Facilitation of morphine withdrawal symptoms and morphine-induced conditioned place preference by a glutamate transporter inhibitor DL-*threo*-β-benzyloxyaspartate in rats

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Abstract

There is a body of evidence implying the involvement of the central glutamatergic system in morphine dependence. In this study, we examined the effect of intracerebroventricular (i.c.v.) administration of a potent glutamate transporter inhibitor, DL-*threo*-β-benzyloxyaspartate (DL-TBOA), on acute morphine-induced antinociception, expression of somatic and negative affective components of morphine withdrawal, and acquisition of morphine-induced conditioned place preference in rats. I.c.v administration of DL-TBOA (10 nmol) to naive rats did not affect the acute antinociceptive effect of morphine. I.c.v. administration of DL-TBOA (10 nmol) to morphine-dependent rats significantly facilitated the expression of naloxone-precipitated somatic signs and conditioned place aversion. DL-TBOA (3 and 10 nmol) significantly facilitated acquisition of morphine-induced conditioned place preference. DL-TBOA itself produced neither conditioned place aversion nor place preference in naive rats. These results suggest that central glutamate transporters play inhibitory roles in the expression of somatic and negative affective components of morphine withdrawal and the reinforcing effect of morphine.
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Keywords: Morphine dependence; Morphine withdrawal; Glutamate transporter; DL-*threo*-β-benzyloxyaspartate; Conditioned place aversion; Conditioned place preference

1. Introduction

Chronic use of opiates such as morphine leads to physical dependence and has a reinforcing effect, and cessation of drug administration precipitates withdrawal symptoms including both somatic and affective components. The mechanisms underlying them have been extensively investigated, and a body of evidence provides support for the essential involvement of excitatory amino acid, particularly glutamatergic, systems. Early studies indicating that a prototypical non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo(*a,d*)cycloheptan-5,10-imine (MK-801), attenuated the development of morphine tolerance and physical dependence without affecting the antinociceptive effect of morphine (Rasmussen et al., 1991;

Trujillo and Akil, 1991) have focused investigators' interest on the involvement of glutamate. Subsequently, other non-competitive and competitive NMDA receptor antagonists (Manning et al., 1996; Bristow et al., 1997; González et al., 1997), non-NMDA receptor antagonists (Rasmussen et al., 1996; McLemore et al., 1997; Rasmussen and Vandergriff, 2003) and metabotropic glutamate receptor antagonists (Fundytus and Coderre, 1994; Fundytus et al., 1997) have been shown to attenuate the development of morphine physical dependence and/or the expression of somatic signs of morphine withdrawal. Furthermore, the negative affective component of morphine withdrawal, characterized by conditioned place aversion in a place-conditioning paradigm, has been reported to be attenuated by NMDA receptor antagonists (Higgins et al., 1992; Popik and Danysz, 1997; Blokhina et al., 2000). The nonselective glutamate receptor antagonist, kynurenic acid, is reported to attenuate the acquisition and expression of morphine-induced conditioned place preference in a place-conditioning paradigm, as well as facilitation of the response in an electrical intracranial

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self-stimulation paradigm (Bespalov et al., 1994). Similarly, it has been shown that morphine-induced conditioned place preference is attenuated by other glutamate receptor antagonists (Tzschentke and Schmidt, 1995; Del Pozo et al., 1996; Popik and Danysz, 1997; Suzuki et al., 1999; Popik and Wróbel, 2002). Taken together, these findings suggest that glutamate receptors could play important roles in the development of physical dependence on morphine and in the reinforcing effect of morphine, and the expression of somatic and negative affective components of morphine withdrawal.

It is well known that extracellular glutamate released from nerve terminals is counterbalanced by glutamate uptake by transporters in neurons and glial cells, thereby terminating glutamatergic signal transmission and protecting neurons from an excitotoxic action of glutamate (Kanai et al., 1993; Seal and Amara, 1999). We recently reported that the expression of a glial glutamate transporter GLT-1 mRNA changed in some brain regions of morphine-dependent rats and naloxone-precipitated withdrawal rats (Ozawa et al., 2001). Furthermore, we have shown that co-administration of (R)-$(-)$-5-methyl-1-nicotinoyl-2-pyrazoline (MS-153), which is reported to accelerate glutamate uptake, with morphine reduced the development of morphine physical dependence in mice (Nakagawa et al., 2001). These results support the possibility that glutamate transporters such as GLT-1 could be involved in morphine dependence.

DL-threo-β-benzyloxyaspartate (DL-TBOA), a newly synthesized derivative of DL-threo-β-hydroxyaspartate, is a potent, non-transported, competitive inhibitor of glutamate uptake (Lebrun et al., 1997; Shimamoto et al., 1998). In the present study, to examine the roles of central glutamate transporters in the expression of somatic and negative affective components of morphine withdrawal and the reinforcing effect of morphine, we investigated the effects of intracerebroventricular (i.c.v.) administration of DL-TBOA on acute morphine antinociception, naloxone-precipitated morphine withdrawal-induced somatic signs and conditioned place aversion, and acquisition of morphine-induced conditioned place preference in rats.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (Japan SLC, Hamamatsu, Japan) initially weighing 180–250 g were used. They were kept at a constant ambient temperature of 24 ± 1 °C under a 12-h light/dark cycle with free access to food and water. The experiments were conducted in accordance with the ethical guidelines of Kyoto University Animal Experimentation Committee, and the guidelines of the Japanese Pharmacological Society.

