Animal Care issued from the Office of the Prime Minister of Japan.

METH hydrochloride (Dainippon Pharmaceutical Ltd., Osaka, Japan) was dissolved in sterile saline and self-administered at the dose of 0.1 mg/kg/infusion over 5 s (infusion volume, 2.1 μl). The unit dose for METH self-administration is based on our previous report [37].

2.2. Apparatus for METH self-administration

METH self-administration, extinction and subsequent tests for reinstatement of METH-seeking behavior were conducted in standard mouse operant conditioning chambers (ENV-307A, Med Associates, Georgia, VT) located within ventilated sound attenuation cubicles as described previously [37]. Briefly, the operant conditioning chambers were equipped with nose-poke sensors (ENV-313M, Med Associates) in two holes located on one side of the chamber 1.0 cm above the floor, cue- and hole-lamps located, respectively, above and in each hole, and a red house light located on the top of the chamber opposite the holes. During the self-administration, one hole was defined as active, and the other as inactive. For all the mice, the active hole was counterbalanced across sides. Nose-poking responses in the active hole resulted in activation of the infusion pump (PHM-100, Med Associates) and inactivation of the cue-lamp and hole-lamp for 5 s followed by a 5-s timeout period. Responses in the active hole during the time-out period and in the inactive hole had no programmed consequences but were recorded by the software MED-PC for Windows (Med Associates). The components of the infusion line were connected to each other from the injector to the exit port of the mouse's catheter by joint FEP tubing (inner diameter, 0.25 mm; outer diameter, 0.55 mm; Eicom Co., Ltd., Japan), which was encased in steel spring leashes (Instech, Plymouth Meeting, PA). Swivels were suspended above the operant conditioning chamber. One pump/syringe set was used for each self-administration chamber located inside of a cubicle. The infusion pump/syringe set was outside of the chambers but inside of the cubicles.

2.3. Catheter implantation

Twenty naïve C57BL/6J mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). Indwelling catheters were constructed of micro-silicone tubing (inner diameter, 0.50 mm; outer diameter, 0.7 mm; IMG, Imamura Co., Ltd., Tokyo, Japan) and polyethylene tubing (inner diameter, 0.50 mm; outer diameter, 0.8 mm). Incisions were made on the skin of the head and ventral neck, and the right jugular vein was externalized. The end of the catheter was inserted into the jugular vein *via* a small incision and was secured to the vein and surrounding tissue with silk sutures. The exit port of the catheter passed subcutaneously to the top of the skull where it was attached to a modified 24-gauge cannula, which was secured to the mouse's skull with quick self-curing acrylic resin (Shofu Inc., Osaka, Japan). To extend catheter patency, the catheters were flushed immediately after surgery, and in the morning and evening of the following days, with 0.03 ml of an antibiotic solution of cefmetazole sodium (20.0 mg/ml; Sankyo Co., Ltd., Tokyo, Japan) dissolved in heparinized saline (70 Unit/ml; Leo Pharmaceutical Products, Ltd., Tokyo, Japan). Patency was usually confirmed once a week prior to operant behavior tests by intravenous injection of pentobarbital sodium solution (6.0 mg/ml, 0.15 ml/mouse). If the mice could not be knocked down within 5 s, the corresponding data were excluded from the statistical analysis.

2.4. Acquisition of METH self-administration

After recovering from the surgery to implant the catheter, the mice ($n = 18$) were subjected to daily 3-h sessions of METH self-administration under a fixed ratio (FR) schedule of reinforcement. During the self-administration, nose-poking responses in the active hole resulted in an infusion of METH at the dose of 0.1 mg/kg/infusion over 5 s (infusion volume, 2.1 μl) followed by a 5-s timeout period. Responses in the active hole during the timeout period and in the inactive hole had no programmed consequences but were recorded. Once the mice could make a minimum of 60% nose-poking responses in the active hole and received no less than 10 infusions of METH over 2 consecutive sessions (on day 6 in the present study), METH reinforcement schedule was changed to FR2. Then, the mice gradually acquired stable METH self-administration behavior (deviations of less than 15% of the mean of active responses in three consecutive training sessions). Eight mice were excluded from the statistical analysis of all the METH self-administration, extinction, and the first drug-primed and cue-induced reinstatement data, but three more mice were excluded from the statistical analysis of the second tests for METH-primed and cue-induced reinstatement behavior, because of a failure of catheter patency or their death from infection.

2.5. Extinction

According to a within-subjects design, the mice ($n = 10$) were then subjected to extinction training before the tests for reinstatement of extinguished drug-seeking behavior as follows: 10–20 daily 3-h sessions of extinction training before the first test for METH-primed reinstatement (1st Primed-reinstatement (RLP)); 6–10 daily 3-h sessions of extinction training before the first test for cue-induced reinstatement (1st Cue-RLP), the second tests for METH-primed and cue-induced reinstatement 2 months after (2nd Primed-RLP or 2nd Cue-RLP), and the third test for cue-induced reinstatement 5 months after (3rd Cue-RLP) withdrawal from METH self-administration. The extinction criterion was as follows: less than 15 active responses or 25% of active responses in the stable phase of self-administration in two consecutive sessions. Throughout the extinction session, the house light was on. The METH-associated cues (cue- and hole-lamps, and the pump noise for an infusion of METH) were unavailable. Nose-poking responses in a previously active hole were counted as active. Nose-poking responses in a previously inactive hole were counted as inactive.

2.6. METH-primed reinstatement

Once the extinction criterion was met, the mice ($n = 10$) were firstly subjected to a daily 3-h session of operant test 30 min after an injection (i.p.) of saline as a control for the METH-primed reinstatement. From the next day, the mice were consecutively subjected to the first test for METH-primed reinstatement (1st Primed-RLP) 30 min after the i.p. injection with ascending doses of METH (0.2, 0.4, 1.0, or 2.0 mg/kg; each dose for one daily 3-h session, without extinction training across the sessions). To investigate the duration of drug-primed reinstatement of extinguished METH-seeking behavior, the mice ($n = 7$) were subjected to extinction training 2 months after withdrawal from METH self-administration. Once the extinction criterion was met, the second test (2nd Primed-RLP) for METH-primed reinstatement was conducted as described above. The tests for METH-primed reinstatement were conducted under the same conditions as in the extinction sessions in which nose-poking responses into a previously active hole resulted in neither infusion of METH nor METH-associated cues. Nose-poking responses in a previously active hole were counted as active. Nose-poking responses in a previously inactive hole were counted as inactive.

2.7. Cue-induced reinstatement

Immediately after the tests for METH-primed reinstatement (1st or 2nd Primed-RLP) or 5 months after withdrawal from METH self-administration, the same group of mice was subjected to the extinction training once again. Once the extinction criterion was met, the mice were subjected to the tests for cue-induced reinstatement (1st, 2nd or 3rd Cue-RLP). The tests for cue-induced reinstatement were conducted under the same conditions as the METH self-administration under the FR2 schedule, except that there was no infusion of METH after nose-poking responses in a previously active hole throughout the testing session. Nose-poking responses in a previously active hole were counted as active. Nose-poking responses in a previously inactive hole were counted as inactive.

