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Midazolam attenuates the antinociception induced by D-serine or morphine at the supraspinal level in rats

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ABSTRACT

Our recent study has shown that the intracerebroventricular administration of D-serine, an endogenous and selective agonist for the glycine site of the *N*-methyl-D-aspartate receptor, alone or in combination with morphine, leads to the potentiation of antinociception on the tail-flick response. Although there is a variety of information concerning the effects of benzodiazepines on opioid-induced antinociception, little is known about the effect of benzodiazepines on the *N*-methyl-D-aspartate receptor agonist-induced antinociception. To clarify the analgesic interactions among the benzodiazepine/$GABA_A$, *N*-methyl-D-aspartate and opioid receptors at the supraspinal level, we investigated the effects of intracerebroventricular administration of midazolam, a benzodiazepine receptor agonist, on the antinociception evoked by the intracerebroventricular application of D-serine or morphine. The intracerebroventricular administration of midazolam alone produced hyperalgesia on the tail-flick response in a benzodiazepine receptor antagonist, flumazenil-reversible manner. The antinociception induced by the intracerebroventricular application of D-serine or morphine was attenuated by the intracerebroventricular administration of midazolam. In addition, this inhibitory effect of midazolam on the antinociception of D-serine or morphine was antagonized by the intracerebroventricular administration of flumazenil. Together with the facts that D-serine and midazolam act as selective agonists for the glycine site of the *N*-methyl-D-aspartate receptor and benzodiazepine/$GABA_A$ receptor, respectively, these observations suggest a functional interaction between the NMDA and benzodiazepine/$GABA_A$ receptors in the regulation of antinociception at the supraspinal level.

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1. Introduction

Midazolam is a benzodiazepine/γ-aminobutyric acid $(GABA)_A$ receptor agonist and is administered along with analgesic agents as a preoperative medication. However, there are several inconsistent reports concerning the effects of benzodiazepines alone or in combination with opioids on the antinociception. The intraperitoneal (i.p.) application of midazolam alone induced both hyperalgesia (Yanez et al., 1990) and antinociception (Coulthard and Rood, 1992). The intrathecal (i.t.) administration of midazolam alone produces antinociception in the spinal cord (Yanez et al., 1990), while the intracerebroventricular (i.c.v.) administration of midazolam alone induces hyperalgesia (Tatsuo et al., 1999) or no effect on the nociception (Mantegazza et al., 1982). The route of application may also contribute to the differing effects of midazolam on the opioid-evoked antinociception. The i.t. administration of midazolam potentiated the antinociception of morphine (Luger et al., 1994, 1995a,b; Mantegazza et al., 1982). In contrast, the i.c.v. administration of midazolam reduced the morphine-induced antinociception, which was blocked by the i.p. administration of flumazenil (a benzodiazepine receptor antagonist) or the i.c.v. administration of bicuculine (a $GABA_A$ receptor antagonist), but not phaclofen (a $GABA_B$ receptor antagonist) in the hot plate test (Luger et al., 1994, 1995a,b; Mantegazza et al., 1982). These results have demonstrated that the opioid-induced antinociception is modulated by the benzodiazepine/$GABA_A$ receptor at the supraspinal level (Luger et al., 1994; Mantegazza et al., 1982; Rady and Fujimoto, 1993). However, the inhibitory effect of midazolam on the morphine-induced antinociception cannot be easily explained by the benzodiazepine/$GABA_A$ receptor (Luger et al., 1995b). It has been suggested that *N*-methyl-D-aspartate (NMDA) receptors might be involved in the analgesic interaction between the benzodiazepine/$GABA_A$ and opioid receptor system at the supraspinal level (Luger et al., 1995b). The i.c.v. administration of midazolam attenuated the antinociception induced by the i.c.v. administration of morphine, which was blocked by the i.c.v. administration of the NMDA antagonist, MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo (a,b) cyclohepton-5,10-imine maleate), in the hot plate test (Luger et al., 1995b).