2.2. Materials

Morphine hydrochloride was purchased from Takeda Chemical Industries (Osaka, Japan). Morphine pellets each containing 75 mg of morphine base were prepared according to the method of Gibson and Tingstad (1970). Naloxone hydrochloride was purchased from Sigma (St. Louis, USA). Morphine hydrochloride and naloxone hydrochloride were dissolved in saline. DL-TBOA was prepared as a stock solution of 100 mM in 50% dimethyl sulfoxide and 100 mM NaOH, and dissolved in phosphate-buffered saline (PBS) or vehicle (1% dimethyl sulfoxide and 2 mM NaOH in PBS) before the experiments.

2.3. Surgery and i.c.v. administration

Under thiamylal sodium (50 mg/kg, intraperitoneally (i.p.)) anesthesia, a stainless steel guide cannula (o.d. 0.7 mm) was implanted on the right side at coordinates of 0.8 mm caudal to the bregma, 1.5 mm lateral to the midline, and 2.0 mm below the surface of the skull according to the atlas of Paxinos and Watson (1998). After surgery, the rats were individually returned to their cages and left to recover for at least 5 days before the experiments. DL-TBOA or vehicle was i.c.v. administered 10 min before i.p. injection of naloxone or subcutaneous (s.c.) injection of morphine. An injection cannula (o.d. 0.35 mm) was inserted into the right lateral ventricle just 5.0 mm below the surface of the skull when attached to the guide cannula. I.c.v. administration was carried out in a volume of 5 μl at a constant rate of 5 μl/30 s by a microinfusion pump. The injection cannula was left in place for an additional 30 s to prevent backflow. During the injection procedure, the experimenter loosely held the animals. Because, in preliminary experiments, i.c.v. administration of DL-TBOA at a dose of 30 nmol to naive rats elicited irritability, motor hyperexcitability and/or convulsion immediately or soon after i.c.v. administration, we used doses lower than 10 nmol. I.c.v. administration of DL-TBOA at a dose of 10 nmol, but not 1 or 3 nmol, elicited teeth chattering and/or passivity (loss of struggle responses from the holding) in some rats, although these behaviors disappeared within a few minutes (experimenter's observation).

2.4. Measurement of nociceptive threshold

Mechanical nociceptive threshold was evaluated by the paw pressure test using an analgesimeter (Ugo Basile, Milan, Italy) with a cuneate piston. The piston was put on the ventral surface of the hind paw. The pressure was loaded at a rate of 32 g/s. The pressure that elicited paw withdrawal was determined as the nociceptive threshold. The procedures for the measurement were carried out three times a day to habituate the animals to the procedure. After 2 days of habituation, the threshold was measured following two additional habituation procedures, and the value

was taken as a control. The control nociceptive threshold was 194.3 ± 3.4 g ($n = 33$). Soon after measuring the control value, vehicle or DL-TBOA (10 nmol) was i.c.v. administered. After 10 min, the nociceptive threshold was measured (time zero), and morphine (0.5 and 3 mg/kg) was s.c. administered. Then, the threshold was measured at 15, 30, 45, 60, 90, 120 and 150 min.

2.5. Measurement of naloxone-precipitated morphine withdrawal-induced somatic signs

Measurement of naloxone-precipitated morphine withdrawal-induced somatic signs was performed as previously described (Nakagawa et al., 2000) with slight modifications. On the first day (day 1), under light ether anesthesia, the rats had a morphine pellet implanted in the back of the neck. Twenty-four hours later (day 2), the rats received a second morphine pellet. After 72 h (day 5), each rat was placed in a Plexiglass cylinder to acclimatize it to the experimental environment. After the 30-min habituation period, DL-TBOA (1, 3 and 10 nmol) or vehicle was i.c.v. administered. Ten minutes after the i.c.v. administration, naloxone (0.1 mg/kg) was i.p. injected without removing the implanted pellets. This dose of naloxone is reported to moderately precipitate somatic signs in morphine-dependent rats (Higgins and Sellers, 1994; Le Guen et al., 2001). Then, the rats were immediately returned to the cylinder and behavior was observed every 5 min for 1 h. Body weight was measured just before and 1 h after naloxone injection, and is presented as the means $\pm$ S.E.M. of percentage body weight loss. The number of occurrences of stretching, wet dog shaking, teeth chattering, jumping, paw shaking, head shaking, ejaculation and backwards walking was counted, and data are presented as means $\pm$ S.E.M. of total numbers. The occurrence of diarrhea, salivation, lacrimation, rhinorrhea and ptosis was monitored and is presented as the number of rats showing positive signs over the total number of rats tested.

2.6. Conditioned place aversion paradigm

2.6.1. Apparatus

Conditioned place aversion was conducted as previously described (Watanabe et al., 2002a,b). A place conditioning apparatus, consisting of a shuttle box ($30 \times 60 \times 30$ cm: width $\times$ length $\times$ height) divided into two equal-sized compartments, was used. The inner surface of one compartment was black with a smooth floor; the other was white with a textured floor. The infrared beam sensors were positioned on each cover, and the time spent in each compartment over a period of 900 s was measured in a blind fashion automatically using a computer system (KN-80; Natsume Seisakusyo, Tokyo, Japan). The shuttle box was enclosed by a sound- and light-attenuated box under conditions of dim illumination (about 40 lx) and white noise masking.

