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The role of NR2B containing NMDA receptor in place preference conditioned with morphine and natural reinforcers in rats

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Abstract

It has been reported that *N*-methyl-D-aspartate (NMDA) receptor is implicated in drug addiction and antagonists of the NMDA receptor complex can inhibit the development and expression of conditioned place preference (CPP) induced by several addictive drugs, implying that this class of compounds might be considered as candidate for the treatment of substance abuse. To explore this possibility, it is important to evaluate whether the inhibitory effect of NMDA receptor antagonists would be confined to behaviors produced by drugs of abuse only, but not by natural reinforcers. According to the quantitative changes of NMDA receptor subunits, including NR1, NR2A, and NR2B, induced by diverse types of reinforcers, we chose NR2B subunit as the target of research. Experimental results showed that (1) an augmented expression of NR2B subunit was revealed by Western blotting in the nucleus accumbens (NAc) and the hippocampus in rats with CPP induced by morphine, but not by natural rewards such as food, novel environment and social interaction. (2) Ifenprodil, an antagonist highly selective for NR2B subunit of the NMDA receptor, produced a dose-dependent reduction in CPP induced by morphine and novel environment, but not that by food consumption and social interaction. Taking together, these findings suggested that NR2B containing NMDA receptor may be more involved with morphine reward rather than natural rewards, and that antagonism of NR2B may have a potential for the treatment of morphine abuse.

Keywords: Conditioned place preference; Morphine; Natural reinforcers; NMDA receptor; Rats

Introduction

The conditioned place preference (CPP) paradigm has been widely used to assess the rewarding properties of a given treatment (Chen et al., 2005; Orsini et al., 2005). In addition to drug rewards, CPP can also be used to study the reinforcing properties of natural rewards, such as food consumption (Bevins et al., 2002; Salamone et al., 2003), sexual encounter (Martinez and Paredes, 2001; Camacho et al., 2004), and some less obvious reinforcers such as social interaction (Douglas et al., 2004) and novelty (Bevins et al., 2002; Parker, 1992).

The aim of the pharmacotherapy for drug addiction is to find a way which selectively interferes with the drug-related responses while leaving other biologically important behaviors such as food consumption, novelty approach and social-interaction intact. For example, experimental medications are required to alter drug self-administration (SA) while having little or no effects on food-seeking and eating (Mello et al., 1993; Soria et al., 2005). Similarly, in place conditioning studies, effects of the potential medications are assessed in both morphine- and nondrug-conditioned animals (Popik and Danysz, 1997; Papp et al., 2002).

One of the commonly accepted views on the mechanisms of compulsive drug seeking and taking are that both drug and natural rewards are mediated by a common neuroanatomical pathway, in which the major components are the nucleus accumbens (NAc), the hippocampus, the prefrontal cortex

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(PFC), and the amygdala (Paredes and Agmo, 2004; Fuchs et al., 2005; Ventura et al., 2005; Sun and Rebec, 2005). However, if a chemical would affect both drug and natural reward at an equal efficiency, it could not be accepted as a useful pharmacological solution. Since NMDA (and other glutamate) receptor antagonists are probed as potential candidates for developing an effective medication to treat drug addiction (Bisaga and Popik, 2000; Herman et al., 1995; Loftis and Janowsky, 2003; Tzschentke and Schmidt, 2003), the specificity of their effects becomes of critical importance.

Most NMDARs in the brain are thought to be heteromeric complexes with NR1 as constructive subunit and NR2 (A–D) as functional subunits to increase the NMDA receptor-mediated current (Narita et al., 2000). Distributions of NR1, NR2A and NR2B, but not NR2C and NR2D, are consistent with brain regions related to reward (Mori and Mishina, 1995). However, it has never been systemically demonstrated which subunit of NMDARs (NR2A or NR2B) dominantly contributes to drug seeking and drug taking, except for the data presented by Narita et al. (Narita et al., 2000).

The present study was designed firstly to establish CPP induced by natural rewards. Then we examined the changes in individual NMDAR subunit proteins, including NR1, NR2A, and NR2B, in the rats induced by diverse types of reinforcers, which would guide us to choose a subunit-selective NMDA receptor antagonist to interfere with rewards induced by drug and non-drug maneuvers.

Materials and methods

Subjects

All experiments were performed on male Sprague–Dawley rats, obtained from the Experimental Animal Center, Peking University, weighing 180–200 g at the beginning of the experiment. Animals were housed 4 per cage in a 12:12 h light/dark cycle (lights on at 07:00 h) with food and water available at all times. The room temperature was maintained at 22±1°C and relative humidity at 45–55%. Animals were conditioned and tested during the light phase of the cycle. They were handled daily during the first week after arrival. All experimental procedures were approved by the Animal Use Committee of Peking University Health Science Center.

Apparatus

Conditioning was conducted in black colored rectangular PVC boxes (795×230×250 mm^3), containing three chambers separated by guillotine doors (Shi et al., 2004). The two large black conditioning chambers (A and C, 280×220×225 mm^3) were separated by a small gray center choice chamber B (135×220×225 mm^3). Chamber A has 4 light-emitting diodes (LEDs) forming a square on the wall and a stainless steel mesh floor (225×225 mm^2), chamber C has 4 LEDs forming a triangle on the wall and a stainless-steel rod floor (15 mm apart), whereas chamber B has a plain floor. Fourteen photobeams were placed across chambers with 47.5 mm apart. Through a computer interface, the time spent for the rat in each chamber was recorded by means of infrared beam crossings.

General place preference procedures

The CPP experiments were conducted using procedures described previously (Shi et al., 2004). Except for minor variations described below, essentially the same CPP paradigm was adopted for all experiments as follows.

Pre-conditioning test phase

On day 0, rats were placed in the center choice chamber with the guillotine doors removed to allow access to the entire apparatus for 15 min and time spent in each side was recorded. These data were used to separate animals into groups with approximately equal biases for each side. Rats with a bias for one side were omitted from the experiments.

Conditioning phase

Beginning on day 1, the animals were allowed for a training period twice a day (09:00 and 15:00) for 4 days. The animals received either drug or natural reward (see below) before confined in one side, and a vehicle injection or no treatment, as control, before confined in the other side. Drug- or nature reward-paired sides were counter balanced among all groups.

Post-conditioning test phase

On day 5, all of the animals were placed in the center choice chamber with the guillotine doors removed to allow access to the entire apparatus for 15 min and time spent in each side was recorded.

Conditioning with morphine

Conditioning consisted of four pairs of 45-min sessions, in which the animals were given morphine (3 mg/kg; i.p.) on one side and saline on the other side. Rats in control group, however, received vehicle (0.9% sodium chloride) injections on both sides of the apparatus.

Conditioning with novelty

The method used in the present study was adopted from Papp et al. (2002). Conditioning consisted of four pairs of 20-min sessions, in which the animals were confined always in the fixed compartments of the CPP apparatus, leaving the other compartment as novel environment during testing. However, rats in the control group were confined in one side during the morning trial and in the other side during the afternoon trial, so that both sides were familiar to the animals.

