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Effect of glutamate receptor antagonists on place aversion induced by naloxone in single-dose morphine-treated rats

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1 The neurobiological mechanism underlying the negative motivational component of withdrawal from acute opiate dependence is far from understood.

2 Our objectives were to determine whether the glutamatergic system is involved in the motivational component of morphine withdrawal in acutely dependent rats and such an involvement is associated with dopaminergic neurotransmission.

3 We examined the effects of various kinds of glutamate receptor antagonists on conditioned place aversion (CPA) induced by naloxone-precipitated withdrawal from a single morphine exposure 24 h before. Furthermore, the influence of pretreatment with the dopamine receptor antagonist haloperidol on those effects of glutamate receptor antagonists was also investigated.

4 CPA was attenuated in a dose-dependent manner by all glutamate receptor antagonists examined including the NMDA receptor antagonists (+)-5-methyl-10,11-dihydro-5H-dibenzo[*a*,*d*]cyclo-hepten-5,10-imine maleate (MK-801) and phencyclidine hydrochloride (PCP), AMPA receptor antagonist 1-(4-aminophenyl)4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride (GYKI 52466), and metabotropic receptor antagonists (±)-2-amino-3-phosphonopropionic acid (AP-3) and (±)-α-methyl-4-carboxyphenylglycine (MCPG). The effects of MK-801, GYKI 52466 and MCPG were blocked by haloperidol.

5 These results suggest that the glutamatergic system involving multiple classes of receptors plays a role in the motivational component of withdrawal from acute morphine dependence, and the function of the glutamatergic system would be closely associated with dopaminergic neurotransmission.

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Abbreviations: AP-3, (±)-2-amino-3-phosphonopropionic acid; CPA, conditioned place aversion; CPP, conditioned place preference; GYKI 52466, 1-(4-aminophenyl)4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride; MCPG, (±)-α-methyl-4-carboxyphenylglycine; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[*a*,*d*]cyclo-hepten-5,10-imine maleate; PCP, phencyclidine hydrochloride

Introduction

Acute opiate dependence is defined as the precipitation of signs characteristic of withdrawal symptoms by an opiate antagonist after short-term infusion or even a single dose of an opiate agonist both in humans and in animals (Martin & Eades, 1964; Bickel *et al*., 1988). Recently, the maintenance of compulsive use of drugs has been suggested to be substantially associated with negative reinforcement, which is related to the aversive properties of withdrawal in dependent subjects (Rodriguez de Fonseca & Navarro, 1998; Koob, 2000). Various behavioral alterations postulated to reflect the aversive aspect of withdrawal in humans can be observed in acutely dependent animals, including suppression of operant responding, conditioned place aversion (CPA), conditioned taste aversion and increased thresholds for intracranial self-stimulation (Adams & Holtzman, 1990; Easterling & Holtzman, 1997; McDonald *et al*., 1997; Schulteis *et al*., 1997; Parker *et al*., 2002; Azar *et al*., 2003).

It is known that acute and chronic opiate dependence are not unitary phenomena. Discrepancies also appear to exist in the mechanisms underlying the negative motivational components of withdrawal that can be seen in both acutely and chronically dependent animals. Three important portions of the extended amygdala including the amygdala, the bed nucleus of the stria terminalis and the shell of the nucleus accumbens have been found to be activated in response to the low-level withdrawal from chronic dependence characterized by motivational signs (Gracy *et al*., 2001; Frenois *et al*., 2002).

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However, we recently demonstrated that only the amygdala among these three regions was sensitive to a negative motivational withdrawal stimulus in acute-dependent rats and appeared to serve as a common anatomic structure involved in the development of dependence from the early to fully developed stages of dependence (Jin *et al*., 2004). It will benefit the development of appropriate therapies for morphine dependence to further clarify the dissociation and similarity in the neuroanatomy, neurochemistry, etc., contributing to the motivational aspect of withdrawal between different stages of dependence.