2.6.2. Preconditioning session

The experimental process consisted of three distinct sessions: a preconditioning session, conditioning session, and test session. On the first day (day 1), under light ether anesthesia, the rats had either a morphine or placebo pellet implanted in the back of the neck. The implanted pellet was left in place until the test session. On day 2, the partition separating the two compartments was raised 12 cm above the floor, and a neutral platform ($5 \times 2 \times 12$ cm) was inserted along the seam separating the compartments. The rats were individually placed on the platform and stepped down to the horizontal floor. The rats were allowed to freely explore the two compartments for 900 s and acclimatized to the apparatus. On day 3 (preconditioning session), the same trial was performed, and the time spent in each compartment was measured over 900 s. There were no significant differences between time spent in the black compartment with a smooth floor (471 ± 16 s, $n = 56$) and the white compartment with a textured floor (429 ± 16 s, $n = 56$), indicating that there was no preference bias before conditioning in the apparatus itself. Nevertheless, in this study, we selected a bias-like protocol in order to nullify each rat's initial slight preference, as previously reported (Watanabe et al., 2002a,b). Thus, we determined the preferred compartment for each rat by establishing the compartment in which the rats spent more than 50% of the total time (i.e. 450 s). Biased rats that spent more than 80% of the time (i.e. 720 s) on one side on day 3 (10 of 86 rats), or that spent more than 600 s on one side on day 2 and more than 600 s on the other side on day 3 (8 of 86 rats) were excluded from further analysis. The time spent in the preferred compartment on day 3 was -543 ± 8.5 s ($n = 56$).

2.6.3. Conditioning session

On day 4, place conditioning was performed without removing the implanted pellet. A pharmacokinetic and pharmacodynamic study by Yoburn et al. (1985) showed that enough morphine remained in the pellet 72 h after implantation. For place conditioning, the rats were i.p. injected with saline and confined to the non-preferred compartment for 1 h. After at least 3 h, the rat was i.p. injected with naloxone (0.003 mg/kg) and then confined to its preferred compartment for 1 h. This dose of naloxone is reported to induce very weak or no conditioned place aversion in morphine-dependent rats (Gracy et al., 2001; Frenois et al., 2002). DL-TBOA (3 and 10 nmol) or vehicle was i.c.v. administered 10 min before the i.p. injection of naloxone.

2.6.4. Test session

On day 5, the partition separating the two compartments was raised, and the rats were individually placed on the neutral platform and allowed to freely explore the two compartments. The time spent in each compartment over 900 s was then measured without any injections. The aversion score represents the time spent in the naloxone-

paired compartment in the test session minus the time spent in the same compartment in the preconditioning session, and is expressed as mean ± S.E.M. Using the same protocol, we found earlier that the time spent in the naloxone-paired compartment in the test session of morphine-dependent rats, but placebo pellet-implanted naive rats, was significantly shorter than that in the pre-conditioning session after conditioning with naloxone at a dose of 0.3 mg/kg (Watanabe et al., 2002a,b).

2.7. Conditioned place preference paradigm

2.7.1. Preconditioning session

The experimental process consisted of three distinct sessions: a preconditioning session, a conditioning session, and a test session. On days 1 and 2, acclimatization to the apparatus was conducted as described above. Biased rats that spent more than 80% of the time (i.e. 720 s) on one side on day 3 (8 of 56 rats), or that spent more than 600 s on one side on day 2 and more than 600 s on the other side on day 3 (3 of 56 rats) were excluded from further analysis. There was no significant difference between time spent in the black compartment with a smooth floor (461 ± 20 s, $n = 37$) and the white compartment with a textured floor (438 ± 20 s, $n = 37$) in the preconditioning session.

2.7.2. Conditioning session

The conditioning session was conducted once daily for 6 consecutive days (day 3–8). The rats were s.c. injected with either morphine (0.5 mg/kg) or saline and confined to one compartment for 1 h. On the next day, the rats were s.c. injected with saline or morphine (0.5 mg/kg), respectively, and confined to the other compartment for 1 h. This dose of morphine is reported to induce very weak or no conditioned place preference in rats (Rezayof et al., 2002; our preliminary data). From days 3 to 8, the conditioning was repeated three times. Assignment of the drug-paired compartment was performed randomly and counterbalanced across the subjects. DL-TBOA (3 and 10 nmol) or vehicle was i.c.v. administered 10 min before the s.c. injection of morphine. Control groups received saline instead of morphine.

2.7.3. Test session

On day 9, the time spent in each compartment for 900 s was measured in the drug-free state as described above. The preference score represents the time spent in the morphine-paired compartment minus the time spent in the saline-paired compartment in the test session, and is expressed as means ± S.E.M.

2.8. Statistical analysis

The antinociceptive effect of morphine was statistically evaluated using a two-way analysis of variance (ANOVA).

In the case of morphine withdrawal, somatic signs (weight loss and symptoms) were analyzed by the one-way ANOVA followed by the Student–Newman–Keuls post hoc test, and the data for the occurrence of diarrhea, salivation, lacrimation, rhinorrhea, and ptosis were compared by the Chi-square test. In the case of place-conditioning experiments, statistical significance was analyzed by the one-way ANOVA followed by the Student–Newman–Keuls post hoc test. Differences with $P < 0.05$ were considered significant.