Conditioning with social interaction

Conditioning consisted of four pairs of 30-min session. On rewarding trials in the morning, a male rat was placed in a side chamber. Twenty min later another male rat was introduced into the same chamber. Different partner was used on each

conditioning trials. On non-rewarding trials in the afternoon, no partner was introduced. Rats in the control group were confined alone in either of the lateral compartments.

Conditioning with food

During this experiment, rats were deprived of food for 23 h per day. During the first 8 days, rats were adapted to the 'feeding cages' and the feeding procedure. Subjects were placed individually for 1 h into the 'feeding cages' (standard plastic laboratory cages measuring $275 \times 215 \times 130$ mm^3, with a bottle of tap water and sawdust bedding). The amount of food consumed was monitored by weighing it before and after feeding. At the end of this 8-day period, rats achieved no less than 80% of their original body weight and consumed an average of 11.12 ± 0.61 g of chow per 1-h period (Fig. 2). There was no significant difference among groups with regard to the amount of food consumed and the loss of body weight. Nine days after the beginning of the food-deprivation procedure, the pre-conditioning test took place, followed by the conditioning sessions as described above. In rewarding trials in the morning, rats were placed in the 'feeding cages' with food for 1 h. The subjects were then transferred into the designated side of the conditioning apparatus for 20 min. On non-rewarding trials in the afternoon, rats were placed in the 'feeding cages' without food (sham feeding) for 1 h. The subjects were then transferred into the other side of the conditioning apparatus for 20 min. Rats in the control group were confined alone in either side of the apparatus. The control groups were feeding for 1 h at random times in each day.

Implantation of microinjection cannulae

One week before pre-conditioning test, the rats were treated with atropine methyl nitrate (0.4 mg/kg, i.p.) and penicillin (1.5×10^5 U/rat), and were anesthetized with chloral hydrate (35 mg/kg, i.p.), and mounted on a Kopf stereotaxic apparatus (Kopf Instruments, Tujunga, CA). The scalp was incised and retracted, and the head position was adjusted to place bregma and lambda in the same horizontal plane. Small burr holes (1 mm in diameter) were drilled on the skull for the placement of stainless steel guide cannulae (0.8 mm in outer diameter) into the lateral ventricle (anteroposterior (AP) +0.8 mm, lateral (L) +0.8 mm, dorsoventral (DV) −2.5 mm), 1 mm above the intended site of injection. Guide cannulae were anchored to the skull with sterile stainless steel screws and the dental acrylic cement. After surgery, stainless steel obturator (0.4 mm in outer diameter) was inserted into the guide in order to prevent cannula occlusion. The obturator was removed and replaced every other day during the 7-day recovery period. To habituate animals to the microinjecting procedures, all rats were given daily 'mock' microinjection for 3 days, with injector placed within but did not extend beyond the guide cannula, prior to their first microinjection. The infusion pump did not engage the syringe plungers during this mock infusion procedure but was allowed to run for 5 min to habituate the rats to the weak noise of the pump.

Lateral ventricular injection

Obturator was removed and infusion cannula (0.4 mm in outer diameter) was inserted, extending 1.0 mm beyond the tip of guide cannula into the right lateral ventricle. Microinjections of vehicle or ifenprodil (2, 6, 20 μg/10 μl per rat; Sigma, St. Louis, MO) were administered into the lateral ventricle. The dosage of ifenprodil was chosen according to the estimate that the dose of microinjection into lateral ventricle should be around 1% dose of systematic injection. Since systematic injection of ifenprodil (10 mg/kg or 2 mg for a rat of 200g) produced complete inhibition of the development of morphine-induced CPP in rats (unpublished data of our lab), the microinjection doses of 2, 6, 20 μg/rat were chosen for lateral ventricle injection. The injection was performed through an infusion pump (1 μl/min) while the rat was gently held. The microinjector was left in place for an additional minute to allow for drug diffusion. The obturator was then replaced, and rats were placed back to their home cages.

Effect of ifenprodil alone

Subjects were confined on one side for 45 min in the morning and on the opposite side for 45 min in the afternoon, without any reinforcers introduced. Four subgroups of animals (ifenprodil administered during CPP development) received vehicle or ifenprodil (2, 6, 20 μg/10 μl per rat, i.c.v.) 30 min before each of the four rewarding conditioning trials in the morning; and another four subgroups (ifenprodil given during CPP expression) were administered with vehicle or ifenprodil (2, 6, 20 μg/10 μl per rat, i.c.v.) 30 min before post-conditioning test.

Effect of ifenprodil on conditioned place preference

In four separate subexperiments, place preference was established as described above, using drug, novelty, social interaction and food, respectively. In each experiment, four subgroups (ifenprodil in CPP development) received vehicle or ifenprodil (2, 6, 20 μg/10 μl per rat, i.c.v.) 30 min before each of the four rewarding conditioning trials, except for food consumption-induced CPP, in which ifenprodil was injected 90 min before each of the four rewarding conditioning trials, which is 30 min before feeding. Another four subgroups (ifenprodil given in CPP expression) were administered vehicle or ifenprodil (2, 6, 20 μg/10 μl per rat, i.c.v.) 30 min before post-test.

Tissue dissection and preparation

Rats were decapitated immediately after the CPP test. The brains were removed and placed on an ice-cooled plate for dissection of the nuclei accumbens, the hippocampus, the amygdala, and the PFC according to stereotaxic atlas of Paxinos and Watson. (1986). Tissue samples were frozen in liquid nitrogen and then stored at −80°C until analysis. The tissue was homogenized in 10 volumes of ice-cold buffer containing 20 mM Tris–HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 1 mM