It has been realized that the glutamatergic system plays a role in the development and maintenance of drug addiction (Tzschentke & Schmidt, 2003). There is evidence suggesting the involvement of the glutamatergic system in the aversive consequences of withdrawal from chronic opiate dependence. Inhibition of glutamatergic neurotransmission was found to attenuate the motivational signs of withdrawal in animals rendered chronically dependent on morphine (Higgins *et al*., 1992; Popik & Danysz, 1997; Watanabe *et al*., 2002; Kratzer & Schmidt, 2003; Maldonado *et al*., 2003). As to acutely dependent subjects, a similar phenomenon began to be reported recently. Blokhina *et al*. (2000) demonstrated that an NMDA receptor antagonist, D-CPPene, attenuated CPA induced by naloxone 4 h after acute morphine treatment in mice. In our most recent study, riluzole (a glutamate release inhibitor) blocked CPA induced by naloxone-precipitated withdrawal from a single morphine exposure 24 h before in rats (Jin *et al*., submitted). All these findings suggest that the glutamatergic system may be another common component in the adaptational changes of acute and chronic morphine dependence. The present study was undertaken to confirm the role of this neurotransmitter system in withdrawal aversion in animals subjected to acute morphine exposure by examining the effects of various kinds of antagonists for glutamate receptors on CPA.

A body of evidence suggests an involvement of the central dopamine system in the expression of opiate dependence. Reduction of the extracellular dopamine level in mesolimbic areas is associated with both spontaneous (Acquas & Di Chiara, 1992) and opiate receptor antagonist-appreciated withdrawal (Pothos *et al*., 1991; Rossetti *et al*., 1992). Furthermore, the role of the dopamine system in the conditioned aversive effects of opiate withdrawal has also been reported. The D2 receptor antagonist raclopride was found to produce CPA in morphine-pelleted rats (Funada & Shippenberg, 1996). In addition, our previous observation showed that the dopamine agonist apomorphine could reverse CPA induced by withdrawal of rats from a single morphine exposure (Araki *et al*., 2004).

There is a report demonstrating that the administration of the NMDA receptor antagonist, MK-801, to rats withdrawn from chronic morphine dependence can readily reverse the fall in the extracellular dopamine concentration in the ventral striatum (Rossetti *et al*., 1992). This implies an interaction between the dopaminergic and glutamatergic systems in opiate withdrawal. It remains unknown whether such an interaction exists in acutely dependent subjects and contributes to the negative motivational component of withdrawal. The present study was conducted to address this issue employing a CPA paradigm.

Methods

Subjects

Male Sprague–Dawley rats (Charles River, Japan; initial weight 205–235 g) were housed two or three per cage. The room temperature was kept at $23 \pm 1^{\circ}C$, and a 12-h light–dark cycle (lights on at 07:00) was maintained throughout the experiment. Food and water were available *ad libitum*. The experimental protocol was conducted according to the Guidelines of the Ethics Review Committee for Animal Experimentation of Okayama University Medical School. The rats were acclimatized to handling every day for about 1 week prior to the experiment.

Drugs

Morphine hydrochloride and naloxone hydrochloride were purchased from Takeda Pharmaceutical Co. Ltd (Osaka, Japan) and Sigma, respectively. Drugs examined were several glutamate receptor antagonists including (+)-5-methyl-10,11-dihydro-5H-dibenzo[*a*,*d*] cyclo-hepten-5,10-imine maleate (MK-801) (NMDA receptor antagonist, Sigma), phencyclidine hydrochloride (PCP) (NMDA receptor antagonist, Fukuyama University, Hiroshima, Japan), 1-(4-aminophenyl)4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride (GYKI 52466) (AMPA receptor antagonist, Sigma), (±)-α-methyl-4-carboxyphenylglycine (MCPG) (metabotropic receptor antagonist, Sigma) and (±)-2-amino-3-phosphonopropionic acid (AP-3) (metabotropic receptor antagonist, Sigma), as well as the dopamine receptor antagonist haloperidol (injection, Dainippon Co. Ltd, Osaka, Japan). All drugs except MCPG were dissolved in or diluted with saline. MCPG was dissolved in 0.1 N NaOH. All drugs were administered subcutaneouly at a volume of $1\,ml\,kg^{-1}$.