Our recent study has demonstrated that the i.c.v. application of D-serine alone produces a dose-dependent antinociception and potentiates the antinociception of morphine in the tail-flick test (Yoshikawa

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et al., 2007). D-Serine acts as an endogenous and obligatory co-agonist for the glycine site of the NMDA receptor in the mammalian brain (Danysz and Parsons, 1998; Hashimoto et al., 1993; Hashimoto and Oka, 1997; Hashimoto et al., 1995; Schell et al., 1995). In addition, the antinociception produced by both D-serine alone or in combination with morphine was dose-dependently attenuated by the i.c.v. administration of L-701,324 (7-chloro-4-hydroxy-3-(3-phenoxy)phenylquinolin-2[1H]-one; a selective antagonist for the glycine site of the NMDA receptor) or naloxone, respectively. These observations have suggested that the activation of the supraspinal NMDA receptors by D-serine leads to the potentiation of the antinociception at the supraspinal level and that endogenous D-serine could modulate the opioid-mediated antinociception via the glycine site of the NMDA receptors at the supraspinal level.

Although there is a variety of information concerning the effects of benzodiazepines on the opioid-induced antinociception, little is known about the analgesic interaction of benzodiazepine/$GABA_A$ and NMDA receptors at the supraspinal level. To clarify the functional interaction among the benzodiazepine/$GABA_A$, NMDA and opioid receptors on the antinociception at the supraspinal level, we investigated the effects of the i.c.v. injection of midazolam on the antinociception of D-serine or morphine. We have also evaluated the effect of the i.c.v. application of midazolam alone or in combination with flumazenil on the antinociception.

2. Materials and methods

2.1. Animals and drugs

The present animal experiments were performed in strict accordance with the guidelines of Tokai University, and were approved by the Animal Investigation Committee of the university. Male Wistar rats (Clea Japan, Inc., Tokyo, Japan) weighing 180–220 g were used. The rats were group-housed in laboratory cages and kept in a temperature-controlled room (23±2 °C) with a 12 h light/dark cycle (light on: 07:00) with food and water freely available. The chemicals were purchased from the following sources: D-serine (Sigma, Tokyo, Japan), midazolam (Astellas, Tokyo, Japan), flumazenil (Astellas, Tokyo, Japan) and morphine hydrochloride (Takeda Chemical Industries, Osaka, Japan). The chemicals were all dissolved in saline.

2.2. Intracerebroventricular (i.c.v.) administration

The infusion of drugs into the third ventricle of the rats was performed via an indwelling cannulae as previously described with some modifications (Hashimoto, 2002). The rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.) and then mounted on a stereotaxic frame (Narishige, Tokyo, Japan). Stainless-steel guide cannulae (outer diameter, o.d.; 0.55 mm) were implanted 3–5 days prior to the day of the experiment. On the day of the experiment, an injection cannula (o.d.; 0.33 mm) was inserted into the third ventricle (AP, −0.8 mm; V, +7.8 mm; L, ±0.0 mm) according to the atlas of Paxinos and Watson through the guide cannula (Paxinos and Watson, 1986). Drugs were infused in a volume of 10 μl for 2 min and the infusion cannulae were left in place for 2 min before removal. The distribution of the drug solution in the cerebroventricular system was verified by the infusion of methylene blue dissolved in saline after the experiment.

2.3. Antinociception assay

The antinociception of the drugs was measured by the tail-flick test using 55 °C water as the nociceptive stimulus (Kanai et al., 2002; Kitamura et al., 2000). The latency to flick the tail from the 55 °C water was measured before and 5, 10, 15, 30, 45, 60, 75, 90, 105 and 120 min after the administration of the drugs. The latency to flick the tail before the administration of the drug was approximately 1 s (0.6 to 1.4 s). A cut-off time of 5 s was used to prevent any injury to the tail. The latencies of the tail-flick responses were converted to the percentage of Maximum Possible Effect (% MPE) for each animal at each time according to the following formula: % MPE = [(test latency − baseline latency)/(5 − baseline latency)] × 100. The area under the curve (AUC) values for the % MPE (0–120 min) were used to evaluate the analgesic effects of the drugs on each rat.

2.4. Data analysis

These results are given as the mean with S.E.M. of the data. A statistical analysis was conducted using computer software (Prism 4, GraphPad Software, Inc., San Diego, CA) for comparison across the experimental conditions. When a significant difference among the groups of AUC data was obtained in the one-way ANOVA, the Dunnett's post-hoc test was used to define which group contributed to these differences. The level of statistical significance was set at $P<0.05$.