3. Results

3.1. Effect of i.c.v. Administration of DL-TBOA on morphine antinociception

The effects of i.c.v. administration of DL-TBOA on the acute antinociceptive effects of morphine were investigated in naive rats not implanted with any pellets (Fig. 1). In the i.c.v. vehicle-treated rats, s.c. administration of morphine at a dose of 3 mg/kg dramatically elevated the mechanical nociceptive threshold, which peaked 30 min and disappeared 150 min after s.c. administration, while a dose of 0.5 mg/kg produced no or a slight elevation of the nociceptive threshold. I.c.v. administration of DL-TBOA (10 nmol) did not alter the mechanical nociceptive threshold 10 min after i.c.v. administration (just before s.c. administration of morphine), or the acute antinociceptive effect of morphine at doses of both 0.5 mg/kg ($F(1,117) = 0.52$, $P = 0.47$) and 3 mg/kg ($F(1,144) = 0.10$, $P = 0.75$).
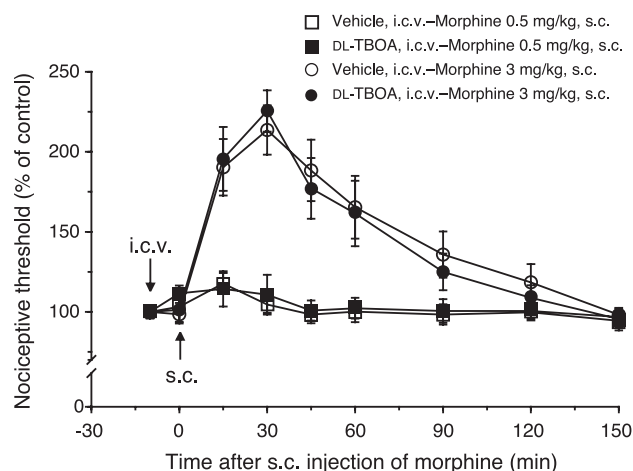

Fig. 1. Effect of i.c.v. administration of DL-TBOA on the acute antinociceptive effect of morphine in the paw pressure test. Animals were i.c.v. injected with vehicle or DL-TBOA (10 nmol) 10 min before s.c. administration of morphine (0.5 and 3 mg/kg). The nociceptive threshold of each animal just before i.c.v. administration served as the control value (100%). The values are presented as the means of the % of controls ± S.E.M. ($n = 7–10$).

Table 1
Effects of i.c.v. administration of DL-TBOA on the somatic signs induced by naloxone-precipitated morphine withdrawal

Withdrawal signs	Placebo (pellets)	Morphine (pellets)	Morphine (pellets)	Morphine (pellets)	Morphine (pellets)	Morphine (pellets)	Placebo (pellets)
	Vehicle (i.c.v.)	Vehicle (i.c.v.)	TBOA (nmol)				
			1	3	10	10	10
	Naloxone (i.p.)	Naloxone (i.p.)	Naloxone (i.p.)	Naloxone (i.p.)	Naloxone (i.p.)	Saline (i.p.)	Naloxone (i.p.)
Weight loss (%)	0.6 ± 0.2	1.6 ± 0.3^a	1.7 ± 0.3	1.8 ± 0.4	2.3 ± 0.3	0.6 ± 0.4	0.4 ± 0.2
Stretching	0.3 ± 0.2	1.6 ± 1.1	2.0 ± 0.8	3.9 ± 1.1	$8.6 \pm 2.9^{b,c}$	0	0.9 ± 0.5
Wet-dog shaking	0.4 ± 0.3	5.1 ± 2.2	4.3 ± 2.3	4.4 ± 1.8	$13.0 \pm 2.3^{b,c,d}$	0.2 ± 0.2	0.4 ± 0.3
Teeth chattering	2.1 ± 0.9	42.0 ± 13.1^e	46.4 ± 12.5	$101.9 \pm 20.0^{b,c}$	$131.7 \pm 16.4^{f,g}$	2.0 ± 1.1	3.7 ± 1.4
Jumping	0	0.6 ± 0.4	0	2.0 ± 1.3	2.4 ± 1.5	0	0
Paw shaking	1.3 ± 0.8	2.3 ± 1.0	3.1 ± 0.6	4.7 ± 2.3	5.6 ± 1.5	0.3 ± 0.2	1.4 ± 0.8
Head shaking	0.9 ± 0.3	1.3 ± 0.7	2.4 ± 1.2	3.3 ± 1.5	4.0 ± 1.4	1.5 ± 0.8	1.9 ± 0.9
Ejaculation	0	0.3 ± 0.2	0.6 ± 0.2	1.0 ± 0.3	1.7 ± 0.6^b	0	0
Backwards walking	0	0.3 ± 0.3	0.3 ± 0.3	0.6 ± 0.3	3.0 ± 1.6	0	0.3 ± 0.3
Diarrhea	0/7	4/7	4/7	3/7	5/7	0/6	0/7
Salivation	0/7	0/7	1/7	4/7	$5/7^h$	0/6	0/7
Lacrimation	0/7	1/7	1/7	5/7	4/7	0/6	0/7
Rhinorrhea	0/7	1/7	2/7	5/7	$6/7^h$	0/6	0/7
Ptosis	0/7	4/7	6/7	5/7	7/7	0/6	0/7

Animals were i.c.v. injected with vehicle or DL-TBOA (1, 3 and 10 nmol) 10 min before i.p. injection of naloxone (0.1 mg/kg). Weight loss is presented as the means ± S.E.M. of percentage body weight loss during 1 h. Signs are shown as the means ± S.E.M. of total numbers for 1 h. The occurrence of diarrhea, salivation, lacrimation, rhinorrhea and ptosis is expressed as the number of rats showing positive signs in the total number of rats tested. $n = 6$–7.