CPA

The method to establish CPA has been described elsewhere (Jin *et al*., 2004). Briefly, the apparatus consisted of two chambers with floors covered with wire mesh and check-pattern sandpaper squares, respectively. The animals experienced a preconditioning habituation to the apparatus and those showing initial bias for either compartment were eliminated from the study. On the first day of the conditioning procedure, all rats were injected with saline, and 5 min later, were confined to one side of the apparatus, either the mesh-floor chamber or the sandpaper-floor chamber in a counterbalanced manner, for 30 min. This chamber will be referred to as the 'nontreatment-paired chamber'. On Day 2, rats were injected with $10\,mg\,kg^{-1}$ of morphine and then returned to their home cages. On Day 3, rats were given naloxone ($0.5\,mg\,kg^{-1}$), and 5 min later, placed in the chamber opposite to that on Day 1 for 30 min. This chamber will be referred to as the 'treatment-paired chamber'. At 48 h after the conditioning trial, all rats were allowed to freely explore the entire apparatus for 15 min and the amount of time spent in each chamber was measured. CPA scores representing the time spent in the treatment-paired chamber minus the time spent in the nontreatment-paired chamber during the place preference test were calculated.

Effects of glutamate receptor antagonists on naloxone-induced CPA in rats acutely treated with morphine

On the third day of the conditioning procedure, rats were given saline or one of the glutamate receptor antagonists prior to receiving naloxone. The intervals between these injections and the naloxone challenge were 20 min, 1 h, 30 min, 20 min, 30 min and 30 min for saline, MK-801, PCP, GYKI 52466, MCPG and AP-3, respectively. The doses used were 0.00625, 0.0125, 0.025 and 0.05 mg kg^{-1} for MK-801; 0.375, 0.75 and 1.5 mg kg^{-1} for PCP; 1.0, 2.0 and 5.0 mg kg^{-1} for GYKI 52466; 1.0, 2.0, 4.0 and 8.0 mg kg^{-1} for MCPG; and 0.75, 1.5 and 3.0 mg kg^{-1} for AP-3. The number of rats in each group was 5.

To determine the effects of these glutamate receptor antagonists alone on place conditioning in either morphine-naïve or morphine-exposed rats, separate groups ($n = 5$–8) received morphine or saline on Day 2. On the next day, they were injected with saline, MK-801 (0.05 mg kg^{-1}), PCP (1.5 mg kg^{-1}), GYKI 52466 (5.0 mg kg^{-1}), MCPG (4.0 mg kg^{-1}) or AP-3 (3.0 mg kg^{-1}) prior to receiving saline instead of naloxone.

Influence of haloperidol on the effects of glutamate receptor antagonists on naloxone-induced CPA in rats acutely treated with morphine

To determine whether there is a glutamatergic–dopaminergic interaction involved in the morphine withdrawal aversion, the influence of haloperidol on the effects of glutamate receptor antagonists on CPA was examined. Rats experienced the same treatments as described above, except that they received a pretreatment with haloperidol (0, 0.1 or 1.0 mg kg^{-1}) 2 h prior to the administration of glutamate receptor antagonists on the third day of the conditioning procedure. The glutamate receptor antagonists employed here were MK-801 (0.05 mg kg^{-1}), GYKI 52466 (5.0 mg kg^{-1}) and MCPG (4.0 mg kg^{-1}). The place conditioning action of haloperidol (1.0 mg kg^{-1}) itself was also examined in both morphine-naïve and morphine-exposed rats. The number of rats in each group was 5–8.

Statistical analysis

Most of the data were analyzed using a one-way analysis of variance followed by Dunnett's multiple comparison test. In the case of comparisons between two groups, Student's *t*-test was carried out. The level of significance was set at $P < 0.05$.