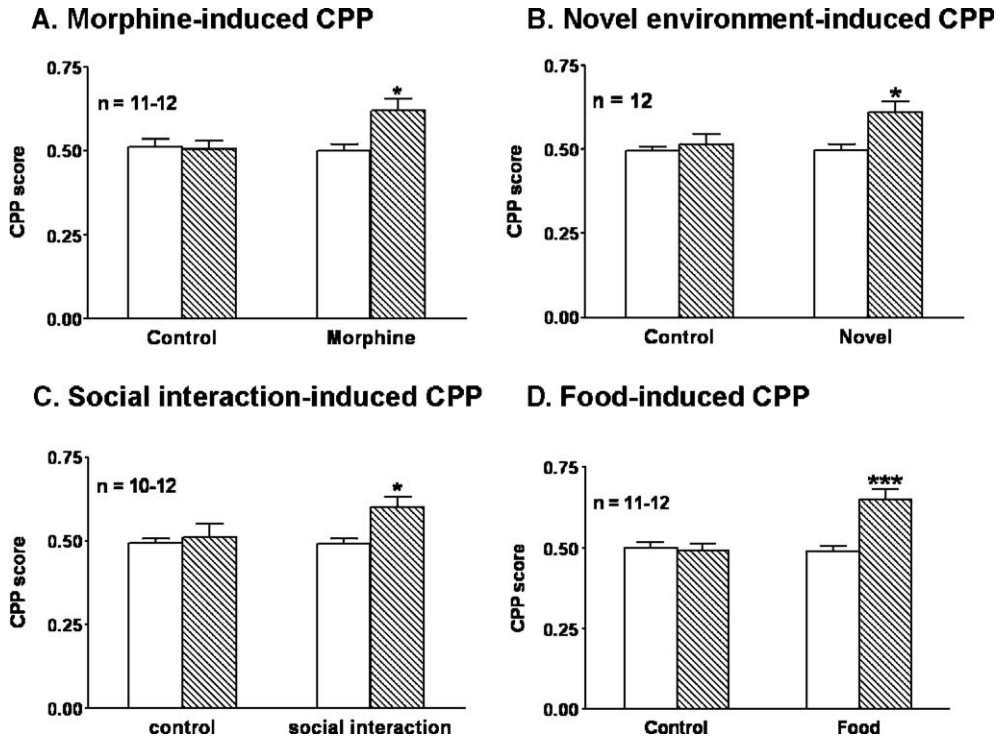

Fig. 1. Drug- and natural reward-induced place preference. Values are expressed as mean (±SEM) of CPP score, $n = 10–12$ in each group. In each panel, the first pair of columns shows the data of animals in control groups, and the second pair of columns shows the data of animals receiving morphine or natural reward training. Blank and striped columns represent data from pre- and post-conditioning test, respectively. $^{*}P < 0.05$, $^{***}P < 0.001$, post- vs. pre-conditioning test.

phenylmethylsulfonyluoride, 25 mg/ml of leupeptin, 0.1 mg/ml of aprotinin and 0.32 M sucrose using a Potter–Elvehjem tissue grinder with Teflon pestle. The homogenate was then centrifuged at 1000 × g for 10 min and the supernatant was centrifuged at 100,000 × g for 30 min at 4°C. The pellet was homogenized in homogenizing buffer containing 0.2% (w/v) Triton X-100. The homogenate was kept at 4°C for 60 min with occasional stirring and then centrifuged at 100,000 × g for 30 min at 4°C. The resulting supernatant was used as the membrane fraction. Protein concentrations were determined using a BCA assay (Pierce, Rockford, IL).

Immunoblotting of NMDA receptor subunits

Equivalent amounts of membrane preparations (50 μg) for each sample were resolved in 7.5% SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were incubated in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBST) containing 5% nonfat milk for 1 h at room temperature with agitation to block nonspecific binding. The membrane was incubated with primary antibody diluted in TBS (NMDAζ1 (NR1), NMDAε1 (NR2A) and NMDAε2 (NR2B), 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) containing 5% nonfat dried milk overnight at 4°C. The membrane was then washed twice for 5 min and then twice for 10 min in TBST followed by 1 h of incubation at room temperature with horseradish peroxidase-conjugated rabbit anti-goat IgG (Zhongshan Biotechnology, Beijing, China) diluted 1:10,000 in TBS containing 5% nonfat dried milk. After this incubation, the membranes were washed twice for 5 min and then three times for 10 min in TBST. The antigen–antibody peroxidase complex was then finally detected by enhanced chemiluminescence (Zhongshan Biotechnology, Beijing, China) according to the manufacturer's instructions and visualized by exposure to Kodak film (Eastman Kodak, Kodak, NJ). The bands on the autoradiogram were quantified with the TotalLab 2.01 Analysis System (Phoretix, UK), and the optical density of the corresponding β-actin band corrected the optical density of each band of the NMDA receptors subunits. The values are presented as a percentage of the control.

Drugs

Morphine hydrochloride was purchased from the first pharmaceutical factory of Shenyang, China. Ifenprodil was purchased from Sigma, USA. Morphine was dissolved in saline, and ifenprodil tartrate was dissolved in DMSO (dimethyl sulfoxide, from Sigma, USA) and diluted in 5% DMSO with 9% Tween 80/saline before use.

Data analysis

CPP score represents the index of place preference (Shi et al., 2004) for each rat, calculated by dividing the time spent in the drug-paired compartment by the time spent in both conditioning compartments. Data were processed by commercially available software Graph Pad Prism 4.0. Results were presented as mean

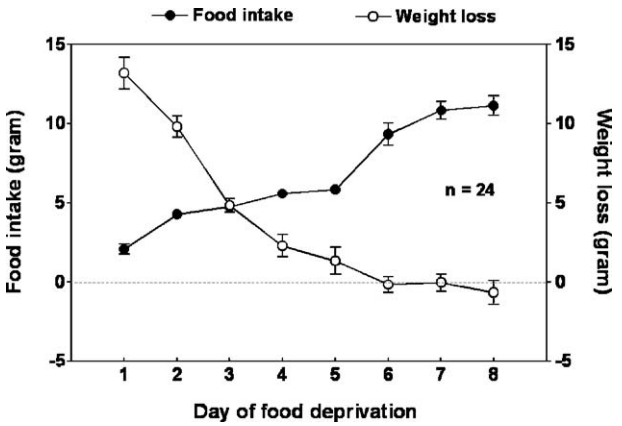

Fig. 2. An 8-day period of daily food deprivation for 23 h was sufficient for rats to adapt to only 1 h feeding per day. The amount of food consumed and the quantity of weight loss became stable by day 7. At the end of this 8-day period, rats achieved no less than 80% of their original body weight and consumed an average of 11.12 ± 0.61 g of chow per 1-h period.

±SEM. Results from behavioral and immunoblotting experiments were analyzed with two-way analysis of variance (ANOVA) followed by Bonferroni post-test. The accepted level of statistical significance is $P<0.05$.

Results

Conditioned place preference induced by morphine or natural rewards

Results of morphine- and natural reward-induced place preference are summarized in Fig. 1. Significant place preference was observed with all four subgroups conditioned by morphine, novelty, social interaction and food, respectively ($P<0.05$ for morphine, novelty, and social interaction; $P<0.001$ for food. post- vs. pre-conditioning test). Four subgroups of control animals (see details in Materials and methods) showed no changes in preference as a result of conditioning ($P>0.05$, post- vs. pre-conditioning test).

In the experiment of novelty-induced CPP, there are two ways to analyze the results. One is the conventional method using the ratio of the time spent in the two chambers, as is shown in Fig. 1B. The other is to count the number of crossings during post-conditioning test. Analysis revealed a significant increase of shuttle between the neutral and novel compartment, and a decrease of crossing between the neutral and familiar compartment. ($P<0.05$, Fig. 7A).

In the experiment of food induced CPP, Fig. 2 showed that 8-day period of food deprivation for 23 h prior to conditioning was sufficient for rats to adapt to only 1 h feeding per day.