2.8. Data analysis

All data were expressed as mean ± S.E.M. A two-way analysis of variance (ANOVA) with repeated measures was performed to analyze the difference of active and inactive nose-poking responses during the METH self-administration, extinction, and tests for METH-primed or cue-induced reinstatement behavior, followed *post hoc* by the Bonferroni/Dunn test. A one-way ANOVA with repeated measures was performed to analyze the change in active nose-poking responses during extinction training and tests for METH-primed and cue-induced reinstatement behavior, followed *post hoc* by the Bonferroni/Dunn test. The correlation between the total amount of METH intake, active nose-poking responses in the first session of extinction, and active nose-poking responses in METH-primed and cue-induced reinstatement was analyzed by a Simple regression. In all cases, a significant difference was set at $P < 0.05$.

3. Results

3.1. Acquisition and retention of METH self-administration

As shown in Fig. 1, naïve C57BL/6J mice could not discriminate active (with an infusion of METH) nose-poking responses from inactive (without an infusion of METH) ones at the early phase of METH self-administration under an FR1 schedule of reinforcement (sessions 1–5). In session 6, the mice showed a tendency to discriminate active from inactive nose-poking responses under the FR1 schedule. Therefore, an FR2 schedule of METH reinforcement was introduced. Thereafter, the mice gradually exhibited stable active nose-poking responses for METH self-administration (with a within-subjects variability of less than 15% in daily active nose-poking responses for at least three consecutive days), suggesting that the mice acquired stable METH self-administration behavior after 10 daily 3-h sessions of METH self-administration under the FR2 schedule (Fig. 1, two-way ANOVA with repeated measures, $F_{(1,288)} = 352.4$, $P < 0.001$). The mean number of active nose-poking responses for METH reinforcement during the stable phase of self-administration was 60.0 ± 5.5 in a daily 3-h session. The average total amount of METH taken in was 24.9 ± 1.8 mg/kg during 16 daily 3-h sessions of self-administration. During the conversion of the FR1 into the FR2 schedule, there was no significant change in the number of METH infusions (day 6–16 in Fig. 1, a repeated one-way ANOVA, $F_{(10,99)} = 1.7$, $P > 0.05$).

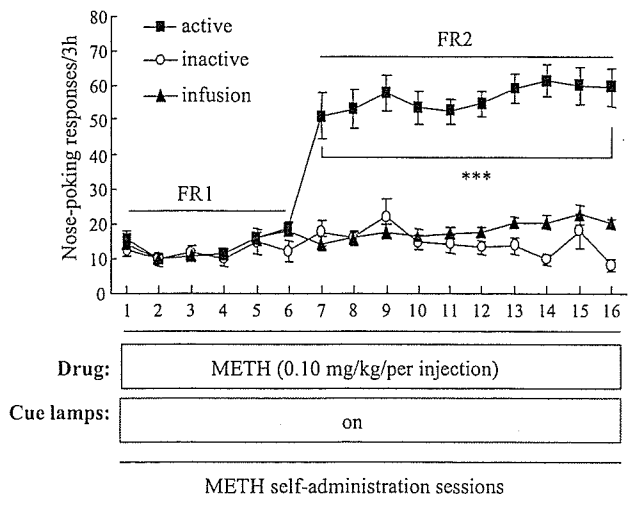

Fig. 1. Nose-poking responses and the number of METH infusions during METH self-administration. METH (0.1 mg/kg/infusion) self-administration was under an FR1 schedule during sessions 1–6 and under an FR2 schedule during sessions 7–16. Data are presented as mean ± S.E.M. $^{***}P < 0.001$ active vs. inactive nose-poking responses.

3.2. Nose-poking responses during extinction

As shown in Fig. 2, during the period of extinction training before the first test for METH-primed reinstatement (1st Primed-RLP), the mice exhibited many more active nose-poking responses (124.2 ± 16.2) than those (52.2 ± 12.5) in the stable phase of METH self-administration at the beginning of extinction training (Ext 1–6, two-way ANOVA with repeated measures, $F_{(1,108)} = 17.2$, $P < 0.001$) in the absence of METH. This phenomenon once again suggests that the mice have developed reliable METH self-administration behavior after 16 daily 3-h sessions of self-administration training. The subsequent daily 3-h sessions of extinction training resulted in a decrease in active nose-poking responses. During the late phase of extinction training (LExt 1–3), the mice met the extinc-

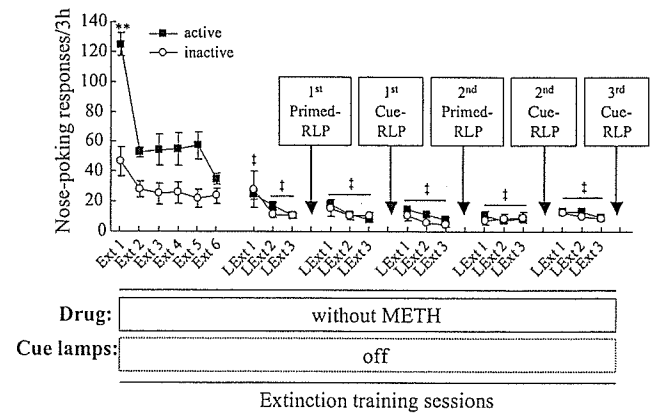

Fig. 2. Active and inactive nose-poking responses during extinction. Prior to the first test for drug-primed reinstatement (1st Primed-RLP), the data were from the first 6 daily 3-h sessions (Ext 1–6) and the last 3 daily 3-h sessions (LExt 1–3) during 10–20 daily 3-h sessions of extinction training. Thereafter, the data were from the last 3 daily 3-h sessions (LExt 1–3) during 6–10 daily 3-h sessions of extinction training. Data are presented as mean ± S.E.M. $^{**}P < 0.01$ active vs. inactive nose-poking responses. $^{\ddagger}P < 0.01$ vs. active nose-poking responses in the first session of extinction. RLP: reinstatement; Ext: extinction; LExt: the last session of extinction.

tion criterion (less than 15 active responses or 25% of active responses in the stable phase of self-administration in two consecutive sessions). The average number of active nose-poking responses (11.1 ± 2.2) was significantly small as compared with that (124.2 ± 16.2) in the first session of extinction (Fig. 2, a repeated one-way ANOVA, $F_{(15,117)} = 35.9$, $P < 0.001$). Accordingly, the mice made active (previously associated with METH self-administration) and inactive (previously without METH self-administration) nose-poking responses in a random manner, suggesting that the purposely made active nose-poking responses acquired during METH self-administration had been extinguished after 10–20 daily 3-h sessions of extinction.

During the period of extinction training before the first test for cue-induced reinstatement (1st Cue-RLP) and the second test for METH-primed or cue-induced reinstatement (2nd Primed-RLP and 2nd Cue-RLP), the mice did not exhibit more active nose-poking responses than in the first session of extinction (Ext 1). After 6–10 daily 3-h sessions of extinction training, the mice met the extinction criteria. During the late phase of extinction training (Fig. 2, LExt 1–3, a repeated one-way ANOVA, $F_{(15,117)} = 35.9$, $P < 0.001$), the mice made active and inactive nose-poking responses in a random manner, suggesting that the purposely made active nose-poking responses had been extinguished after 6–10 daily 3-h sessions of extinction.