Results

Effects of glutamate receptor antagonists on naloxone-induced CPA in rats acutely treated with morphine

As presented in Figure 1, CPA was dose dependently attenuated by both NMDA receptor antagonists, MK-801 (Figure 1a) ($F(4, 20) = 7.062$, $P < 0.01$) and PCP (Figure 1b) ($F(3, 16) = 15.059$, $P < 0.01$). The *post hoc* comparisons demonstrated a significant difference for each dose (0.00625–0.05 mg kg^{-1}) of MK-801 ($P < 0.01$, each) and two higher doses (0.75 and 1.5 mg kg^{-1}) of PCP ($P < 0.01$, each) compared with the control group.

The AMPA receptor antagonist GYKI 52466 showed a similar dose-dependent inhibitory effect on CPA (Figure 2) ($F(3, 16) = 4.474$, $P < 0.05$). Significant differences compared with the control group were seen in the 2.0 and 5.0 mg kg^{-1} groups ($P < 0.05$, each).

The metabotropic receptor antagonists MCPG (Figure 3a) and AP-3 (Figure 3b) were also effective against CPA. Significant intergroup differences were present for both MCPG ($F(4, 20) = 4.010$, $P < 0.05$) and AP-3 ($F(3, 16) = 4.700$, $P < 0.05$). The *post hoc* comparisons revealed that CPA was significantly attenuated by MCPG at doses of 4.0 and 8.0 mg kg^{-1} ($P < 0.05$ and 0.01, respectively) and by AP-3 at a dose of 3.0 mg kg^{-1} ($P < 0.01$).

None of the agents examined including MK-801 (0.05 mg kg^{-1}), PCP (1.5 mg kg^{-1}), GYKI 52466 (5.0 mg kg^{-1}), MCPG (4.0 mg kg^{-1}) and AP-3 (3.0 mg kg^{-1}) showed place conditioning ability (aversion or preference) on their own in either morphine-exposed (Figure 4a) or morphine-naïve (Figure 4b) rats.

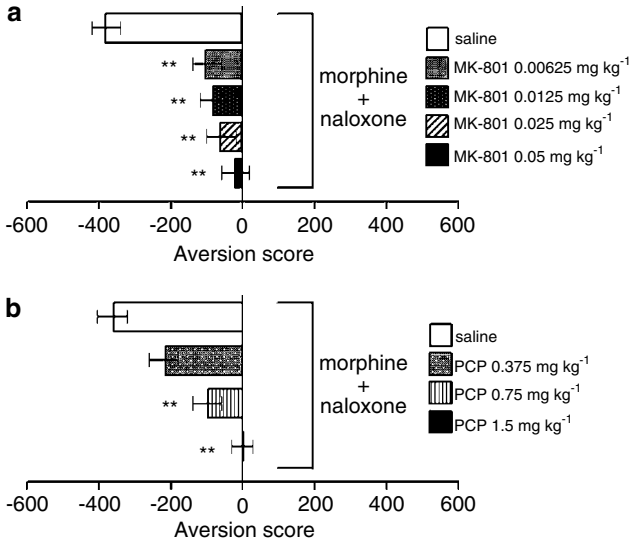

Figure 1 Effects of NMDA receptor antagonists on CPA induced by naloxone in rats exposed to a single dose of morphine. (a) MK-801; (b) PCP. Aversion scores are expressed as the mean time (±s.e.m.) in seconds spent in the treatment-paired chamber of the apparatus minus the mean time in seconds spent in the nontreatment-paired chamber during the place preference test. **$P < 0.01$ vs saline.