3. Results

3.1. Hyperalgesia induced by the i.c.v. administration of midazolam alone

The analgesic effects by the i.c.v. administration of midazolam alone (10, 15, 20, 30, or 50 nmol) or saline are shown in Fig. 1A and B. There were significant differences in the AUC values for the %MPE (0–120 min), $AUC_{0-120\ min}$, among the six groups (Fig. 1B; $F=35.66$, $P<0.0001$; Kruskal–Wallis H test). The Dunn's multiple comparison test showed a significant decrease at doses of 15, 20 and 30 nmol of midazolam compared to the saline-treated group (Fig. 1B). These results demonstrate that the i.c.v. application of midazolam alone induces a significant hyperalgesia (15–30 nmol) on the tail-flick

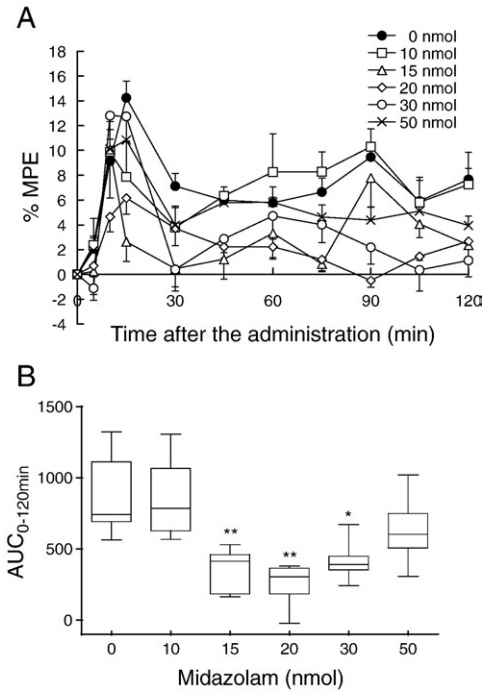

Fig. 1. Hyperalgesia of i.c.v. administration of midazolam (10–50 nmol) alone. The upper panel (A) indicates the time course of %MPE of midazolam. Results are means with S.E.M. of data obtained from five to eleven rats in each group. The lower panel (B) shows the $AUC_{0-120\ min}$ for the value of %MPE indicated in the upper panel (A). As determined by the Kruskal–Wallis H test, P was <0.0001. Significantly different from the values of a group that received saline (*$P<0.05$, **$P<0.01$) using Dunn's multiple comparison tests.

response. However, there was no significant difference between the $AUC_{0-120\ min}$ at doses of 10 and 50 nmol of midazolam and that of the saline-treated group. Because the high dose (50 nmol) of midazolam produced a sedation, no significant difference in the AUC values due to high dose of midazolam could be ascribed to the sedation. Therefore, 20 nmol of midazolam was selected and used in the following study. In addition, other drugs in combination with midazolam (20 nmol) failed to produce the sedation.

3.2. Antagonism of midazolam-induced hyperalgesia by flumazenil

To evaluate the effect of flumazenil, a benzodiazepine receptor antagonist, on the hyperalgesia induced by 20 nmol of midazolam, flumazenil (5–20 pmol, i.c.v.) was administered. At 10 min following the application of flumazenil, midazolam (20 nmol, i.c.v.) was administered (Fig. 2). There were significant differences in the $AUC_{0-120\ min}$ among the six groups (Fig. 2B; $F=22.30$, $P=0.0005$; Kruskal–Wallis H test). Flumazenil (20 pmol, $P<0.05$) significantly attenuated the hyperalgesia of midazolam (20 nmol)(Fig. 2B). Therefore, 20 pmol of flumazenil was selected and used in the following study. In addition, the i.c.v. administration of flumazenil (20 pmol) alone had no effect on the antinociception (Fig. 2B).

3.3. Inhibitory effect of midazolam on antinociception induced by D-serine

To evaluate the effect of midazolam on the antinociception induced by different doses of D-serine or saline, midazolam (20 nmol, i.c.v.) was administered. At 10 min following the i.c.v. administration of 20 nmol midazolam, the i.c.v. administration of D-serine (1, 5, 10 or 20 μmol) was administered (Fig. 3A). Midazolam significantly inhibited the D-serine-evoked antinociception (1 μmol, $P<0.01$; 10 μmol, $P<0.01$; Mann–Whitney U test) (Fig. 3A). There was a tendency to reduce the D-serine-induced antinociception by midazolam at doses of 5 μmol and 20 μmol of D-serine, whereas there were no significant differences between the saline/D-serine-treated and midazolam/D-serine-treated groups at these doses. The AUC curve was shifted to the right by the midazolam administration.