Change of brain NMDA receptor subunits levels during the development of conditioned place preference

To investigate the molecular mechanism of the CPP behavior, we assessed changes in the levels of NMDA receptor subunits in brain regions associated with reward and drug craving. Western blotting analysis with a goat polyclonal immunoglobulin G specific to each NMDA receptor subunit indicated a significant increase in the levels of NR2B subunit in the NAc ($P<0.001$ vs. control group, Fig. 3A) and the

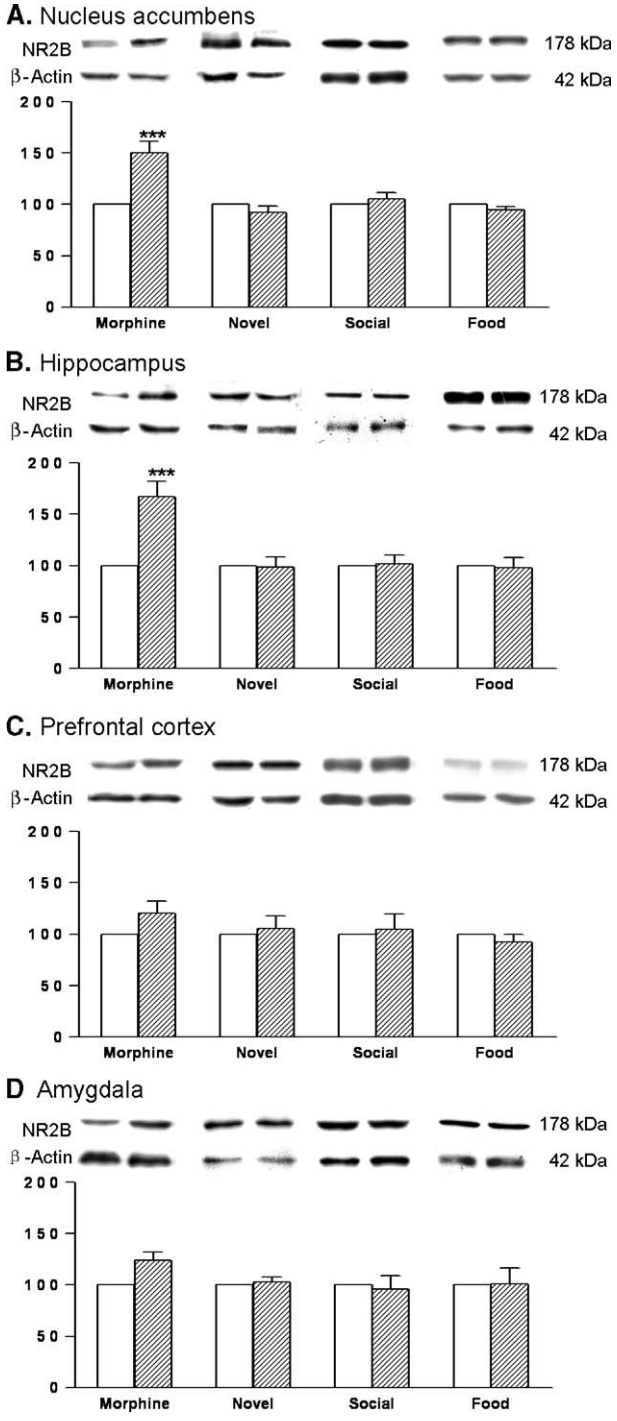

Fig. 3. Change of NR2B levels in brain regions related to rewards. Representative results of Western blotting are shown on the top of each panel. Statistical data are expressed as mean (±SEM). In each panel, the first pair of columns shows the data of animals conditioned by morphine (A), the second pair by novel environment (B), the third pair by food consumption (C), and the last pair by novel environment (D). Striped and blank columns represent data from the conditioning and the corresponding control group, respectively. ***$P<0.001$, compared with the control group conditioned by saline (two-way ANOVA, Bonferroni post-test).

hippocampus ($P<0.001$ vs. control group, Fig. 3B) in CPP rats conditioned by morphine, but not by any natural rewards ($P>0.05$ vs. control group Fig. 3C, D). No change in the protein levels of NR1, NR2A subunits was seen in the NAc, hippocampus, PFC, and amygdala (data about NR1, NR2A in morphine-induced CPP rats were shown in Fig. 4).

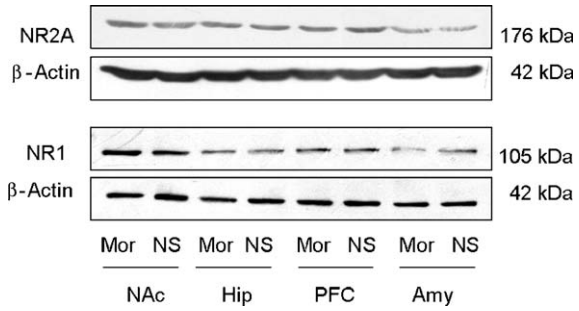

Fig. 4. No change of NR1 and NR2A subunits levels in brain regions of CPP rats induced by morphine Mor means receiving alternative injections of morphine and saline in the CPP training; NS means saline and saline in the CPP training. NAc, Hip, PFC, and Amy mean the nucleus accumbens, the hippocampus, the prefrontal cortex, and the amygdale, respectively.

Effects of ifenprodil alone

Subjects were confined to one side for 45 min in the morning and to the opposite side for 45 min in the afternoon, without any reinforcers introduced. In order to explore the effect of ifenprodil alone on the development of CPP, four subgroups of animals were administered with vehicle or ifenprodil (2, 6, 20 μg/10 μl/rat, i.c.v.) 30 min before each of the four rewards conditioning trials (Table 1, row 1). Two-way ANOVA indicated no significant interaction between treatment and time point [$F(3, 88) = 1.54$, $P = 0.2099$], nor any significant difference between pre- and post-conditioning test [$F(1, 88) = 0.81$, $P = 0.3695$], and among the groups of animals that were injected with different doses of vehicle or ifenprodil (2, 6, 20 μg/10 μl/rat, i.c.v.) [$F(3, 88) = 0.81$, $P = 0.3695$]. In order to explore the effect of ifenprodil alone on the expression of CPP, another four subgroups were administered with vehicle or ifenprodil (2, 6, 20 μg/10 μl/rat, i.c.v.) 30 min before post-test (Table 1, row 2). Two-way ANOVA indicated no significant interaction [$F(3, 88) = 0.04$, $P = 0.9906$] between treatment and time point, nor any significant difference [$F(1, 88) = 0.07$, $P = 0.7982$] between pre- and post-conditioning test, and [$F(3, 88) = 0.10$, $P = 0.9589$] among the groups of animals that were injected with different doses of ifenprodil (2, 6, 20 μg/10 μl/rat or vehicle 10 μl/rat, i.c.v.).