3.3. Transient METH-primed reinstatement

As shown in Fig. 3A, during the first test (1st Primed-RLP) for METH-primed reinstatement of extinguished drug-seeking behavior, the priming injection with saline or the lower doses of METH (0.2 and 0.4 mg/kg, i.p.) failed to reinstate drug-seeking behavior in mice. In contrast, the priming injection with 1.0 mg/kg of METH (i.p.) reliably provoked reinstatement of extinguished drug-seeking behavior (a repeated one-way ANOVA, $F_{(4,45)} = 2.73$, $P < 0.001$). The priming injection with 2.0 mg/kg of METH (i.p.) did not evoke extinguished active nose-poking responses. During the 1st test for METH-primed reinstatement behavior, there was significant difference between active and inactive nose-poking responses (Fig. 3A and B, two-way ANOVA with repeated measures, $F_{(1,90)} = 4.1$, $P < 0.05$), suggesting that the priming effects of METH on previously extinguished active nose-poking responses are specific. Two months after withdrawal from METH self-administration (2nd Primed-RLP), however, the same group of mice failed to show a reinstatement of extinguished METH-seeking behavior at any of the doses of METH examined (Fig. 3A, 0.2–2.0 mg/kg, i.p.). These results suggest that the METH-primed reinstatement of extinguished drug-seeking behavior in mice was transient. In addition, as shown in Fig. 3B, there was no significant difference in inactive nose-poking responses between saline and METH priming at any dose examined during the 1st and 2nd tests for METH-primed reinstatement behavior.

3.4. Persistent cue-induced reinstatement

As shown in Fig. 4, during the first test (1st Cue-RLP) for cue-induced reinstatement of extinguished drug-seeking behavior, the mice displayed many more active nose-poking responses after being exposed to METH-associated cues (a repeated one-way ANOVA, $F_{(5,42)} = 34.10$, $P < 0.001$). In contrast, there was no change in inactive nose-poking responses after the exposure to METH-associated cues. To investigate the duration of cue-induced reinstatement of extinguished drug-seeking behavior, the same group of mice was subjected to second (2nd Cue-RLP) and third (3rd Cue-RLP) tests for cue-induced reinstatement 2 and 5 months after withdrawal from METH self-administration, respectively. A typical METH-associated cue-induced reinstatement of extinguished active nose-poking responses remained 5 months after withdrawal from METH self-administration (a repeated one-way ANOVA, $F_{(5,42)} = 34.10$, $P < 0.05$), although the cue-induced reinstatement was attenuated after repeated extinction training (a repeated one-way ANOVA, $F_{(5,42)} = 34.10$, $P < 0.05$). During all the tests for cue-induced reinstatement (1st, 2nd or 3rd Cue-RLP), there was no significant change in inactive nose-poking responses after the exposure to METH-associated cues. These results suggest that the mice exhibited a long-lasting (at least 5 months) cue-induced reinstatement of extinguished drug-seeking behavior.

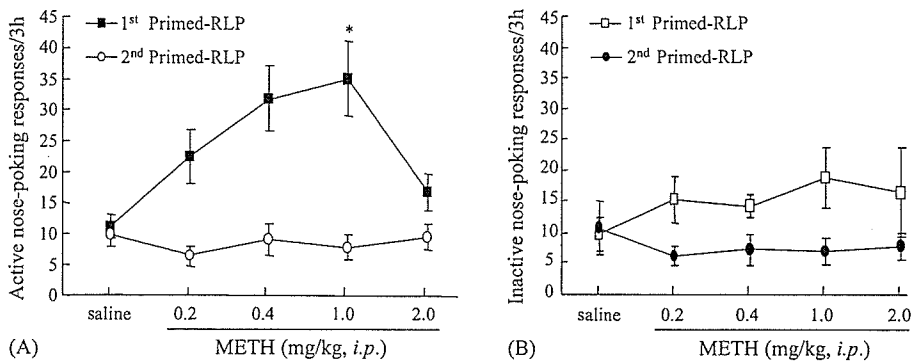

Fig. 3. Active (A) and inactive (B) nose-poking responses during METH-primed reinstatement. The first test for METH-primed reinstatement (1st Primed-RLP) was performed immediately after withdrawal from METH self-administration. The second test for METH-primed reinstatement (2nd Primed-RLP) was performed 2 months after withdrawal from METH self-administration. Data are presented as mean ± S.E.M. $^{*}P < 0.05$ vs. active nose-poking responses after the priming injection (i.p.) with saline in the first test for METH-primed reinstatement.

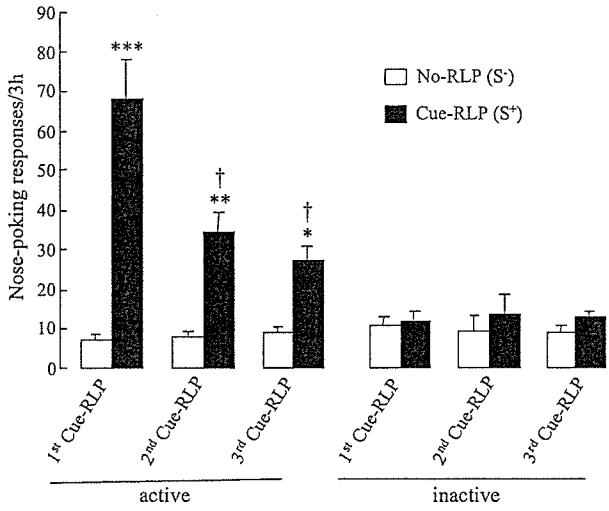

Fig. 4. Nose-poking responses during METH-associated cue-induced reinstatement. The first (1st Cue-RLP), second (2nd Cue-RLP), and third (3rd Cue-RLP) tests for METH-associated cue-induced reinstatement of extinguished METH-seeking behavior were performed immediately, 2 months, and 5 months after withdrawal from METH self-administration. Data are presented as mean ± S.E.M. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. active nose-poking responses during an additional session of extinction (No-RLP (S^{-}) group) in the same test for cue-induced reinstatement. $^{\dagger}P<0.05$ vs. active nose-poking responses in the first test for cue-induced reinstatement (1st Cue-RLP). RLP: reinstatement.

3.5. METH-primed, but not cue-induced, reinstatement behavior is correlated to the total amount of METH intake during self-administration

A correlation study revealed that the number of active nose-poking responses during the first test (1st Primed-RLP) for METH-primed reinstatement (at 1.0 mg/kg of METH, i.p.) was positively correlated with the total amount of METH taken by individual mouse during self-administration training (Fig. 5A, $R=0.73$, $P<0.05$), probably suggesting that METH self-administration and METH-primed reinstatement share some common neural pathways. There was no correlation between the total amount of METH taken by individuals during self-administration and the number of active nose-poking responses during the first test for cue-induced reinstatement (Fig. 5B, 1st Cue-RLP), or the first session of extinction (Fig. 5C, 1st Ext). Also, there was no correlation in the number of active nose-poking responses between the first test (1st Primed-RLP) for METH-primed reinstatement and the first test (1st Cue-RLP) for cue-induced reinstatement (Fig. 5D), probably suggesting that METH-primed and METH-associated cue-induced reinstatement involve different neural pathways.