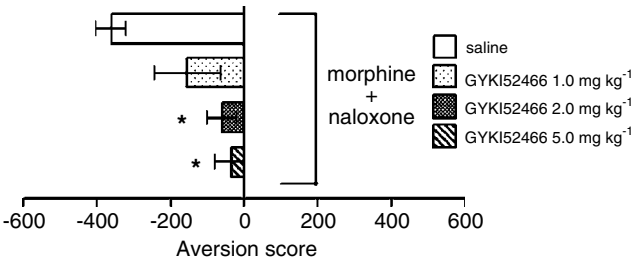

Figure 2 Effect of AMPA receptor antagonist GYKI 52466 on CPA induced by naloxone in rats exposed to a single dose of morphine. Aversion scores are expressed as the mean time (±s.e.m.) in seconds spent in the treatment-paired chamber of the apparatus minus the mean time in seconds spent in the nontreatment-paired chamber during the place preference test. *$P < 0.05$ vs saline.

Influence of haloperidol on the effects of glutamate receptor antagonists on naloxone-induced CPA in rats acutely treated with morphine

The inhibition of CPA by three kinds of glutamate receptor antagonists was blocked by the dopamine receptor antagonist haloperidol in a dose-dependent manner. Significant intergroup differences were present for 0.05 mg kg^{-1} of MK-801 (Figure 5a) ($F(2, 12) = 12.492$, $P < 0.01$), 5.0 mg kg^{-1} of GYKI 52466 (Figure 5b) ($F(2, 12) = 4.923$, $P < 0.05$) and 4.0 mg kg^{-1} of MCPG (Figure 5c) ($F(2, 12) = 8.935$, $P < 0.01$). The *post hoc* comparisons showed that haloperidol exerted its effect significantly at the dose of 0.1 and/or 1.0 mg kg^{-1} for each glutamate receptor antagonist ($P < 0.05$ or 0.01, respectively).

Haloperidol (1.0 mg kg^{-1}) itself produced no place bias in either morphine-exposed or morphine-naïve rats (Figure 6).

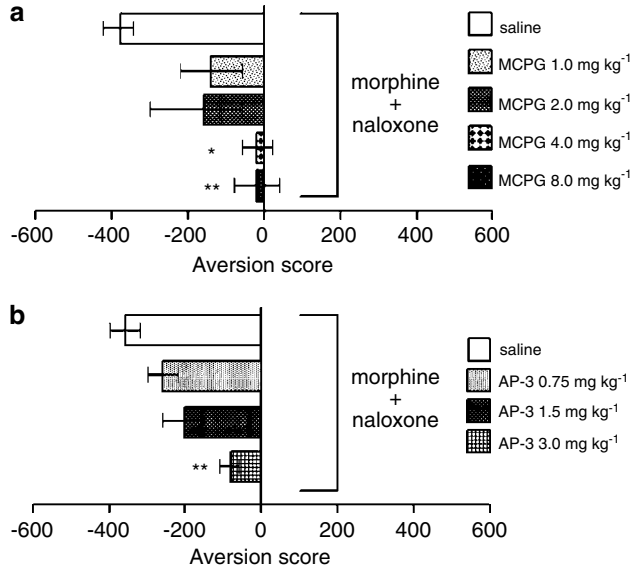

Figure 3 Effects of metabotropic receptor antagonists on CPA induced by naloxone in rats exposed to a single dose of morphine. (a) MCPG; (b) AP-3. Aversion scores are expressed as the mean time (±s.e.m.) in seconds spent in the treatment-paired chamber of the apparatus minus the mean time in seconds spent in the nontreatment-paired chamber during the place preference test. *$P < 0.05$, **$P < 0.01$ vs saline.