Effects of ifenprodil on the development of conditioned place preference

In four separate experiments, place preference was established as described above, using morphine, novelty, social interaction and food as reinforcers. In order to test the effects of ifenprodil on the development of CPP, four subgroups of each experiment were given vehicle or ifenprodil (2, 6, 20 μg/10 μl/rat, i.c.v.) 30 min before each of the four rewards conditioning trials. Morphine-induced place preference was attenuated by ifenprodil in a dose-dependent manner (Fig. 5A), while novelty-induced shift in preference was blocked only by high dose of ifenprodil (20 μg/10 μl/rat) (Fig. 5B). Using another method of analysis for the novelty CPP, one can also find that ifenprodil produced a marked attenuation of the number of shuttles between the neutral and novel compartment in a dose dependent way (Fig. 7B). No significant effects of ifenprodil were observed on social- and food-induced place preference (Figs. 5C, D).

Table 2 showed that ifenprodil had no significant effect on food intake during conditioning trials, although a tendency of decrease in food intake was seen in rats injected with ifenprodil.

Effects of ifenprodil on the expression of conditioned place preference

In order to clarify the effects of ifenprodil on the expression of CPP, another four subgroups in each experiment were administered with vehicle or ifenprodil (2, 6, 20 μg/10 μl/rat, i.c.v.) 30 min before post-conditioning test. Expression of place preference induced by morphine was blocked by ifenprodil in all of the three doses used in the present experiment (Fig. 6A), while expression of place preference induced by novelty was inhibited only by middle and high doses of ifenprodil (Fig. 6B). Analysis of the number of crossings during post-conditioning test revealed that ifenprodil, injected 30 min prior to post-conditioning test, attenuated the increased number of shuttles between the neutral and novel compartment in all of the three doses

Table 1
Effect of ifenprodil alone in two separate experiments

Phase of CPP	Vehicle (10μl/rat, i.c.v.)		Ifenprodil (μg/10 μl/rat, i.c.v.)					
			2		6		20	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Development	0.49 (±0.02)	0.50 (±0.03)	0.53 (±0.02)	0.47 (±0.04)	0.49 (±0.02)	0.50 (±0.02)	0.50 (±0.02)	0.50 (±0.02)
Expression	0.50 (±0.01)	0.50 (±0.02)	0.50 (±0.03)	0.49 (±0.02)	0.49 (±0.03)	0.49 (±0.02)	0.50 (±0.02)	0.51 (±0.02)

Values are expressed as mean (±SEM) of CPP score, $n = 12$ in each group. In the 'development' experiment, vehicle or ifenprodil was administered 30 min prior to the confinement of the rat in one side of apparatus in the morning and no injections were administered prior to confinement in the opposite side in the afternoon. In the 'expression' experiment, vehicle or ifenprodil was administered 30 min prior to the post-conditioning test. Pre: CPP score in the pre-conditioning test; post: CPP score in the post-conditioning test.

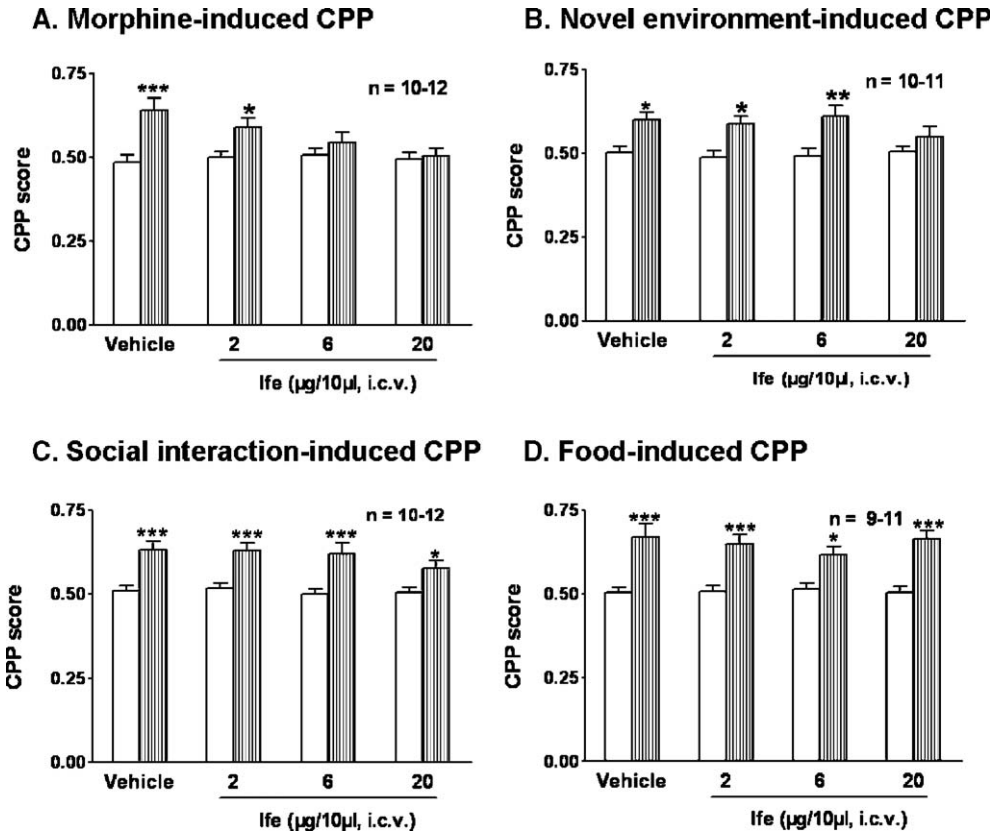

Fig. 5. Effect of ifenprodil (Ife) on the development of CPP. Values are expressed as mean (±SEM) of CPP score, $n = 9–12$ in each group. In each panel, the first pair of columns shows the data of animals receiving vehicle (10 μl/rat, i.c.v.), and the other three pairs of columns show the data of animals receiving ifenprodil (2, 6, 20 μg/10 μl, i.c.v.) 30 min before each of the four rewarding conditioning trials. Blank and striped columns represent data from pre- and post- conditioning test, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, post- vs. pre-conditioning test (two-way ANOVA, Bonferroni post-test).

adopted in the present study (Fig. 7B). No significant effects of ifenprodil on social- and food-induced place preference were observed (Fig. 6C, D).

Discussion

Conditioned place preference induced by drug or natural rewards

As expected, addictive drugs such as morphine could induce place preference. In the present study significant CPP was also observed in experiments using food consumption, social interactions, and novel environment as reinforcers. It is interesting to note that these three types of natural rewards are different in their conditioning properties. In contrast to previous studies using food place conditioning (Popik and Danysz 1997; Papp et al., 2002) where food was provided in the conditioning compartment, in our study, rats were allowed to consume food for 1 h in a 'feeding cage' (corresponding to the injection of morphine given prior to the confinement of rats in the conditioning environment) and were then exposed to the conditioning environment immediately after feeding. This is

Table 2
Ifenprodil has no effect on food intake during conditioning trials

Conditioning	Random feeding	Regular feeding				
		Blank	Vehicle (10μl/rat, i.c.v.)	Ifenprodil (μg/10 μl/rat, i.c.v.)		
				2	6	20
	$n = 12$	$n = 12$	$n = 9$	$n = 10$	$n = 10$	$n = 10$
Day 1	12.18 (±1.00)	12.89 (±1.20)	11.23 (±2.30)	9.29 (±2.87)	9.01 (±2.40)	10.35 (±2.39)
Day 2	13.09 (±0.89)	12.04 (±0.89)	12.56 (±2.54)	10.18 (±2.43)	10.78 (±2.96)	11.94 (±2.78)
Day 3	13.94 (±1.12)	14.07 (±1.13)	14.04 (±1.97)	12.03 (±3.03)	12.39 (±1.98)	11.38 (±3.01)
Day 4	15.01 (±1.11)	14.12 (±1.09)	13.89 (±2.25)	12.44 (±2.09)	12.29 (±2.70)	12.03 (±2.93)

Values are expressed as mean (±SEM) of the amount of food consumed (g/day). Random feeding values are the mean of the group feeding for 1 h at random times in each day without injection. Regular feeding values are the mean of the groups injected with a, nothing (Blank column), b, vehicle (10 μl/rat, i.c.v.), and c, ifenprodil (2, 6, 20 μg/10 μl/rat, i.c.v.) 30 min before feeding for 1 h in each morning trial.