4. Discussion

In the present study, we established, for the first time, a typical mouse model of drug-primed reinstatement of extinguished drug-seeking behavior. Furthermore, there was a significant

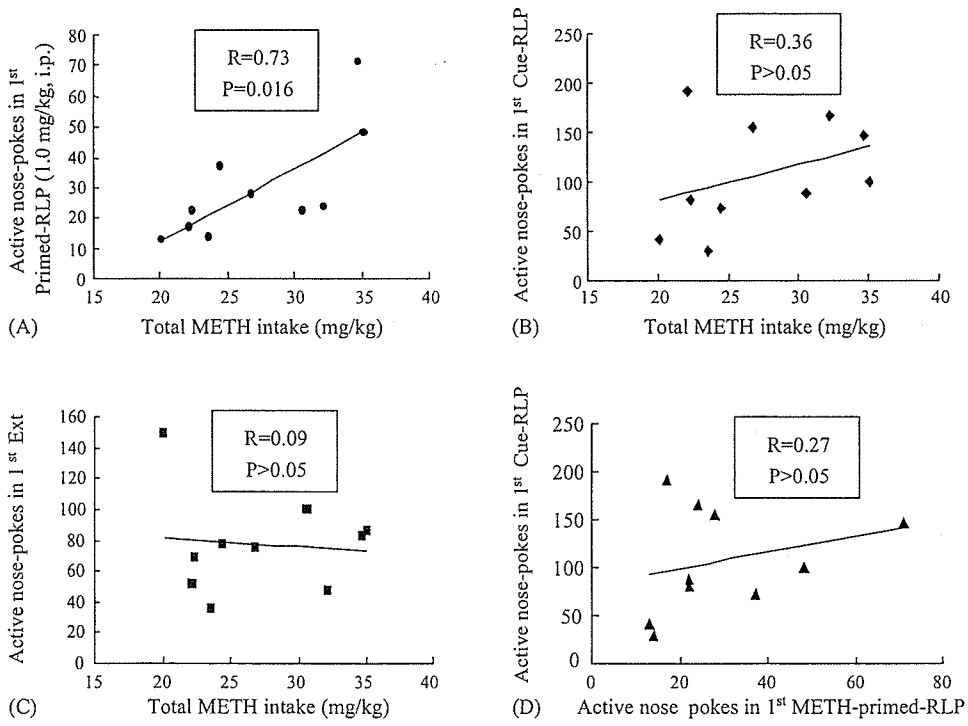

Fig. 5. Correlation between total METH intake during self-administration and the number of active nose-poking responses in the first test for METH-primed reinstatement (1st Primed-RLP) (A), the first test for METH-associated cue-induced reinstatement (1st Cue-RLP) (B), and the first session of extinction (1st Ext) (C), and the relationship in active nose-poking responses between METH-primed reinstatement (1st Primed-RLP) and METH-associated cue-induced reinstatement (1st Cue-RLP) (D). Data are presented as mean ± S.E.M. RLP: reinstatement; Ext: extinction.

difference in the duration between METH-primed and cue-induced reinstatement of extinguished drug-seeking behavior in mice. Our observations indicated that mice display a transient drug-primed, but a long-lasting cue-induced, reinstatement of extinguished drug-seeking behavior, suggesting that different neural pathways are involved in drug-primed and cue-induced reinstatements.

4.1. METH self-administration and extinction

Consistent with previous reports [4,9,17,25,27], our study indicated that C57BL/6J mice are an inbred strain susceptible to drug self-administration. Without nose-poking training with reinforcement using food pellets, naïve C57BL/6J mice could acquire stable METH self-administration behavior under the FR schedule of reinforcement within 16 daily 3-h sessions in the present study (Fig. 1).

Immediately after the end of METH self-administration, the mice exhibited many more active nose-poking responses for METH during the first session of extinction, consistent with our previous report [37]. This phenomenon strongly suggests that the mice developed reliable METH self-administration behavior during 16 daily 3-h sessions of self-administration training. Interestingly, there is no correlation between the active nose-poking responses for METH during the first session of extinction and total METH intake during METH self-administration (Fig. 5C). The sessions required to reach the extinction criterion were not correlated to the total amount of METH taken in during METH self-administration (data not shown). Following prolonged withdrawal from METH self-administration, the mice showed a decrease in the number of active nose-pokes for METH in the absence of both METH and METH-associated cues (cue- and hole-lamps, and the pump noise for an infusion of METH), suggesting that the propensity for relapse to drug-seeking behavior is reduced by the extinction training [31]. Previous reports have shown that the mice are considerably resistant to extinction training (18.3 ± 2.7 days) to reach a criterion of less than 25 active responses in a 2-h test session [12,14]. In the present study, the mice met a stricter extinction criterion (less than 15 active responses or 25% of active responses in the stable phase of self-administration in two consecutive sessions) after 10–20 daily 3-h sessions of extinction training. This difference may result from different procedural factors between previous reports and the present study (e.g. in the present study, both METH and METH-associated cues were absent during the extinction training), although we could not exclude the possibility that the mechanisms at work may differ between cocaine- and METH-conditioning effects.

4.2. Drug-primed and cue-induced reinstatement of extinguished drug-seeking behavior

With a "between-sessions" reinstatement procedure, Fuchs et al. [12] has demonstrated that C57BL/6 mice fail to exhibit a reinstatement of cocaine-seeking behavior after a priming injection with a wide range of doses of cocaine (0, 1, 2.5, 5, 10, 20, and 40 mg/kg, i.p.) according to a between-subjects design. With a "between-within sessions" reinstatement procedure, Highfield et al. [14] has reported that 129X1/SvJ mice show modest drug-primed reinstatement behavior after a priming injection with cocaine at a dose of 6.0 mg/kg (i.v.), although lower doses of cocaine (1.5 and 3.0 mg/kg, i.v.) also fail to reinstate cocaine-seeking behavior. Similar to these two reports, we previously demonstrated that C57BL/6J mice failed to show a reinstatement of METH-seeking behavior after a priming injection with METH at doses of 0.5 and 1.0 mg/kg (i.p.) (37). In the present study, however, we have successfully demonstrated that a priming injection with METH at a dose of 1.0 mg/kg (i.p.) reliably reinstates drug-seeking behavior in mice, by modifying the experimental procedure as follows: First, besides the absence of cue- and hole-lamps previously associated with an infusion of METH, pump noise (for an infusion of METH during METH self-administration) was avoided during daily 3-h sessions of extinction training by turning the infusion pump off. Thus, the absence of all the response-contingent cues reduced the number of extinction training sessions (days) needed to achieve the extinction criterion. Given that METH-primed reinstatement is transient as observed in the present study, the decreased number of extinction training sessions to achieve the criterion may be critical to detect the METH-primed reinstatement behavior in mice. Second, a stricter extinction criterion was introduced ("less than 15 active responses or 25% of active responses in a stable phase of self-administration in two consecutive sessions" in the present study versus "less than 25 active responses or 30% of active responses in stable self-administration on two consecutive days" in the previous study). Thus, a more strict criterion for extinction training may be useful for the subsequent METH-primed reinstatement. Lastly, a possible delay in drug-primed reinstatement behavior in mice could be detected by consecutively testing for reinstatement behavior according to a within-subjects design. Together with previous studies in rats [10,18], these findings suggest that the reinstatement procedure itself may be important to detect the drug-primed reinstatement of extinguished drug-seeking behavior in mice.