[a] $P < 0.01$ compared with the placebo pellet-i.c.v. vehicle-i.p. naloxone-treated group.

[b] $P < 0.05$ compared with the morphine pellet-i.c.v. vehicle-i.p. naloxone-treated group.

[c] $P < 0.05$ compared with the morphine pellet-i.c.v. DL-TBOA (1 nmol)-i.p. naloxone-treated group.

[d] $P < 0.05$ compared with the morphine pellet-i.c.v. DL-TBOA (3 nmol)-i.p. naloxone-treated group.

[e] $P < 0.05$ compared with the placebo pellet-i.c.v. vehicle-i.p. naloxone-treated group.

[f] $P < 0.01$ compared with the morphine pellet-i.c.v. vehicle-i.p. naloxone-treated group.

[g] $P < 0.01$ compared with the morphine pellet-i.c.v. DL-TBOA (1 nmol)-i.p. naloxone-treated group by the Student–Newman–Keuls post hoc test.

[h] $P < 0.05$ compared with the vehicle-administered group individually by the Chi-square test ($df = 1$).

3.2. Effect of i.c.v. administration of DL-TBOA on the expression of somatic signs induced by naloxone-precipitated morphine withdrawal

In the placebo pellet-implanted naive rats i.c.v. injected with vehicle, i.p. injection of naloxone (0.1 mg/kg) did not precipitate any characteristic somatic signs. In the morphine pellet-implanted morphine-dependent rats i.c.v. injected with vehicle, i.p. injection of naloxone precipitated characteristic somatic signs. The one-way ANOVA showed that there were significant differences between naive and morphine-dependent rats in weight loss ($F(1, 12) = 12.37$, $P < 0.01$) and teeth chattering ($F(1,12) = 9.20$, $P < 0.05$). I.c.v. administration of DL-TBOA (1, 3 and 10 nmol) dose dependently facilitated various somatic signs induced by naloxone-precipitated morphine withdrawal. The one-way ANOVA showed that DL-TBOA significantly increased stretching ($F(3,24) = 3.59$, $P < 0.05$), wet-dog shaking ($F(3,24) = 3.87$, $P < 0.05$), teeth chattering ($F(3, 24) = 7.65$, $P < 0.001$), and ejaculation ($F(3,24) = 3.15$, $P < 0.05$), and the Chi-square test showed significant differences in salivation ($df = 3$, $\chi^2 = 10.58$, $P < 0.05$) and rhinorrhea ($df = 3$, $\chi^2 = 9.71$, $P < 0.05$). However, DL-TBOA had no significant effect on other somatic signs such as weight loss, jumping, paw shaking, head shaking, backwards walking, diarrhea, lacrimation and ptosis. The facilitative effects of DL-TBOA (10 nmol) were observed at 0–45 min after the i.p. injection of naloxone, and disappeared toward
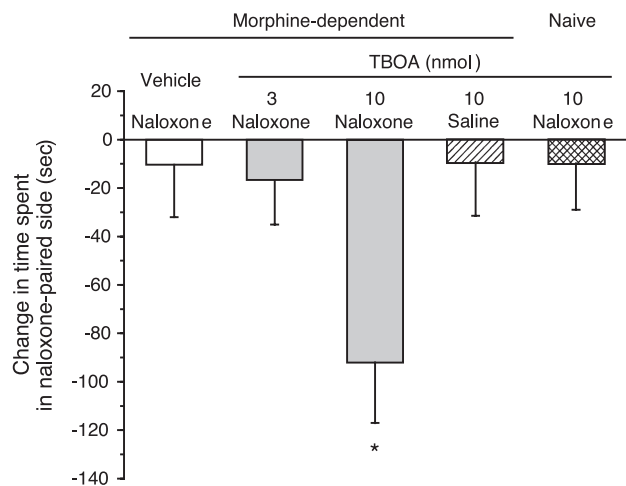

Fig. 2. Effect of i.c.v. administration of DL-TBOA on naloxone-precipitated morphine withdrawal-induced conditioned place aversion. Animals were given either a placebo or morphine pellet and then were i.c.v. injected with vehicle or DL-TBOA (3 or 10 nmol) 10 min before i.p. injection of naloxone (0.003 mg/kg) or saline. Each column represents the aversion scores expressed as means ± S.E.M. ($n = 9$–14). *$P < 0.05$ compared with the i.c.v. vehicle-injected morphine-dependent rats by the Student–Newman–Keuls post hoc test.

the end of the session (data not shown). In the morphine-dependent rats, i.p. injected with saline instead of naloxone and in the placebo pellet-implanted naive rats which were i.p. injected with naloxone, i.c.v. administration of DL-TBOA (10 nmol) did not produce somatic signs (Table 1).