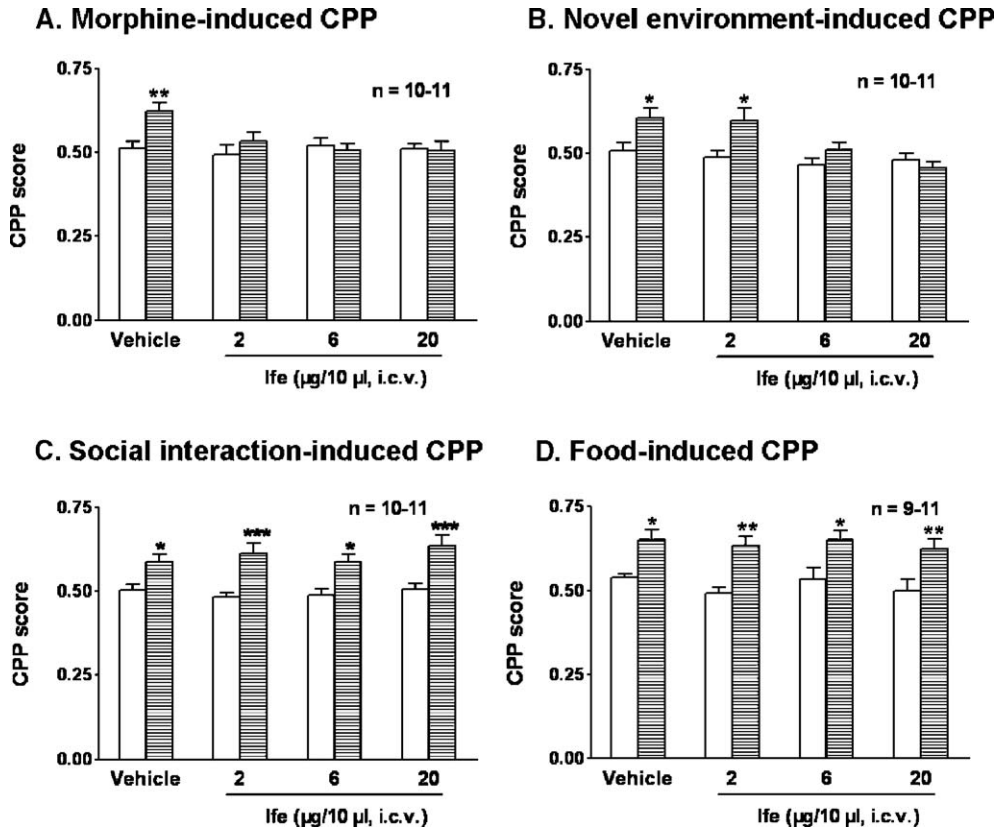

Fig. 6. Effect of ifenprodil (Ife) on the expression of place preference. Values are expressed as mean (±SEM) of CPP score, $n = 9–12$ in each group. In each panel, the first pair of columns shows the data of animals receiving vehicle (10 μl/rat, i.c.v.), and the other three pairs of columns show the data of animals receiving ifenprodil (2, 6, 20 μg/10 μl, i.c.v.) 30 min before post-conditioning test. Blank and striped columns represent data from pre- and post-conditioning test, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, post- vs. pre-conditioning test (two-way ANOVA, Bonferroni post-test).

termed backward conditioning paradigm, similar to those used by Lett et al. (2000). To establish social interaction-induced place preference, we adopted a forward conditioning paradigm, in which a male rat was exposed to the positive compartment for 20 min alone until a novel, male partner was introduced. This is the same as the procedure used by Papp et al. (2002). In contrast with the previous two procedures, novel environment-induced place preference established in the present study was an atypical conditioning paradigm (Papp et al., 2002), neither backward nor forward. During the conditioning, rats experienced repeated exposures to the very same lateral compartments and never had a chance to visit the opposite compartment. Therefore these rats would prefer to visit the compartments novel to them (Fig. 7A). The successful development of CPP using the three natural reinforcers made the comparison between characteristics of drug and natural rewards feasible.

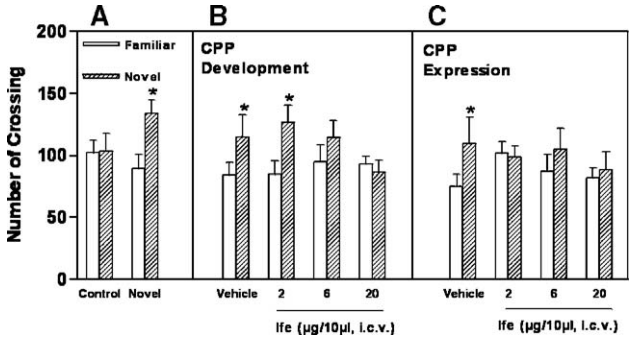

Fig. 7. An alternative way showing the novel environment-induced place preference, by counting the number of crossings between two adjacent compartments in the 15-min post-conditioning test. Values are expressed as mean ± SEM, $n = 10–11$ in each group. The blank columns represent the number of crossings between the familiar and the neutral compartment, and the striped columns represent the number of crossings between the novel and neutral compartment. $^{*}P < 0.05$, striped vs. blank column.

Augmented expression of NR2B subunits induced by drug, but not natural rewards

The levels of NMDA receptor subunits were detected using Western blotting. Rats conditioned by morphine show robust increase of NR2B subunits in the NAc and the hippocampus, without significant change in the PFC and the amygdala. On the contrary, rats conditioned by all three varieties of natural rewards show no change of NR2B subunits in any of the four brain regions known to be related with rewards. It can thus be speculated that separate neural circuits may be involved in the mediation of behaviors induced by drug (represented by morphine in the present study) or non-drug rewards. Indeed, results of animal experiments have offered evidence that in subjects self-administering either cocaine or a natural reinforcer (food or water), the two types of rewards activated different populations of cells in the NAc, with fewer than 10%

of cells responding to both cocaine and natural rewards (Carelli et al., 2000).