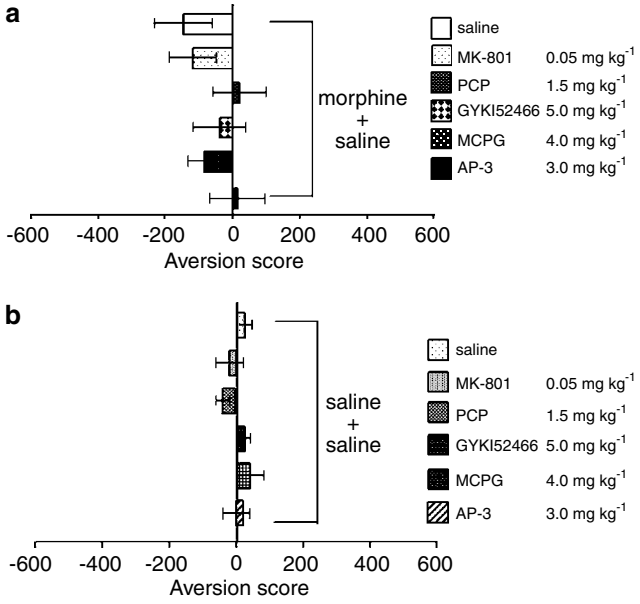

Figure 4 Effect of glutamate receptor antagonists alone on place conditioning in either morphine-exposed (a) or morphine-naïve (b) rats. Aversion scores are expressed as the mean time (±s.e.m.) in seconds spent in the treatment-paired chamber of the apparatus minus the mean time in seconds spent in the nontreatment-paired chamber during the place preference test.

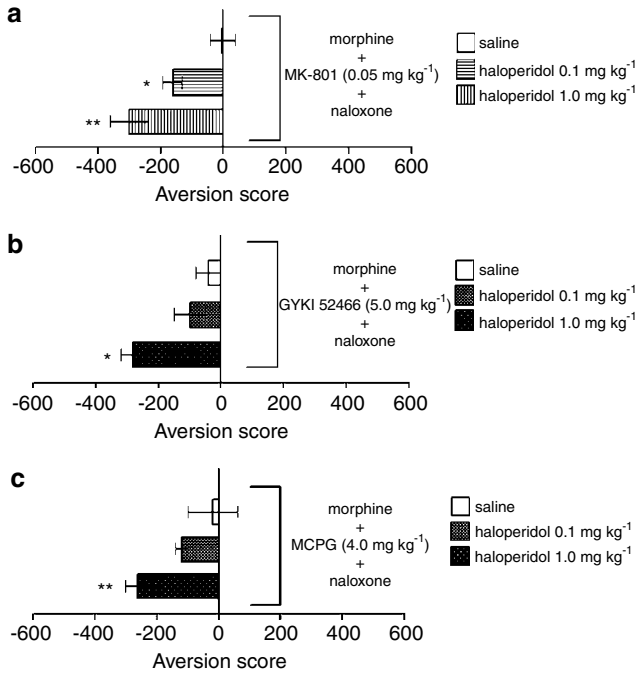

Figure 5 Influence of haloperidol on the effects of glutamate receptor antagonists on CPA induced by naloxone in rats exposed to a single dose of morphine. (a) MK-801; (b) GYKI 52466; c, MCPG. Aversion scores are expressed as the mean time (±s.e.m.) in seconds spent in the treatment-paired chamber of the apparatus minus the mean time in seconds spent in the nontreatment-paired chamber during the place preference test. *$P < 0.05$, **$P < 0.01$ vs saline.

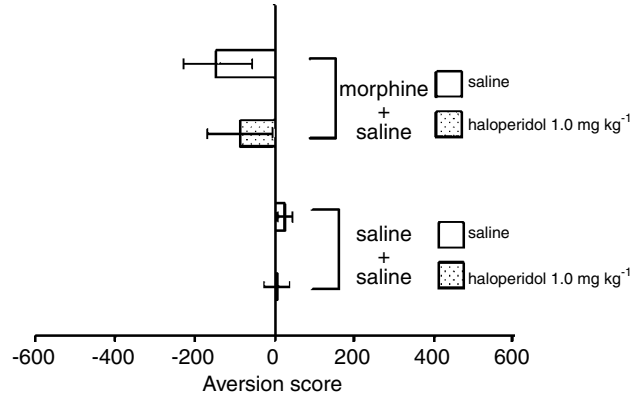

Figure 6 Effect of haloperidol alone on place conditioning in either morphine-exposed or morphine-naïve rats. Aversion scores are expressed as the mean time (±s.e.m.) in seconds spent in the treatment-paired chamber of the apparatus minus the mean time in seconds spent in the nontreatment-paired chamber during the place preference test.