3.3. Effect of i.c.v. administration of DL-TBOA on naloxone-precipitated morphine withdrawal-induced conditioned place aversion

In the i.c.v. vehicle-injected morphine-dependent rats, i.p. injection of naloxone (0.003 mg/kg) did not change the time spent in the naloxone-paired compartment in the test session compared with that in the pre-conditioning session, and the aversion score was -10 ± 22 s. I.c.v. administration of DL-TBOA (3 and 10 nmol) to morphine-dependent rats lowered the aversion score (-17 ± 18 and -92 ± 25 s, respectively). The one-way ANOVA demonstrated a significant difference among groups ($F(2,33) = 4.32$, $P < 0.05$). Post hoc comparison by the Student–Newman–Keuls test revealed that DL-TBOA at a dose of 10 nmol, but not 3 nmol, produced a significant facilitation of the naloxone-precipitated morphine withdrawal-induced conditioned place aversion, compared with the effect of vehicle ($P < 0.05$). However, in neither the morphine-dependent rats i.p. injected with saline instead of naloxone, nor the placebo pellet-implanted naive rats i.p. injected with naloxone, did i.c.v. administration of DL-TBOA (10 nmol) change the aversion score (-10 ± 19 and -9 ± 22 s, respectively) (Fig. 2).
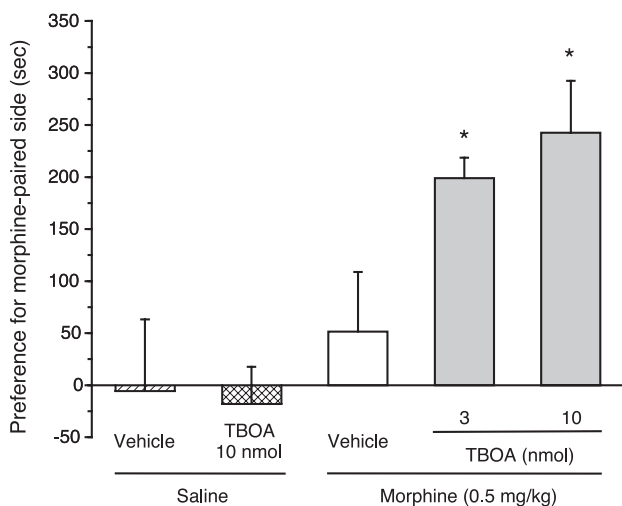

Fig. 3. Effect of i.c.v. administration of DL-TBOA on morphine-induced conditioned place preference. Animals were i.c.v. injected with vehicle or DL-TBOA (3 or 10 nmol) 10 min before conditioning by s.c. injection of morphine (0.5 mg/kg) or saline. Each column represents the preference scores expressed as means $\pm$ S.E.M. ($n = 7–8$). *$P < 0.05$ compared with the i.c.v. vehicle-administered morphine-paired rats by the Student–Newman–Keuls post hoc test.

3.4. Effect of i.c.v. administration of DL-TBOA on morphine-induced conditioned place preference

In the i.c.v. vehicle-injected rats, the preference score of the morphine (0.5 mg/kg)-paired group was 52 ± 57 s, which was slightly higher than that of the saline-paired control group (-6 ± 69 s), although there was no significant difference. I.c.v. administration of DL-TBOA (3 and 10 nmol) dose dependently increased the preference score (199 ± 20 and 243 ± 50 s, respectively). The one-way ANOVA demonstrated a significant difference among groups ($F(2,19) = 4.78$, $P < 0.05$). Post hoc comparison revealed that DL-TBOA at doses of 3 and 10 nmol produced a significant facilitation of morphine-induced conditioned place preference, compared with the effect of vehicle ($P < 0.05$). However, in the saline-paired control group, DL-TBOA (10 nmol) did not change the preference score (-18 ± 36 s) (Fig. 3).

4. Discussion

To date, five subtypes of Na^+-dependent high-affinity glutamate transporters have been cloned and characterized, namely, GLT-1, GLAST, EAAC1, EAAT4 and EAAT5. GLT-1 and GLAST are mainly expressed in astrocytes, whereas EAAC1 and EAAT4 are mainly found in neurons and EAAT5 exists predominantly in the retina (Seal and Amara, 1999). A novel glutamate transporter inhibitor, DL-TBOA, is reported to potently block all of them, while it shows no significant effects on either ionotropic or metabotropic glutamate receptors (Shimamoto et al., 1998; Shigeri et al., 2001). In contrast to L-trans-pyrrolidine-2,4-dicarboxylic acid (L-trans-PDC) and DL-threo-β-hydroxyaspartate, well-known inhibitors of glutamate transporters that evoke transport currents by themselves (Mennerick and Zorumski, 1994), DL-TBOA does not evoke currents in neuronal or glial cells, indicating that it is a nonsubstrate blocker of glutamate transporters. These properties suggest that DL-TBOA could be a suitable compound for behaviorally studying the involvement of central glutamate transporters in morphine dependence and withdrawal.