Interestingly, METH-primed reinstatement of extinguished drug-seeking behavior disappeared within 2 months after withdrawal from METH self-administration. In contrast, cue-induced reinstatement of extinguished drug-seeking behavior lasted for at least 5 months after withdrawal from METH self-administration. It seems unlikely that the consecutive five tests (saline plus four doses of METH) lead to transient METH-primed reinstatement. First, the same mice were subsequently subjected to extinction training, METH-primed reinstatement test, and cue-induced reinstatement test in the present study. Second, as shown in Fig. 2, the cue-induced reinstatement tests were subsequent to the corresponding METH-primed instatement tests. Third, different from cue-induced reinstatement, the mice received METH injection (i.p.) prior to each test for METH-primed reinstatement behavior. In addition, there was no extinction training session across all the tests for METH-primed reinstatement. In addition, there was a decrease in drug-seeking behavior induced by METH-associated cues with prolonged withdrawal and repeated sessions of extinction training. This observation seems inconsistent with previous reports that cue-

induced reinstatement often shows evidence for incubation of drug-craving behavior following a delay of several weeks in rats [19]. This discrepancy may be because the cue-induced reinstatement behavior in the present study was examined after repeated cycles of extinction training (a with-subjects design), since repeated training of extinction decreases the propensity for a relapse of extinguished drug-seeking behavior [31]. The positive correlation between METH self-administration and drug-primed reinstatement behavior in mice suggests that there are some common neural mechanisms underlying drug self-administration and drug-primed reinstatement. For example, it has been shown that dopamine transmission and the cortico-limbic system in the brain play an important role in drug self-administration and drug-primed reinstatement of drug-seeking behavior [28,36,38]. In contrast, different neural mechanisms may be attributable to drug-primed and cue-induced reinstatement since there is no correlation between cue-induced reinstatement and METH-primed reinstatement or self-administration. This observation is also consistent with previous report [10]. Although the number of samples analyzed in Fig. 5 are relatively small, we argue that the correlation analysis shown in Fig. 5 is reliable, since the mice analyzed in Fig. 5 underwent a long period of conditioning, including self-administration, extinction, and reinstatement of drug-seeking behavior [1].

In conclusion, the present drug-primed reinstatement procedure and distinct characterizations of drug-primed and cue-induced reinstatement of extinguished drug-seeking behavior in mice may provide a new platform with which to identify specific genes involved in relapse of drug-seeking behavior by using genetically modified strains of mice. However, the modifications made in the present study to improve the effectiveness of the drug-primed reinstatement procedure have to be tested further by extending the present approach to other addictive drugs such as cocaine or other inbred strains such as 129X1/SvJ mice.

Acknowledgements

This study was supported in part by a Grant-in-aid for Scientific Research and Special Coordination Funds for Promoting Science and Technology, the Target-Oriented Brain Science Research Program, from the Ministry of Education, Culture, Sports, Science and Technology of Japan; by a Grant-in-aid for Health Science Research on Regulatory Science of Pharmaceuticals and Medical Devices, and Dementia and Fracture from the Ministry of Health, Labor and Welfare of Japan; by the Japan Society for the Promotion Science Joint Research Project under the Japan-Korea Basic Scientific Cooperation Program; by a Smoking Research Foundation Grant for Biomedical Research; by a Grant-in-aid for Scientific Research (B) and Young Scientists (A); by an Uehara Memorial Foundation Research Fellowship; and in part by the 21st Century Center of Excellence Program "Integrated Molecular Medicine for Neuronal and Neoplastic Disorders" from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and by the Brain Research Center from 21st Century Frontier Research Program funded by the Ministry of Science and Technology, Republic of Korea.

References

[1] Ahmed SH, Cador M. Dissociation of psychomotor sensitization from compulsive cocaine consumption. Neuropsychopharmacology 2006;31:563–71.
[2] Anglin MD, Burke C, Perrochet B, Stamper E, Dawud-Noursi S. History of the methamphetamine problem. J Psychoactive Drugs 2000;32:137–41.
[3] Caine SB, Negus SS, Mello NK, Patel S, Bristow L, Kulagowski J, et al. Role of dopamine D2-like receptors in cocaine self-administration: studies with D2 receptor mutant mice and novel D2 receptor antagonists. J Neurosci 2002;22:2977–88.
[4] Carney JM, Landrum RW, Cheng MS, Seale TW. Establishment of chronic intravenous drug self-administration in the C57BL/6J mouse. Neuroreport 1999;2:477–80.
[5] Chiamulera C, Epping-Jordan MP, Zocchi A, Marcon C, Cottiny C, Tacconi S, et al. Reinforcing and locomotor stimulant effects of cocaine are absent in mGluR5 null mutant mice. Nat Neurosci 2001;4:873–4.
[6] Cohen JB, Dickow A, Horner K, Zweben JE, Balabis J, Vandersloot D, et al. Methamphetamine Treatment Project: Abuse and violence history of men and women in treatment for methamphetamine dependence. Am J Addict 2003;12:377–85.
[7] Comings DE, Muhleman D, Ahn C, Gysin R, Flanagan SD. The dopamine D2 receptor gene: a genetic risk factor in substance abuse. Drug Alcohol Depend 1994;34:175–80.
[8] Cossu G, Ledent C, Fattore L, Imperato A, Bohme GA, Parmentier M, et al. Cannabinoid CB1 receptor knockout mice fail to self-administer morphine but not other drugs of abuse. Behav Brain Res 2001;118:61–5.
[9] Deroche V, Caine SB, Heyser CJ, Polis I, Koob GF, Gold LH. Differences in the liability to self-administer intravenous cocaine between C57BL/6 × SJL and BALB/cByJ mice. Pharmacol Biochem Behav 1997;57:429–40.
[10] Deroche-Gamonet V, Martinez A, Le Moal M, Piazza PV. Relationships between individual sensitivity to CS- and cocaine-induced reinstatement in the rat. Psychopharmacology 2003;168:201–7.
[11] Elmer GI, Pierper JO, Rubinstein M, Low MJ, Grandy DK, Wise RA. Failure of intravenous morphine to serve as an effective instrumental reinforcer in dopamine D2 receptor knock-out mice. J Neurosci 2002;22:1–6.
[12] Fuchs RA, See RE, Middaugh LD. Conditioned stimulus-induced reinstatement of extinguished cocaine seeking in C57BL/6 mice: a mouse model of drug relapse. Brain Res 2003;973:99–106.
[13] Hart CL, Ward AS, Haney M, Foltin RW, Fischman MW. Methamphetamine self-administration by humans. Psychopharmacology 2001;157:75–81.
[14] Highfield DA, Mead AN, Grimm JW, Rocha BA, Shaham Y. Reinstatement of cocaine seeking in 129X1/SvJ mice: effects of cocaine priming, cocaine cues and food deprivation. Psychopharmacology 2002;161:417–24.
[15] Kendler KS, Jacobson KC, Prescott CA, Neale MC. Specificity of genetic and environmental risk factors for use and abuse/dependence of cannabis, cocaine, hallucinogens, sedatives, stimulants, and opiates in male twins. Am J Psychiatry 2003;160:687–95.
[16] Kreek MJ, Nielsen DA, Butelman ER, LaForge KS. Genetic influences on impulsivity, risk taking, stress responsivity and vulnerability to drug abuse and addiction. Nat Neurosci 2005;8:1450–7.
[17] Kuzmin A, Johansson B. Reinforcing and neurochemical effects of cocaine: differences among C57, DBA, and 129 mice. Pharmacol Biochem Behav 2000;65:399–406.
[18] Lenoir M, Ahmed SH. Heroin-induced reinstatement is specific to compulsive heroin use and dissociable from heroin reward and sensitization. Neuropsychopharmacology 2006; doi:10.1038/sj.npp.1301083.
[19] Lu L, Grimm JW, Hope BT, Shaham Y. Incubation of cocaine craving after withdrawal: a review of preclinical data. Neuropharmacology 2004;47(Suppl):214–26.
[20] Morgan AD, Carroll ME, Loth AK, Stoffel M, Wickman K. Decreased cocaine self-administration in Kir3 potassium channel subunit knockout mice. Neuropsychopharmacology 2003;28:932–8.
[21] Nestler EJ. Genes and addiction. Nat Genetics 2000;26:277–81.