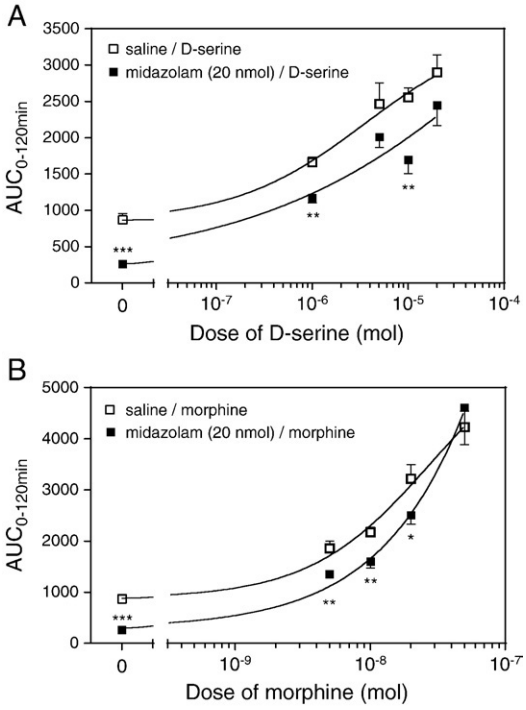

Fig. 3. Dose-dependent inhibitory effects of i.c.v. administration of midazolam (20 nmol) on antinociception induced by i.c.v. administration of (A) D-serine (1, 5, 10, and 20 μmol) or (B) morphine (5, 10, 20, and 50 nmol). Significantly different from the values of a group that received saline using Mann–Whitney U test; *$P<0.05$, **$P<0.01$, ***$P<0.001$. Results are means with S.E.M. of data obtained from four to ten rats in each group.

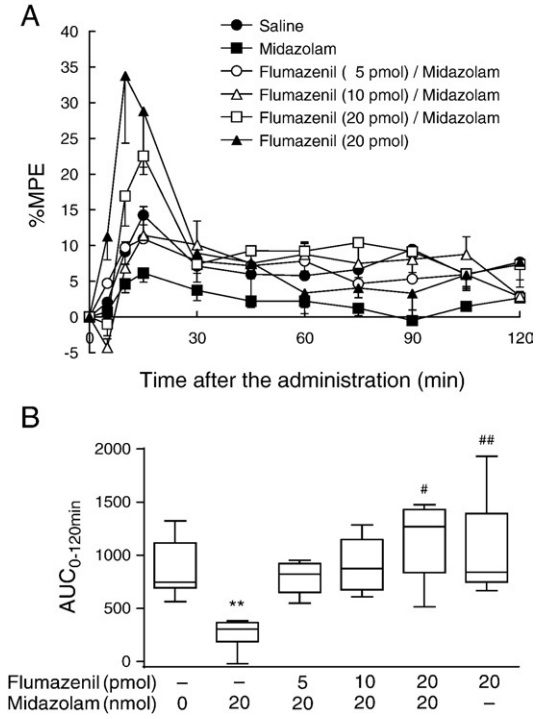

Fig. 2. Effect of i.c.v. administration flumazenil (5, 10 and 20 pmol) on hyperalgesia induced by i.c.v. administration of midazolam (20 nmol). Flumazenil was also injected 10 min before the administration of midazolam. The upper panel (A) indicates the time course of %MPE after the administration of saline or midazolam. The vertical bars represent the S.E.M. of data from four to ten rats. The lower panel (B) shows the $AUC_{0-120\ min}$ for the value of %MPE indicated in the upper panel (A). As determined by the Kruskal–Wallis H test, P was 0.0003. Significantly different from the values of a group that received saline (**$P<0.01$) and received midazolam alone (20 nmol; $^{\#}P<0.05$, $^{\#\#}P<0.01$) using Dunn's multiple comparison tests.

3.4. Inhibitory effect of midazolam on antinociception induced by morphine

To evaluate the effect of midazolam on the antinociception induced by different doses of morphine or saline, midazolam (20 nmol, i.c.v.) was administered. At 10 min following the i.c.v. administration of 20 nmol midazolam, the i.c.v. administration of morphine (5, 10, 20 or 50 nmol) was administered. Midazolam significantly inhibited the antinociception of morphine (5 nmol, $P<0.01$; 10 nmol, $P<0.01$; 20 nmol, $P<0.05$; Mann–Whitney U test) (Fig. 3B). The AUC curve was shifted to the right by the midazolam administration.