In the present study, we showed that i.c.v. administration of DL-TBOA facilitated the expression of various somatic signs induced by naloxone-precipitated morphine withdrawal. In the place-conditioning paradigm, it facilitated the naloxone-precipitated morphine withdrawal-induced conditioned place aversion. However, i.c.v. administration of DL-TBOA did not induce somatic signs or conditioned place aversion in either the placebo-pellet implanted naive rats i.p. injected with naloxone or the morphine-dependent rats i.p. injected with saline. These results suggest that DL-TBOA facilitated naloxone-precipitated morphine withdrawal symptoms, but DL-TBOA itself did not precipitate somatic signs or conditioned place aversion even in the morphine-dependent state; however, i.c.v. administration of DL-TBOA

facilitated the morphine-induced conditioned place preference. DL-TBOA had no significant effects on the preference score in the control rats even in the conditioned place preference paradigm, indicating DL-TBOA itself produced no preferential effects. These facilitative effects are unlikely to be due to the facilitation of an acute morphine effect by DL-TBOA, because it did not affect the antinociceptive effect of a single s.c. injection of morphine. Consistent with the present study, it has been reported that neither blockade of glutamate receptors (Trujillo and Akil, 1991) nor activation of glutamate transporters (Nakagawa et al., 2001) affects acute morphine antinociception.

A growing body of evidence suggests that the central glutamatergic system could be involved in the development of morphine physical dependence and/or the expression of morphine withdrawal-induced somatic signs. Many behavioral studies have reported that non-competitive and competitive NMDA receptor antagonists (Rasmussen et al., 1991; Trujillo and Akil, 1991; Manning et al., 1996; Bristow et al., 1997; González et al., 1997), non-NMDA receptor antagonists (Rasmussen et al., 1996; McLemore et al., 1997; Rasmussen and Vandergriff, 2003) and metabotropic glutamate receptor antagonists (Fundytus and Coderre, 1994; Fundytus et al., 1997) attenuate these signs. The neural substrates involved have been extensively investigated, and it is suggested that one of them is the locus coeruleus (Maldonado et al., 1992), although this still remains debatable (Christie et al., 1997). It was shown that injection of several glutamate receptor antagonists into the locus coeruleus inhibited morphine withdrawal-induced somatic signs, as well as activation of locus coeruleus neurons (Akaoka and Aston-Jones, 1991; Rasmussen et al., 1996; Rasmussen and Vandergriff, 2003). Neurochemical studies using in vivo microdialysis have directly demonstrated an elevation of extracellular glutamate levels within the locus coeruleus during morphine withdrawal (Aghajanian et al., 1994). Furthermore, direct injection of glutamate or NMDA into the locus coeruleus precipitated opioid withdrawal-like signs in opioid-dependent, but not non-dependent, rats (Tokuyama et al., 2001). These previous findings strongly suggest that activation of the glutamatergic system during morphine withdrawal in brain areas such as the locus coeruleus contributes to the expression of somatic signs (Zhu et al., 1998). Taken together, it is conceivable that the reduction of glutamate uptake by the inhibitory effect of DL-TBOA on glutamate transporters could further enhance the elevation of extracellular glutamate levels at the synaptic cleft during naloxone-precipitated morphine withdrawal in brain areas such as the locus coeruleus, to facilitate the expression of somatic signs. Consequently, the present results suggest that central glutamate transporters play an inhibitory role in the expression of morphine withdrawal-induced somatic signs. Recently, we have reported that co-administration of MS-153, a glutamate transporter activator, during repeated morphine treatment suppresses naloxone-precipitated morphine withdrawal-induced somatic signs in mice (Nakagawa et al., 2001). It is suggested that glutamate transporters might be involved in the development of morphine-induced physical dependence as well as the expression of morphine withdrawal-induced somatic signs.

It has been suggested that the neural mechanisms underlying the physical and negative affective components of morphine withdrawal could be dissociated. It was reported that morphine withdrawal precipitated by low doses of naloxone (>7.5 μg/kg) induced conditioned place aversion as well as c-*fos* gene expression in specific limbic areas such as the central nucleus of the amygdala, nucleus accumbens shell and bed nucleus of the stria terminalis, while it did not induce somatic signs (Higgins and Sellers, 1994; Gracy et al., 2001; Frenois et al., 2002). Furthermore, microinjection of methylnaloxonium, a hydrophilic opioid antagonist, in morphine-dependent rats revealed that the most sensitive structures for the induction of conditioned place aversion were the amygdala and nucleus accumbens (Stinus et al., 1990), which were less sensitive with regard to the induction of somatic signs (Maldonado et al., 1992). Nevertheless, it has been reported that systemic administration of NMDA receptor antagonists such as MK-801, D-CPPene and memantine attenuates the negative affective and physical components of morphine withdrawal (Higgins et al., 1992; Popik and Danysz, 1997; Blokhina et al., 2000). Recently, we reported that microinjection of MK-801 or D-CPPene into the central nucleus of the amygdala significantly attenuated the naloxone-precipitated morphine withdrawal-induced conditioned place aversion, but not somatic signs (Watanabe et al., 2002a). These findings suggest that activation of the glutamatergic system in specific areas such as the central nucleus of the amygdala during morphine withdrawal might contribute to the negative affective component. Taken together, it is conceivable that the facilitative effect of DL-TBOA on morphine withdrawal-induced conditioned place aversion might be due to a further enhancement of activation of the glutamatergic system during naloxone-precipitated morphine withdrawal by inhibition of glutamate transporters in areas such as the central nucleus of the amygdala.