[22] Picciotto MR, Zoli M, Rimondini R, Lena C, Marubio LM, Pich EM, et al. Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. Nature 1998;391:173–7.
[23] Prescott CA, Kendler KS. Genetic and environment contributions to alcohol abuse and dependence in a populated-based sample of male twins. Am J Psychiatry 1999;156:34–40.
[24] Roberts AJ, McDonald JS, Heyser CJ, Kieffer BL, Matthes HW, Koob GF, et al. mu-Opioid receptor knockout mice do not self-administer alcohol. J Pharmacol Exp Ther 2000;293:1002–8.
[25] Rocha BA, Odom LA, Barron BA, Ator R, Wild SA, Forster MJ. Differential responsiveness to cocaine in C57BL/6J and DBA/2J mice. Psychopharmacology 1998;138:82–8.
[26] Rocha BA, Scearce-Levie K, Lucas JJ, Hiroi N, Castanon N, Crabbe JC, et al. Increased vulnerability to cocaine in mice lacking the serotonin-1B receptor. Nature 1998;393:175–8.
[27] Ruiz-Durántez E, Hall SK, Steffen C, Self DW. Enhanced acquisition of cocaine self-administration by increasing percentages of C57BL/6J genes in mice with a nonpreferring outbred background. Psychopharmacology 2006;186:553–60.
[28] Self DW. Regulation of drug-taking and -seeking behaviors by neuroadaptations in the mesolimbic dopamine system. Neuropharmacology 2004;47:242–55.
[29] Soria G, Mendizabal V, Tourino C, Robledo P, Ledent C, Parmentier M, et al. Lack of CB1 cannabinoid receptor impairs cocaine self-administration. Neuropsychopharmacology 2005;30:1670–80.
[30] Szumilinski KK, Dehoff MH, Kang SH, Frys KA, Lominac KD, Klugmann M, et al. Homer proteins regulate sensitivity to cocaine. Neuron 2004;43:401–13.
[31] Sutton MA, Schmidt EF, Choi KH, Schad CA, Whisler K, Simmons D, et al. Extinction-induced upregulation in AMPA receptors reduces cocaine-seeking behaviour. Nature 2003;421:70–5.
[32] Tsuang MT, Lyons MJ, Doyle T, Eisen SA, Goldberg J, True W, et al. Co-occurrence of abuse of different drugs in men: the role of drug-specific and shared vulnerabilities. Arch Gen Psychiatry 1998;55:967–72.
[33] Uhl GR, Grow RW. The burden of complex genetics in brain disorders. Arch Gen Psychiatry 2004;61:223–9.
[34] Ujike H, Sato M. Clinical features of sensitization to methamphetamine observed in patients with methamphetamine dependence and psychosis. Ann NY Acad Sci 2004;1025:279–87.
[35] Volkow ND, Li TK. Drug addiction: the neurobiology of behaviour gone awry. Nat Rev Neurosci 2004;5:963–70.
[36] Weiss F, Maldonado-Vlaar CS, Parsons LH, Kerr TM, Smit DL, Ben-Shahar O. Control of cocaine-seeking behavior by drug-associated stimuli in rats: effects on recovery of extinguished operant-responding and extracellular dopamine levels in amygdala and nucleus accumbens. Proc Natl Acad Sci USA 2000;97:4321–6.
[37] Yan Y, Nitta A, Mizoguchi H, Yamada K, Nabeshima T. Relapse of methamphetamine seeking behavior demonstrated by a reinstatement procedure involving self-administration. Behav Brain Res 2006;168:137–43.
[38] Zavala AR, Weber SM, Rice HJ, Alleweireldt AT, Neisewander JL. Role of the prelimbic subregion of the medial prefrontal cortex in acquisition, extinction, and reinstatement of cocaine-conditioned place preference. Brain Res 2003;990:157–64.

Available online at www.sciencedirect.com
ScienceDirect
Behavioural Brain Research xxx (2007) xxx–xxx
BEHAVIOURAL BRAIN RESEARCH
www.elsevier.com/locate/bbr

Research report

Involvement of glial cell line-derived neurotrophic factor in inhibitory effects of a hydrophobic dipeptide Leu-Ile on morphine-induced sensitization and rewarding effects

Minae Niwa [a], Atsumi Nitta [a], Liya Shen [b], Yukihiro Noda [a,c], Toshitaka Nabeshima [a,*]

[a] *Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8560, Japan*
[b] *Laboratory of Cellular Carcinogenesis and Tumor Promotion, Center for Cancer Research, NCI, Bethesda, MD 20983, USA*
[c] *Division of Clinical Science in Clinical Pharmacy Practice, Management and Research, Faculty of Pharmacy, Meijo University, Nagoya 468-8503, Japan*

Received 13 December 2006; received in revised form 24 January 2007; accepted 30 January 2007

Abstract

There are few efficacious medications for drug dependence at present. We have previously demonstrated that Leu-Ile, which induces the expression of not only tumor necrosis factor-α (TNF-α) but also glial cell line-derived neurotrophic factor (GDNF), inhibits methamphetamine (METH) and morphine (MOR)-induced sensitization and rewarding effects by regulating extracellular dopamine levels *via* the induction of TNF-α expression, and indicated the potential of Leu-Ile as a novel therapeutic agent for METH and MOR-induced dependence. In the present study, we investigated the involvement of GDNF in inhibitory effects of Leu-Ile on MOR-induced sensitization and rewarding effects. Repeated treatment with MOR for 9 days, which results in an enhancement of the locomotor-stimulating effects (sensitization) of MOR, increased GDNF levels in the nucleus accumbens compared with those in saline-treated mice. Repeated pre-treatment with Leu-Ile for 9 days potentiated the MOR-induced increase in GDNF levels. MOR at a low dose (3 mg/kg) produced place preference in GDNF heterozygous knockout (GDNF-(+/−)) mice, but not in littermate GDNF-(+/+) mice. No inhibitory effect of Leu-Ile on MOR-induced place preference was observed in GDNF-(+/−) mice. These results suggest that GDNF is involved in the inhibitory effects of Leu-Ile on MOR-induced sensitization and rewarding effects.

Keywords: Morphine (MOR); Glial cell line-derived neurotrophic factor (GDNF); Sensitization; Rewarding effects; Leu-Ile; Mice

1. Introduction

Drugs of abuse are able to elicit compulsive drug-seeking behaviors upon repeated administration, which ultimately leads to the phenomenon of addiction. Evidence indicates that the susceptibility to develop addiction is influenced by sources of reinforcement, variable neuroadaptive mechanisms, and neurochemical changes that together lead to altered homeostasis of the brain reward system [7].