3.5. Attenuation of D-serine-induced antinociception by midazolam

To examine the effect of midazolam on the antinociception induced by 10 μmol of D-serine, midazolam (20 nmol, i.c.v.) was administered. Ten minutes later, 10 μmol of D-serine was i.c.v. administered (Fig. 4). There were significant differences in the $AUC_{0-120\ min}$ among the three groups (Fig. 4B; $F=13.64$, $P<0.01$; Kruskal–Wallis H test). The i.c.v. administration of 20 nmol ($P<0.01$) of midazolam significantly antagonized the D-serine-induced antinociception (Fig. 4B). At 10 min following the i.c.v. application of 20 pmol of flumazenil, the i.c.v. administration of 20 nmol of midazolam and

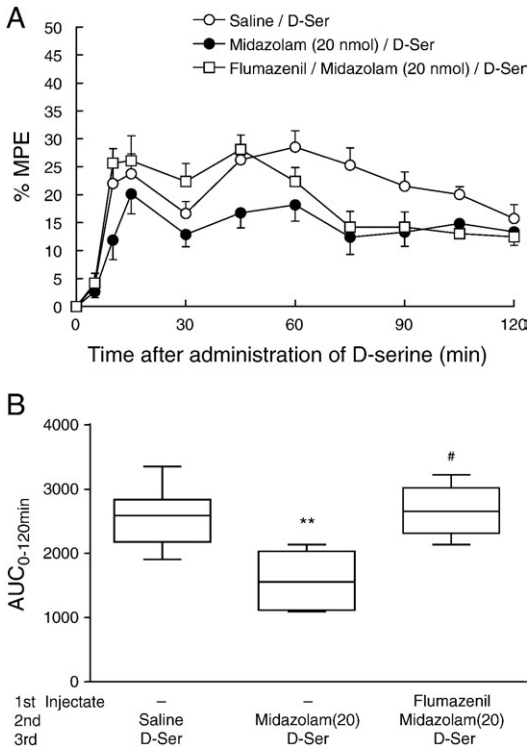

Fig. 4. Inhibitory effect of i.c.v. administration of midazolam (20 nmol) on antinociception induced by i.c.v. administration of D-serine (10 μmol). The upper panel (A) indicates the time course of %MPE. The vertical bars represent the S.E.M. of data from four to ten rats. The lower panel (B) shows the $AUC_{0-120\ min}$ for the value of %MPE indicated in the upper panel (A). As determined by the Kruskal–Wallis *H* test, *P* was 0.0011. Significantly different from the values of a group that received D-serine (10 μmol; $^{**}P<0.01$) and received midazolam plus D-serine (20 nmol; $^{\#}P<0.05$) using Dunn's multiple comparison tests.

10 μmol of D-serine were continuously administered at intervals of 10 min. The i.c.v. application of flumazenil ($P<0.05$) significantly reversed the attenuation of the D-serine-induced antinociception by midazolam (Fig. 4B).

3.6. Attenuation of morphine-induced antinociception by midazolam

To examine the effect of midazolam on the antinociception induced by 10 nmol of morphine, midazolam (20 nmol, i.c.v.) was administered. Ten minutes later, 10 nmol of morphine was i.c.v. administered (Fig. 5). There were significant differences in the $AUC_{0-120\ min}$ among the three groups (Fig. 5B; $F=15.24$, $P<0.001$; Kruskal–Wallis *H* test). The i.c.v. application of 20 nmol ($P<0.01$) of midazolam significantly antagonized the morphine-induced antinociception (Fig. 5B). At 10 min following the i.c.v. administration of 20 pmol flumazenil, 20 nmol midazolam and 10 nmol morphine were continuously administered at intervals of 10 min. The i.c.v. application of flumazenil ($P<0.05$) significantly reversed the attenuation of the morphine-induced antinociception by midazolam (Fig. 5B).