The central glutamatergic system has also been suggested to contribute to the reinforcing effect of morphine as well as other abuse drugs (Bisaga and Popik, 2000). It was reported that systemic administration of NMDA receptor antagonists (Bespalov et al., 1994; Tzschentke and Schmidt, 1995; Del Pozo et al., 1996; Popik and Danysz, 1997; Suzuki et al., 1999) and metabotropic glutamate receptor antagonists (Popik and Wróbel, 2002) attenuated morphine-induced conditioned place preference. Importantly, some of these drugs affected neither learning nor memory retrieval (Popik and Danysz, 1997; Suzuki et al., 1999; Popik and Wróbel, 2002). Several lines of evidence suggest that the mesolimbic dopaminergic neurons, which project from the ventral tegmental area to the nucleus accumbens and other forebrain regions, play an important role in the rewarding effect of

morphine (Koob, 1992). It has been shown that the meso-limbic dopaminergic system could be modulated by gluta-matergic systems (Kalivas, 1993). For example, numerous electrophysiological studies indicate that glutamate and NMDA excite mesolimbic dopaminergic neurons (Johnson et al., 1992; Zhang et al., 1992; Seutin et al., 1994). In vivo microdialysis studies showed that glutamate injection into the ventral tegmental area elevated dopamine release in the nucleus accumbens (Kalivas et al., 1989), and the elevation of dopamine release in the striatum by systemic administration of cocaine was attenuated by NMDA receptor antago-nists (Moghaddam and Bolinao, 1994). The dopamine release elicited by glutamate in the striatum was reported to be mediated through the activation of presynaptic NMDA receptors on dopaminergic nerve terminals (Krebs et al., 1991). In addition, increased locomotor activity as a result of glutamate injection into the ventral tegmental area (Kalivas et al., 1989) or nucleus accumbens (Donzanti and Uretsky, 1983) was blocked by dopamine receptor antagonists. Popik and Kolasiewicz (1999) reported that injection of an NMDA receptor antagonist, NPC17742, into the ventral tegmental area and nucleus accumbens reduced the expression of morphine-induced conditioned place preference. Further-more, recent studies have suggested the involvement of GluR1, an AMPA glutamate receptor subunit, in the ventral tegmental area (Fitzgerald et al., 1996; Carlezon et al., 1997) and the NR2B subunit of NMDA receptors in the nucleus accumbens (Suzuki et al., 1999; Narita et al., 2000) in morphine-induced conditioned place preference. These find-ings provide evidence that glutamatergic systems in the ventral tegmental area and nucleus accumbens contribute to the reinforcing effect of morphine by affecting mesolimbic dopaminergic neurons. It was shown that a glutamate trans-porter inhibitor, L-*trans*-PDC, increased extracellular gluta-mate as well as dopamine levels in the striatum (Del Arco et al., 1999). These findings suggest that the elevated glutamate level caused by the inhibition of glutamate transporters might cause dopamine release from the striatal dopaminergic nerve terminals, probably via presynaptic NMDA receptors (Krebs et al., 1991). Taken together, it is conceivable that inhibition of glutamate transporters by DL-TBOA activates glutamatergic systems in areas such as the ventral tegmental area and nucleus accumbens, and further enhances activation of the mesolimbic dopaminergic system during repeated morphine treatment, to facilitate morphine-induced condi-tioned place preference.

Recently, we and the other investigators have suggested the involvement of changes in the glutamate transporter expression levels in morphine tolerance and dependence. We have previously reported that the expression of a glial glutamate transporter, GLT-1 mRNA, was decreased in the striatum and thalamus of morphine-dependent rats (Ozawa et al., 2001). Mao et al. (2002) have shown that chronic morphine treatment decreases EAAC1- and GLAST-immu-noreactivity in the rat spinal cord dorsal horn, and intrathecal administration of a glutamate transporter inhibitor, L-*trans*-PDC, facilitates the development of morphine tolerance, suggesting that a decrease in spinal glutamate transporters might contribute to the development of morphine tolerance. The enhancement of central glutamatergic transmission following a decrease in glutamate transporter expression levels might contribute to the development of morphine tolerance and dependence, and the expression of morphine withdrawal.

Because DL-TBOA is reported to inhibit all subtypes of Na$^+$-dependent glutamate transporters (GLAST, GLT-1, EAAC1, EAAT4 and EAAT5) (Shimamoto et al., 1998; Shigeri et al., 2001), it is difficult to determine which subtype is involved in the facilitative effects of i.c.v. DL-TBOA on the expression of morphine-withdrawal induced-somatic signs and conditioned place aversion, and acquisi-tion of morphine-induced conditioned place preference. However, GLT-1 is reported to play the most important role in maintaining low extracellular glutamate levels (Rothstein et al., 1996; Tanaka et al., 1997), and furthermore, we have shown changes in the expression of mRNA for GLT-1, but not GLAST, in the brain of morphine-dependent and nal-oxone-precipitated withdrawal rats (Ozawa et al., 2001). Taken together, we consider that a glial glutamate trans-porter, GLT-1, might be involved in the facilitative effects of DL-TBOA in the present experiments. Additional investiga-tions are needed to fully explore this.

In conclusion, we have shown that i.c.v. administration of DL-TBOA facilitates naloxone-precipitated morphine withdrawal-induced somatic signs and conditioned place aversion, and the acquisition of morphine-induced condi-tioned place preference, without affecting acute morphine-induced antinociception. These findings suggest that central glutamate transporters play inhibitory roles in the expression of somatic and negative affective components of morphine withdrawal and the reinforcing effect of morphine. The present study may provide a new strategy for preventing morphine dependence.

Acknowledgements

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