Although all functional NMDA receptors appear to contain NR1 subunits, we failed to observe any changes in NR1 subunits following morphine or natural reinforcers. Existing data are not enough to clearly explain why the increase in NR2B proteins was not accompanied by a similar increase in NR1 subunits. One possibility is that some NMDA receptors composed of NR1 subunit and another subunits such as NR3A (Ma and Sucher, 2004; Narita et al., 2000) may be decreased, so that the total amount of NR1 subunits are apparently unchanged following morphine treatment.

It was interesting to note that the change of NR2B subunit levels in rats with morphine injection following the same schedule as in the CPP experiment but without the behavioral component of place preference was not significantly different from that in control rats with alternative saline injection only (Fig. 8). It was suggested that the changes in the concentration of the NR2B subunit were due to the combination of the activation of the reward circuit by morphine and the CPP paradigm but not by pharmacological actions of morphine alone. The mechanisms determining CPP appear to follow the principles of classical (Pavlovian) conditioning. An up-regulation of NR2B containing NMDA receptor was postulated to be involved in the process of association learning between morphine rewarding effect and the previously neutral set of environmental cues.

Analysis of the effect of ifenprodil on the place preference

In the present study, ifenprodil was found to inhibit the development and expression of place preference induced by morphine as well as by novel environment in a dose-dependent manner, with little or no effect on that induced by food consumption and social interaction. Before drawing a conclusion favoring the notion that ifenprodil selectively blocks the rewarding effects of morphine, one should first consider some alternative explanations.

First, attenuation of place preferences conditioned with both morphine and novel environment suggests that ifenprodil might impair discrimination between the environments, resulting in a general motivational deficits and/or nonspecific disruption of the test performance. This could occur as a result of loss of visual discrimination ability. NMDA antagonism was reported

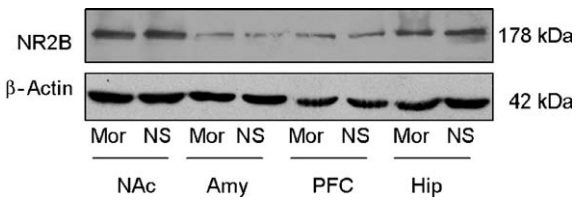

Fig. 8. No change of NR2B subunit levels in rats with morphine injection following the same schedule as in the CPP experiment but without the behavioral component (Mor) compared with that in control rats with alternative saline injection only (NS). NAc, Hip, PFC, and Amy mean the nucleus accumbens, the hippocampus, the prefrontal cortex, and the amygdale, respectively.

to impair attention to exteroceptive stimuli (Dai and Carey, 1994), but no direct evidence can be obtained that NMDA antagonists, especially selective antagonist for NR2B containing NMDARs such as ifenprodil, would impair visual discrimination. Moreover, any effect of this kind would appear equally in all behavioral procedures, and not selective for drug and novelty.

Secondly, it has been suggested that treatments aiming at attenuating the rewarding aspects of drugs of abuse may have aversive properties by themselves. Such aversive effects have been demonstrated for the L-type calcium channel blockers (Pizzi and Cook, 1996), which inhibit cocaine-induced CPP (Pani et al., 1991). To rule out this interpretation, experiment was performed in which ifenprodil (2, 6, 20 μg/10 μl/rat, i.c.v.) was administered 30 min before the morning trials in conditioning phase. If ifenprodil would have aversive actions, rats would avoid the compartment paired with ifenprodil. The data presented under Results demonstrate a lack of aversive effects of ifenprodil, making the "aversive" interpretation unlikely.

Furthermore, a marked augmentation of locomotor activity may also lead to an equalization of time spent in the two lateral compartments, which in the post-conditioning test means failed to develop or express place preference (Bozarth, 1987). Thus, a "blockade" of CPP could arise if ifenprodil would increase the locomotor activity. However, morphine-conditioned rats co-injected with ifenprodil did not show any increase of the number of crossings between adjacent compartments (data not shown). In the novel environment-induced CPP paradigm, co-injecting of ifenprodil did increase the number of crossings from novel compartment to neutral compartment, but in the same time it decreased the number of crossings from familiar compartment to neutral compartment. As a result, the total crossing number was not significantly changed.

From the above analysis of alternative explanations, we may conclude that the most tenable account of our data is that ifenprodil selectively blocks morphine-induced place preference owing to its selective blockade of the rewarding properties of morphine.

It is interesting to find that ifenprodil also prevented the development and expression of place preference induced by novel environment, a result consistent with Bevins and Bardo (1999), while contrary to Papp et al. (2002). It has been suggested that there is a very close interrelation between the behavioral and physiological mechanisms underlying an animal's sensitivity to novelty as well as to the drugs of abuse (Cervo and Samanin, 1996; Bevins et al., 2002; Kim et al., 2004). Place-conditioning procedures have been used extensively to study both the novelty and drugs of abuse. The use of common procedures would allow within and between experiment comparisons of the behavioral and biological substrates underlying their appetitive effects. Animal's sensitivity to novelty appears to be a powerful predictive variable for later sensitivity to drugs of abuse. For example, rats that are more activated by a novel environment are also more likely to self-administer amphetamine. These rats are also more sensitive to the locomotor stimulant effects of amphetamine (Piazza et al.,

1989, 1990; Paredes and Agmo, 2004). Similarly, human research suggests a positive relation between novelty-sensation seeking and drug taking (Sutker et al., 1978; Morita et al., 1994; Scourfield et al., 1996).

Mechanisms of inhibitory effect of ifenprodil on morphine-induced place preference

Ifenprodil is an antagonist of NMDA receptor, highly selective for the NR2B subunit (Kew et al., 1996; Kohl and Dannhardt, 2001; Perin-Dureau et al., 2002; Kiss et al., 2005). Earlier studies have demonstrated that non-subunit-selective NMDA receptor antagonists such as non-competitive (dizocilpine, memantine) and competitive (CGP 37,849) NMDA receptor antagonists block the development and expression of place preferences conditioned with morphine (Tzschentke and Schmidt, 1995; Popik et al., 1998). However, non-subunit-selective NMDA receptor antagonists were found to support CPP in their own right (Papp et al., 1996; Tzschentke and Schmidt, 1998). In contrast with that, ifenprodil was demonstrated to block the development and expression of morphine-induced place preference without supporting place conditioning by its own. To the best of our knowledge, the present study is the first to demonstrate the involvement of NR2B containing NMDA receptor in the expression of place preferences conditioned to morphine.

The rewarding properties of drugs of abuse are thought to derive primarily from the enhancement of dopaminergic (DA) activity within the mesolimbic DA system. Morphine can increase the firing rate of mesolimbic DA neurons by its actions within the VTA (Koob et al., 1998; Nestler, 2001; Wise, 2002). Non-specific DA receptor antagonists such as haloperidol or relatively D1-receptor-specific antagonists such as SCH-23390 or 31966 block the acquisition of place preferences conditioned with psychostimulants, morphine or nicotine (Acquas and Di Chiara, 1994; Acquas et al., 1989).