Neurotrophic factors and cytokines, which are known to influence synaptic transmission and neuronal morphology [1,2,12], may be involved in alterations of the morphology of dendrites and dendritic spines in the nucleus accumbens (NAc) and prefrontal cortex after repeated injections of psychostimulants [18,19]. Glial cell line-derived neurotrophic factor (GDNF) inhibits the cocaine-induced upregulation of tyrosine hydroxylase (TH) activity in the ventral tegmental area (VTA) and blocks behavioral responses to cocaine [10]. GDNF would be a candidate for therapeutic agents against drug dependence. However, there are serious obstacles to its therapeutic application: it is difficult to deliver GDNF from the periphery to the brain, since it is a macromolecule that cannot penetrate the blood–brain barrier

Abbreviations: CPP, conditioned place preference; DA, dopamine; EIA, enzyme immunoassay; GDNF, glial cell line-derived neurotrophic factor; METH, methamphetamine; MOR, morphine; NAc, nucleus accumbens; TNF-α, tumor necrosis factor-α; TH, tyrosine hydroxylase; VTA, ventral tegmental area

* 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 © 2007 Published by Elsevier B.V.
doi:10.1016/j.bbr.2007.01.026

[8], and is easily broken down by proteases in the blood stream. Therefore, GDNF cannot be used directly as a therapeutic tool for drug dependence. We hypothesized that a low-molecular-weight compound which induces production of GDNF in the brain could be a novel therapeutic agent for drug dependence.

Recently, we have demonstrated that Leu-Ile, which induces the expression of tumor necrosis factor-α (TNF-α) and GDNF, inhibits methamphetamine (METH)-induced sensitization and rewarding effects by negating the METH-induced inhibition of dopamine (DA) uptake as well as attenuating the METH-induced increase in extracellular DA levels in the NAc *via* the induction of TNF-α and GDNF expression [15]. Moreover, we have demonstrated that Leu-Ile inhibits MOR-induced sensitization and rewarding effects by regulating extracellular DA levels *via* the induction of TNF-α expression [14].

In the present study, to extend our findings, we examined the involvement of GDNF in addition to TNF-α in inhibitory effects of Leu-Ile on MOR-induced sensitization and rewarding effects.

2. Materials and methods

2.1. Reagents

GDNF as a standard for the enzyme immunoassay (EIA) was donated by Amgen (CA, USA). Leu-Ile was purchased from Kokusan Chemical Co., Ltd. (Tokyo, Japan). All other materials used were of reagent grade.

2.2. Animals

Animals were housed in plastic cages and kept in a temperature-, humidity-, and light-controlled room (23 ± 1 °C; 50 ± 5% humidity; 12:12 h light/dark cycle starting at 8:00 a.m.) and had free access to food and water, except during behavioral experiments. All animals' care and use were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine. Animals were treated according to the Guidelines of Experimental Animal Care issued from the Office of the Prime Minister of Japan.

The wild-type C57BL/6J mice were obtained from Slc Japan (Hamamatsu, Japan).

Male C57BL/6J-GDNF heterozygous knockout (GDNF-(+/−)) mice, 8–12 weeks of age, were used in the experiments. GDNF-(+/−) were generated as described previously [17]. GDNF (−/−) homozygous knockout mice die shortly after birth (postnatal 7 days), but GDNF (+/−) mice are viable. GDNF levels in the frontal cortex, NAc, caudate putamen, and hippocampus of GDNF-(+/−) mice are 54.8, 65.4, 59.0, and 66.8%, respectively, of those in littermate GDNF-(+/+) mice [15]. Littermate GDNF-(+/+) mice were used as controls in the behavioral experiments.

2.3. Locomotor activity

Locomotor activity was measured using an infrared detector (Neuroscience Co., Ltd., Tokyo, Japan) in a plastic box (32 cm × 22 cm × 15 cm high) [11,14]. Mice were administered Leu-Ile (1.5 and 15 μmol/kg, i.p.) or vehicle, and habituated for 1 h in the box. Mice were administered MOR (10 mg/kg, s.c.) or saline 1 h after the Leu-Ile administration, and the locomotor activity was measured for 2 h immediately after the MOR or saline administration [14]. Leu-Ile and MOR were injected once a day for 9 days.

2.4. Enzyme immunoassay

GDNF levels were measured using an EIA with a minor modification [13]. Mice were administered Leu-Ile (1.5 and 15 μmol/kg, i.p.) once a day 1 h before MOR (10 mg/kg, s.c.) treatment for 9 days and decapitated 24 h after the last administration of MOR (Fig. 1A). Homogenate buffer (0.1 M Tris–HCl [pH 7.4] containing 1 M NaCl, 2% bovine serum albumin, 2 mM EDTA, and 0.2% Na_3N) was added to brain tissue at a ratio of 1 g wet weight per 19 ml of buffer, pulse-sonicated for 100 s, and centrifuged at 100,000 × *g* for 30 min. The supernatant was collected and used for the EIA.

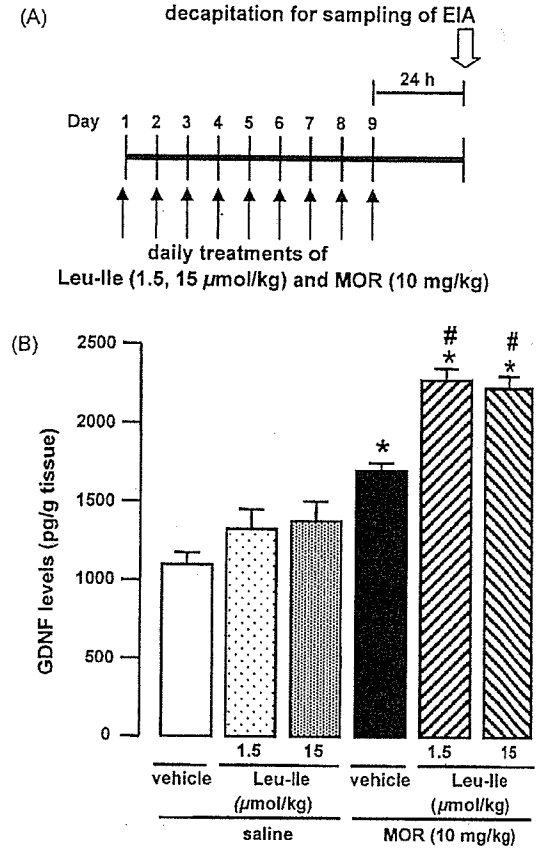

Fig. 1. Effect of Leu-Ile on morphine (MOR)-induced increase in glial cell line-derived neurotrophic factor (GDNF) levels. (A) Experimental schedule for measurement of GDNF levels using the enzyme immunoassay (EIA) method. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, i.p.) or vehicle 1 h before MOR (10 mg/kg, s.c.) or saline once a day for 9 days and decapitated 24 h after the last MOR or saline administration. (B) Change of GDNF levels in the nucleus accumbens after the administration of Leu-Ile and/or MOR. Values are mean ± S.E. ($n = 6$–8). $^{*}p < 0.05$ vs. vehicle/saline-treated mice. $^{\#}p < 0.05$ vs. vehicle/MOR-treated mice.

2.5. Conditioned place preference

The apparatus used for the place conditioning task consisted of two compartments: a transparent Plexiglas box and a black Plexiglas box (both 15 cm × 15 cm × 15 cm high). To enable mice to distinguish easily the two compartments, the floors of the transparent and black boxes were covered with white plastic mesh and black frosting Plexiglas, respectively. Each box could be divided by a sliding door (10 cm × 15 cm high). The place conditioning paradigm was performed by using a previously established procedure [14,16]. The experimental schedule for the conditioned place preference (CPP) task is shown in Fig. 2A. In the pre-conditioning test, the sliding door was opened, and the mouse was allowed to move freely between both boxes for 15 min once a day for 3 days. On the third day of the pre-conditioning test, we measured the time that the mouse spent in the black and transparent boxes by using a Scanet SV-20 LD (Melquest Co., Ltd., Toyama, Japan). The box in which the mouse spent the most time was referred to as the "preferred side", and the other box as the "non-preferred side". Conditioning was performed during six successive days.