Discussion

In the present study, all the glutamatergic antagonists examined significantly attenuated naloxone-induced CPA, a conditioned aversive behavior well established in rats experiencing a single morphine exposure. Furthermore, the effects of MK-801, GYKI 52466 and MCPG were blocked by haloperidol.

None of the glutamatergic antagonists produced any place conditioning by themselves in either morphine-naïve or morphine-treated subjects, suggesting that their effects on CPA induced by withdrawal from acute morphine exposure may not be due to a counteraction against withdrawal aversion of morphine *via* intrinsic reinforcing effects. Nevertheless, a considerable body of literature has demonstrated that some glutamatergic antagonists have some motivational properties, particularly two NMDA receptor antagonists PCP and MK-801. PCP is a widely abused drug in humans. Its rewarding effect has been shown in animals with certain indexes of reward including the conditioned place preference (CPP) paradigm (Marglin *et al*., 1989; Kitaichi *et al*., 1996; Nabeshima *et al*., 1996). However, PCP produces such a rewarding behavior only under appropriate experimental conditions. No place bias or CPA would occur with different doses and pretreatment experience (Barr *et al*., 1985; Acquas *et al*., 1989; Kitaichi *et al*., 1996; Nabeshima *et al*., 1996; Noda & Nabeshima, 1998). Here in this study, PCP failed to produce any place conditioning on its own under the present conditions: no pretreatment experience, $1.5\,mg\,kg^{-1}$ and one-cycle conditioning training. MK-801 is another agent having some rewarding properties and eliciting CPP (Layer *et al*., 1993; Hoffman, 1994; Steinpreis *et al*., 1995; Del Pozo *et al*., 1996; Papp *et al*., 1996; Sukhotina *et al*., 1998; Panos *et al*., 1999). Similar to PCP, however, MK-801-induced CPP also appears to be dependent on the experimental conditions. Consistent with our observation, there are several reports demonstrating that this agent alone produces neither place preference nor aversion in rodents (Tzschentke & Schmidt, 1995; 1998; Kim *et al*., 1996; Ribeiro Do Couto *et al*., 2004). More importantly, MK-801 was reported to produce no motivational effects in morphine-dependent animals (Maldonado *et al*., 2003). All these findings suggest that a more specific mechanism rather than a simple additive one would mediate the effects of the glutamatergic antagonists examined to attenuate CPA in animals acutely dependent on morphine.

It may be argued that the effects of glutamatergic antagonists observed in the present study are a result of impairment in the rats' ability to associate the environmental cues to naloxone-precipitated opiate withdrawal. Indeed, it is well known that NMDA receptors are involved in some types of learning and memory, and NMDA receptor antagonists can interfere with these processes (Riedel *et al*., 2003). However, this explanation appears unlikely. If this is the case, it is difficult to interpret the phenomenon that these antagonists can produce CPP under suitable experimental conditions as mentioned above. In addition, the effective doses of MK-801 ($0.00625–0.05\,mg\,kg^{-1}$) in the present study are lower than those (0.06 and $0.12\,mg\,kg^{-1}$) required to impair passive avoidance learning (Venable & Kelly, 1990), implying that the rats employed here were capable of developing certain conditioned associations under the MK-801 exposure. Furthermore, although MK-801 exerts an inhibitory influence on both CPA induced by opiate withdrawal (Higgins *et al*., 1992; Watanabe *et al*., 2002; Maldonado *et al*., 2003) and CPP induced by morphine (Tzschentke & Schmidt, 1995; Del Pozo *et al*., 1996; Kim *et al*., 1996) and cocaine (Cervo & Samanin, 1995), it had no effect on CPP induced by amphetamine (Hoffman, 1994). This phenomenon would be difficult to understand if the effect of MK-801 is due to a general nonselective disruption of place conditioning as the establishment of both CPA and CPP involves associative learning. In contrast to the well-established contribution of NMDA receptors to learning and memory, the contribution of AMPA receptors is less clearcut (Riedel *et al*., 2003). With respect to metabotropic receptors (Riedel *et al*., 2003), numerous studies have examined the functions of this kind of glutamatergic receptor with various agonists and antagonists (including MCPG) employing many different forms of learning. The functions of metabotropic receptors appear variable and dependent on the learning task (Riedel *et al*., 2003). So far, the data suggest little or no involvement of metabotropic receptors in the actual acquisition of new information (Riedel *et al*., 2003).