Intracranial mapping studies have identified the mesolimbic DA system as a major (though not exclusive) circuitry supporting drug-induced place preference conditioning (Koob et al., 1998, Nestler, 2001; Wise, 2002). A major role of glutamate in drug addiction is directly or indirectly related to the modification of the activity of the DA system (Tzschentke and Schmidt, 2003). Both the cell body region in the VTA and the terminal region in the NAc receive massive glutamatergic inputs from several forebrain sites, such as hippocampus and amygdala, which are known to be involved in reinforcing learning (Sun and Rebec, 2005; Everitt et al., 1991, 1999). The interaction between glutamate and dopamine in VTA and NAc is rather complex, but in simplified terms, glutamatergic inputs to the VTA increases the activity of DA cells and enhances DA release in the NAc. A substantial literature suggests that the role of DA in reinforcing learning, as typified by place preference conditioning, is mediated by its action at the D1 receptor subtype (Valjent et al., 2004; Noda and Nabeshima, 2004; Nazarian et al., 2004). Due to some excitatory actions of DA at D1 receptors (Gerfen, 2000), D1 antagonists tend to decrease the activity of target neurons in the mesolimbic DA system. In the meantime, as NMDA receptors are also excitatory, these two receptors may share common properties in mediating CPP effects. The blockade of morphine-induced CPP by ifenprodil might be attributed to NR2B containing NMDA receptors located in the same NAc cells carrying D1-receptors.

Western blotting analysis showed that in rats conditioned by morphine, overexpression of NR2B was observed not only in the NAc, but also in the hippocampus. NMDA receptors, especially the NR2B subunits (Clayton and Browning 2001; Clayton et al., 2002; Mamiya et al., 2003; Yaka et al., 2003; Liu et al., 2004), are responsible for most excitatory neurotransmission in the hippocampus and have been clearly involved in the learning and memory processes since NMDA antagonists do impair memory in a wide variety of tasks and species (Riedel et al., 2003). In accordance with this hypothesis, another alternative explanation for the inhibition of the development of morphine-induced CPP observed in this study is that ifenprodil may interfere with the associating ability between the rewarding effect of morphine and the related cue in the drug-paired compartment, or the retrieval capability of this association.

Effect of ifenprodil on the natural reward-induced place preference

Contrary to a large number of evidence concerning drug induced place conditioning, surprisingly little information is available concerning the involvement of glutamate systems, especially the NR2B containing NMDA receptors, in place conditioning using natural rewards. However, a large body of evidence suggests that, like drug conditioning, the development and expression of place preferences conditioned with natural rewards, such as food, novelty, or sucrose, requires intact DA transmission, probably via D1 receptors (Beninger et al., 1989; Pierce et al., 1990; Agmo et al., 1995; Imaizumi et al., 2000). There are only several studies on the involvement of glutamate receptors in place conditioning with natural reward. In these studies, the acquisition of a novel object-induced place preference was blocked by dizocilpine, a noncompetitive NMDA receptor antagonist (Bevins et al., 2002); and the expression of a sexual interaction-induced place preference was blocked by memantine, an NMDA receptor channel blocker (Popik et al., 2003). Thus, the available evidence would not immediately support a distinction between drug, novel rewards and other natural rewards in terms of the involvement of D1 and NMDA receptors in place preference conditioning.

Mechanisms of differential effects of ifenprodil on the place preferences induced by different types of reinforcers

Ifenprodil inhibits both the development and expression of place preferences induced by morphine and novel environment, while having little or no effect on the food or social interaction conditioned responses. The degree to which DA synapses in the NAc shell are activated may be quite different by drug or natural rewards. As mentioned above, a common feature of drugs of abuse is that they increase extracellular levels of DA in the NAc

shell, either by activating the firing of DA neurons or by blocking the reuptake of released DA. Brain microdialysis studies have revealed that this drug-induced effect does not undergo tolerance with repeated exposure (Di Chiara, 1995). On the other hand, DA release in the NAc shell can only be phasically activated by the presentation of novel natural rewards, and this effect rapidly undergoes habituation, becoming absent on the second and subsequent presentations (Di Chiara, 1995; Schultz, 1997; Suri and Schultz, 1999).

DA was known to activate D1 receptors and inhibit D2 receptors (Gerfen, 2000). The increase in extracellular DA during drug conditioning was postulated to change the balance of activity in medium-sized spiny accumbens neurons by increasing D1-related activity and decreasing D2-related activity. Thus, drug rewards might result in an increase in the activity of predominantly D1-bearing cells, at the expense of predominantly D2-bearing cells, during CPP conditioning. However natural rewards, due to its phasic action, may not produce the same effect. As the NR2B containing receptor antagonists are hypothesized to act on D1-bearing cells (see above), the differential activation of DA synapses by drug rewards as contrast to non-drug natural rewards, and the consequent activation of D1-bearing medium spiny neurons, may provide a mechanism to explain the selective action of ifenprodil on the drug-conditioned place preferences. The signal arising from stimulation of NMDA receptors can be amplified by activating D1 receptors on the cells where D1 and NMDA receptors are co-localized can amplify (Onn et al., 2000); co-activation of D1 and NMDA receptors was proved to induce the expression of immediate early genes such as c-*fos* in striatal neurons (Wang et al., 1994; Konradi et al., 1996; Gerfen, 2000). Calabresi et al. (2000) also observed the long term potentiation (LTP) of cortico-striatal excitatory transmission by prolonged co-activation of D1 and NMDA receptors. We hypothesize that NR2B containing NMDA receptor antagonists may block drug-induced CPP by damping down NMDA activity sufficiently to prevent the D1-NMDA receptor synergism necessary for the development of synaptic plasticity in cortico-striatal pathways.

It should be mentioned that, in addition to their rewarding properties, natural reinforcers also have exteroceptive visual, gustatory, and/or olfactory features, which are processed in areas of the brain that may be independent of those involved in drug induced CPP.

Conclusions

The current results indicate that the NR2B containing NMDA receptor plays a critical role in morphine-induced place preference. Ifenprodil, an antagonist of NMDA receptor highly selective for the NR2B subunit, can block the development and expression of morphine-induced place preference. The following considerations imply a potential use of ifenprodil for the treatment of opiate addicts. First, as ifenprodil itself does not support a CPP, the probability of abuse to itself is low. Second, ifenprodil does not seem to affect the place preference induced by natural rewards, such as food consumption and social interaction. Third, considering the potent inhibitory effect on the development and expression of morphine CPP, ifenprodil is expected to prevent the relapse to opioid drugs. Finally, psychostimulant- and psychotomimetic-like effects, frequently occurring with non-selective NMDA receptor antagonists, had never been reported when using ifenprodil (Narita et al., 2001). This cocktail of effects could prove a promising maneuver serving not only as an adjunct to therapy in counteracting the further development of addiction, but also a means of counteracting relapse to drugs.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.expneurol.2006.02.117.

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