4. Discussion

The present study demonstrates for the first time that the antinociception evoked by the NMDA/glycine site agonist D-serine was attenuated by the i.c.v. application of the benzodiazepine/$GABA_A$ receptor agonist, midazolam, at the supraspinal level in the tail-flick test. This attenuation was reversed by the i.c.v. administration of a benzodiazepine receptor antagonist, flumazenil. The present and previous data suggest functional interactions among the benzodiazepine/$GABA_A$, NMDA and μ-opioid receptors in the regulation of the antinociception at the supraspinal level (Fields, 2004; Millan, 2002). Several lines of evidence support this possibility. First, neurons immunoreactive for the NMDA receptors and glutamate are identified in the PAG, and a subset of these projects to the RVM (Beitz, 1990; Commons et al., 1999; Wiklund et al., 1988). Neurons immunoreactive for the $GABA_A$ receptor and GABA are also present in the PAG (Williams and Beitz, 1990). Second, the i.c.v. administration of midazolam alone causes hyperalgesia on the tail-flick response in a flumazenil-reversible manner (Tatsuo et al., 1999) (Figs. 1 and 2). The antinociception induced by the μ-opioid receptor agonist, morphine, was also antagonized by the i.c.v. administration of midazolam in a flumazenil-reversible manner at the supraspinal level (Luger et al., 1995a) (Fig. 5). These data suggest that midazolam may inhibit μ-opioid-evoked antinociception by interacting with $GABA_A$ receptor at the supraspinal level (Fields, 2004; Millan, 2002). Third, we have recently shown that the activation of the supraspinal NMDA receptors by D-serine alone or in combination with morphine leads to the potentiation of the antinociception in the tail-flick test (Yoshikawa et al., 2007). Previous data also demonstrated that the i.c.v. injection of the NMDA receptor agonists, such as D-serine, NMDA, or glutamate, enhanced the morphine-induced antinociception at the supraspinal level, indicating the analgesic interaction between the NMDA and μ-opioid receptors at the supraspinal level (Hunter et al., 1994; Jacquet, 1988; van Praag and Frenk, 1990; Yoshikawa et al., 2007). Finally, midazolam attenuated the morphine-induced antinociception, an effect blocked by the non-competitive NMDA antagonist, MK-801, in the hot plate test at the supraspinal level, suggesting an analgesic link between the benzodiazepine/$GABA_A$ and NMDA receptors at the supraspinal level (Luger et al., 1995b). However, we have recently

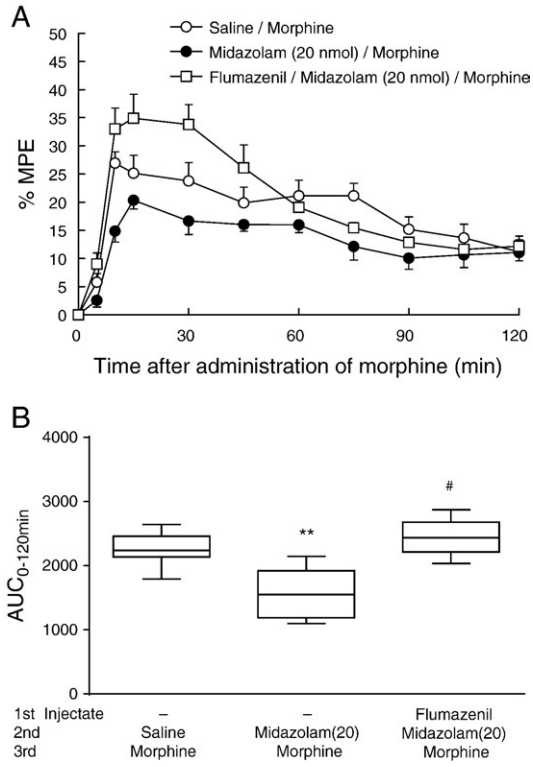

Fig. 5. Inhibitory effect of i.c.v. administration of midazolam (20 nmol) on antinociception induced by i.c.v. administration of morphine (10 nmol). The upper panel (A) indicates the time course of %MPE. The vertical bars represent the S.E.M. of data from four to twelve rats. The lower panel (B) shows the $AUC_{0-120\ min}$ for the value of %MPE indicated in the upper panel (A). As determined by Kruskal–Wallis *H* test, *P* was 0.0005. Significantly different from the values of a group that received morphine (10 nmol; $^{**}P<0.01$) and received midazolam plus morphine (20 nmol; $^{\#}P<0.05$) using Dunn's multiple comparison tests.

shown that the NMDA receptor-glycine site antagonist L-701,324 inhibited the morphine-evoked antinociception and the i.c.v. administration of D-serine produced antinociception in the tail-flick test at the supraspinal level in an L-701,324-reversible manner (Yoshikawa et al., 2007). This discrepancy might be derived from the different assay method (hot plate test vs. tail-flick test) and the different binding sites of NMDA antagonists (channel site vs. glycine site). Further studies are needed to clarify the analgesic interactions between the benzodiazepine/$GABA_A$ and NMDA receptors at the supraspinal level.