Taken together, although the possibility of impairing learning and memory processes could not be explicitly ruled out for all agents examined in the present study, such an impairment may not be of major importance for the antagonism of withdrawal-induced CPA in acutely dependent animals. The effects of glutamatergic antagonists might be attributable, at least in part, to a reduction in aversive or negative motivational aspects of opiate withdrawal. This is supported by the similar effect of riluzole observed in our most recent study using the same place-conditioning paradigm (Jin *et al*., submitted) and the inhibitory effect of D-CPPene on naloxone-induced CPA in mice acutely treated with morphine (Blokhina *et al*., 2000). These findings imply that the glutamatergic system may be involved in the motivational component of morphine withdrawal in acutely dependent animals and this involvement appears to include multiple classes of receptors. The existing literature has demonstrated that the neurobiological mechanisms underlying the aversive consequences of withdrawal from chronic opiate dependence also involve the glutamatergic system (Higgins *et al*., 1992; Popik & Danysz, 1997; Watanabe *et al*., 2002; Kratzer & Schmidt, 2003; Maldonado *et al*., 2003). Therefore, it is presumable that the glutamatergic system would serve as a common substrate for the negative motivational aspects of both chronic- and acute-dependent subjects.

Another finding in the present study is that the inhibition by MK-801, GYKI 52466 and MCPG of morphine withdrawal-induced CPA was blocked by the dopamine receptor antagonist haloperidol. The result that haloperidol itself showed no action to produce place conditioning in morphine-naïve or morphine-treated rats suggests a specific interactive effect of glutamatergic and dopaminergic neurotransmission in acute-dependent animals, and the inhibitory effects of glutamatergic antagonists on CPA may be related to the activation of the dopaminergic system. In animals rendered chronically dependent on opiate, withdrawal has been found to be associated with increased extracellular glutamate release (Aghajanian *et al*., 1994; Zhang *et al*., 1994; Sepulveda *et al*., 1998; Tokuyama *et al*., 2001) and a reduction of the dopamine level (Pothos *et al*., 1991; Acquas & Di Chiara, 1992; Rossetti

et al., 1992) in the brain. Such a fall in the extracellular dopamine concentration in the ventral striatum could be reversed by MK-801, implying that the modification to the dopamine level caused by opiate withdrawal is related to NMDA-mediated glutamatergic activity (Rossetti *et al*., 1992). This glutamatergic–dopaminergic interaction is supported by an observation made in brain slices from normal rats. That is, glutamate mediated an inhibitory postsynaptic potential in dopamine neurons (Fiorillo & Williams, 1998). As we demonstrated above, the glutamatergic system may be involved in the negative motivational aspects of opiate withdrawal from both chronic and acute dependence. The dopaminergic system appears to play a similar role. The D2 receptor antagonist raclopride was found to induce CPA in morphine-pelleted rats (Funada & Shippenberg, 1996). The dopamine agonist apomorphine was shown to attenuate CPA in animals withdrawn from acute morphine exposure (Araki *et al*., 2004). The results of the present study suggest that the negative motivational aspects of opiate withdrawal involve an interaction between the glutamatergic and dopaminergic systems at least in acutely dependent subjects.

In conclusion, the present study suggests that the glutamatergic system involving multiple classes of receptors plays a role in the motivational component of withdrawal from acute dependence on morphine, and the function of the glutamatergic system would be closely associated with dopaminergic neurotransmission.

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