Please cite this article in press as: Niwa M, et al., Involvement of glial cell line-derived neurotrophic factor in inhibitory effects of a hydrophobic dipeptide Leu-Ile on morphine-induced sensitization and rewarding effects, Behav Brain Res (2007), doi:10.1016/j.bbr.2007.01.026

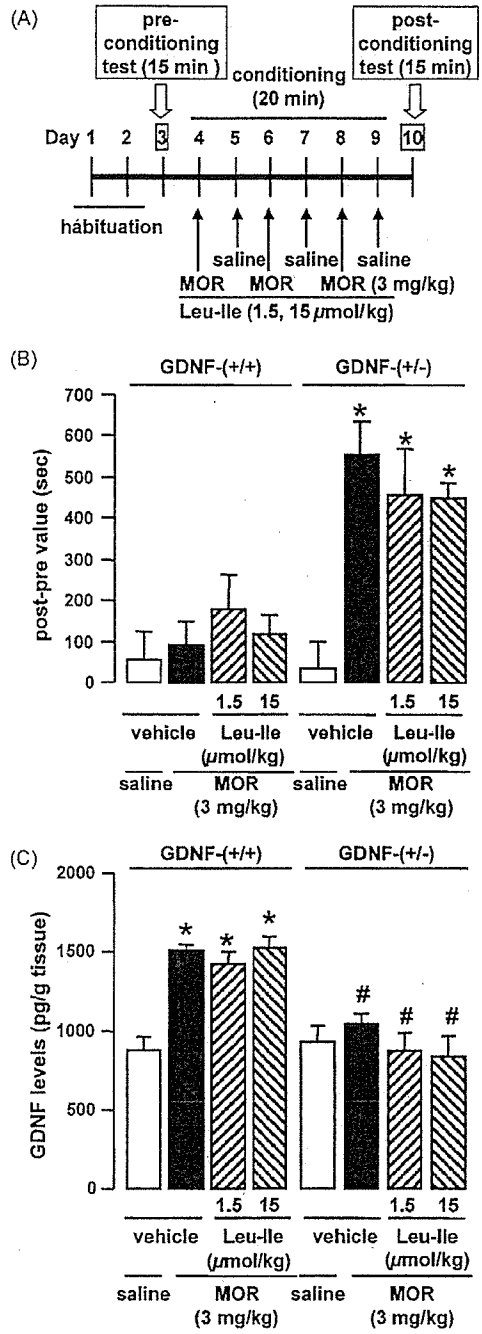

Fig. 2. Effect of Leu-Ile on MOR-induced place preference in GDNF-(+/−) mice. (A) Experimental schedule for the conditioned place preference task. On the third day of the pre-conditioning test, we measured the time that the mouse spent in the black and transparent boxes. Mice were subcutaneously given MOR (3 mg/kg, s.c.) and put in its non-preferred side for 20 min. On the next day, the mouse was given saline and placed opposite the drug conditioning site for 20 min. These treatments were repeated for three cycles (6 days). In the post-conditioning test, the sliding door was opened, and we measured the time that the mouse spent in the black and transparent boxes for 15 min. Closed arrows indicate the days of MOR or saline administration. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, i.p.) or vehicle 1 h before MOR (3 mg/kg, s.c.) or saline administration. (B) Effect of Leu-Ile treatment on MOR-induced place preference in GDNF-(+/−) mice. Mice were co-treated with Leu-Ile and MOR in the conditioning phase. Mice were treated with Leu-Ile (1.5 and 15 μmol/kg, i.p.) 1 h before MOR (3 mg/kg, s.c.) or saline administration. Values are means ± S.E. ($n = 10$–14). $^{*}p < 0.05$ vs. vehicle/MOR-treated GDNF-(+/+) mice. (C) Change of GDNF levels in the NAc after post-conditioning test in conditioned place preference paradigm. Mice were co-treated with Leu-Ile (1.5 and 15 μmol/kg, i.p.) and MOR (3 mg/kg, s.c.) in the conditioning period and decapitated 24 h after post-conditioning test. Values are means ± S.E. ($n = 5$). $^{*}p < 0.05$ vs. vehicle/saline-treated GDNF-(+/+) mice. $^{\#}p < 0.05$ vs. vehicle/MOR-treated GDNF-(+/+) mice. Abbreviations as in Fig. 1.

Mice were given MOR or saline in the apparatus with the sliding door closed. That is, a mouse was subcutaneously given MOR and put in its non-preferred side for 20 min. On the next day, the mouse was given saline and placed opposite the drug conditioning site for 20 min. These treatments were repeated for three cycles (6 days). In the post-conditioning test, the sliding door was opened, and we measured the time that the mouse spent in the black and transparent boxes for 15 min, using the Scanet SV-20 LD. Place conditioning behavior was expressed by post–pre, which was calculated as: [(post-value) − (pre-value)], where post- and pre-values were the difference in time spent at the drug conditioning and the saline conditioning sites in the post-conditioning and pre-conditioning tests, respectively.

2.6. Statistical analysis

All data were expressed as means ± S.E. Statistical differences among more than three groups were determined using a one-way analysis of variance (ANOVA), followed by the Bonferroni multiple comparison test. $p < 0.05$ was regarded as statistically significant.

3. Results

3.1. Effect of Leu-Ile on MOR-induced increase in GDNF levels

Single MOR treatment at the dose of 10 mg/kg increases locomotor activity, and repeated administration for 9 days results in an enhancement of the locomotor-stimulating effect of MOR (sensitization) [14]. Leu-Ile (1.5 and 15 μmol/kg, i.p.) inhibits the MOR-induced hyperlocomotion and sensitization [14]. The sensitization to the locomotor-stimulating effects is argued to reflect one neuroadaptive process associated with dependence. To confirm the involvement of GDNF in the inhibitory effects of Leu-Ile on MOR-induced sensitization, GDNF levels in the NAc were determined after the co-administration of Leu-Ile and MOR using the EIA method. MOR (10 mg/kg) increased GDNF levels in the NAc compared with those in the vehicle/saline-treated mice. GDNF levels after the co-administration of Leu-Ile (1.5 and 15 μmol/kg, i.p.) and MOR (10 mg/kg) were much more increased compared with those in the vehicle/MOR-treated mice ($F_{(5,38)} = 28.1$, $p < 0.05$, one-way ANOVA) (Fig. 1B). These results suggest that GDNF is involved in the effects of Leu-Ile on the sensitization.

3.2. Effect of Leu-Ile on MOR-induced place preference in GDNF-(+/−) mice

We have investigated the effects of Leu-Ile on the rewarding effects of MOR in the CPP paradigm, in which animals learn the association of an environment paired with drug exposure. Therefore, CPP is considered a measure of the rewarding properties of drugs of abuse. Leu-Ile (1.5 μmol/kg, i.p.) inhibits

Please cite this article in press as: Niwa M. et al., Involvement of glial cell line-derived neurotrophic factor in inhibitory effects of a hydrophobic dipeptide Leu-Ile on morphine-induced sensitization and rewarding effects, Behav Brain Res (2007), doi:10.1016/j.bbr.2007.01.026