It has been proposed that the morphine-mediated antinociception in the PAG and RVM is produced by the direct inhibition of the GABAergic interneurons which disinhibit off-cells (Fields, 2004). In fact, GABA-releasing inputs are presynaptically attenuated by morphine in the neurons of PAG and RVM (Heinricher et al., 1992; Vaughan and Christie, 1997). The activation of RVM off-cells is also induced by the local application of either morphine or the $GABA_A$ receptor antagonist bicuculline (Heinricher et al., 1994; Heinricher and Tortorici, 1994). Furthermore, activation of the supraspinal NMDA receptor by agonists could directly lead to the off-cell activation and produce antinociception (Fields, 2004; Heinricher et al., 1999; Jacquet, 1988). The iontophoretic administration of glutamate into the RVM induces the off-cells activation, suggesting that the off-cells possess functional glutamate receptors (Heinricher and Roychowdhury, 1997). The administration of a competitive NMDA receptor antagonist, D-(−)-2-amino-5-phosphosophonopentanoic acid (AP5), into the RVM also inhibited the morphine-evoked activation of the off-cells, and significantly decreased the antinociceptive effect in the tail-flick test (Fields, 2004; Heinricher et al., 2001).

Another possibility is that the antinociceptive effect of the NMDA receptor agonists could be related to the release of the endogenous opioid peptides at the supraspinal level. The application of NMDA and glutamate into the hypothalamus or striatum induces the release of methionine-enkephalin or β-endorphin in an NMDA receptor antagonist-reversible manner (Bach and Yaksh, 1995; Ruzicka and Jhamandas, 1991; Yang et al., 1995) and the administration of NMDA or glutamate into the PAG, nucleus raphe magnus or hypothalamus enhanced the antinociceptive effect in the tail-flick test (Bach and Yaksh, 1995; Jacquet, 1988; van Praag and Frenk, 1990). Further support for this possibility stems from observations that the D-serine-evoked antinociception is inhibited by the i.c.v. injection of naloxone, suggesting that activation of the opioid receptors is required for the D-serine-evoked antinociception (Yoshikawa et al., 2007). Interestingly, our laboratory has indicated that inhibition of the degradation of the endogenous opioid peptides by the i.c.v. pretreatment with the mixture of the three peptidase inhibitors enhances the antinociceptive effect in the tail-flick test, suggesting that endogenous opioid peptides at the supraspinal level may be involved in the regulation of the antinociception in the tail-flick test (Kanai et al., 2002; Kitamura et al., 2000).

Hyperalgesia was induced by the i.c.v. administration of midazolam in a flumazenil-reversible manner (Figs. 1 and 2). Although the i.c.v. administration of midazolam alone has been reported to induce both hyperalgesia (Tatsuo et al., 1999) or no effect on nociception (Mantegazza et al., 1982), these contradictory results could be due to the dose of midazolam used. Indeed, high doses of midazolam in the present (20 nmol) and previous study (approximately 77 nmol) (Tatsuo et al., 1999) induced hyperalgesia, while a low dose of midazolam (approximately 1.5 nmol) showed no effect on the nociception (Mantegazza et al., 1982). Contrary to the midazolam-induced hyperalgesia, the combination of midazolam and ketamine is clinically used as a postoperative analgesia (Visser and Schug, 2006). These contradictory results could be derived from the different route of administration (i.c.v. vs. systemic). In fact, midazolam was administered by the i.c.v. injection in the present study, whereas the midazolam–ketamine combination is clinically administered by the intravenous injection (Visser and Schug, 2006).

In conclusion, the present studies demonstrated that the i.c.v. application of midazolam inhibits the antinociceptive effect of D-serine or morphine. In addition, this inhibitory effect of midazolam on the antinociception of D-serine or morphine was antagonized by the i.p. administration of flumazenil. Together with the facts that D-serine and midazolam act as selective agonists for the glycine site of the NMDA receptor and benzodiazepine/$GABA_A$ receptor (Hashimoto and Oka, 1997; Mantegazza et al., 1982), respectively, these findings suggest a functional interaction between the NMDA and benzodiazepine/$GABA_A$ receptors in the regulation of antinociception at the supraspinal level.

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