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# Involvement of the opioid system in the anxiolytic effect of diazepam in mice

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**Abstract**

In the present study, the anticonflict effect of diazepam was significantly abolished by pretreatment with naloxone, β-funaltrexamine or nor-binaltorphimine but not naltrindole, using a Vogel-type conflict paradigm in mice. However, naloxone alone had a significant proconflict effect, and β-funaltrexamine alone tended to produce a proconflict effect. Spontaneous drinking behavior was not affected by treatment with diazepam and nor-binaltorphimine. In addition, nor-binaltorphimine had no effect on diazepam-induced motor incoordination, hypothermia or anticonvulsant action, respectively. Moreover, the stable dynorphin analog E2078 ([*N*-methyl-Tyr[1],*N*-α-methyl-Arg[7],D-Leu[8]]dynorphin A-(1–8) ethylamide) and the highly selective κ-opioid receptor agonist U50,488H (trans-3,4-dichloro-*N*-(2-(1-pyrrolidinyl)cyclohexyl)benzenacetamide methanesulfonate hydrochloride) produced a significant anticonflict effect, which was completely antagonized by pretreatment with nor-binaltorphimine. These findings suggested that the κ-opioid system may play an important role in the anxiolytic effect of benzodiazepines and the regulation of anxiety.

*Keywords:* κ-Opioid receptor agonist; Anticonflict effect; Diazepam; Vogel-type conflict test; (Mouse)

## 1. Introduction

Recently, several investigators have indicated that neuropeptides in the brain are associated with the regulation of anxiety in animals and humans. For example, selective cholecystokinin B ($CCK_B$) receptor antagonists have an anxiolytic effect (Hughes et al., 1990; Singh et al., 1991), and corticotropin-releasing factor (CRF) produces an anxiogenic state (Britton et al., 1985, 1986). Wahlestedt et al. (1993) also reported that animals treated with antisense oligodeoxynucleotides of the neuropeptide Y $Y_1$ receptor display behavioral signs of anxiety. Furthermore, these neuropeptides can modulate the anxiolytic effect of benzodiazepines (Britton et al., 1988).

The role of endogenous opioid peptides, one of the most abundant peptide transmitters in the mammalian brain, in the anxiolytic effects of benzodiazepines was first demonstrated by Billingsley and Kubena (1978), who reported that the anticonflict effect of chlordiazepoxide was antagonized by naloxone (20–100 mg/kg). Furthermore, Duka et al. (1981) found that naloxone (1.0 mg/kg) could block the anticonflict effect of diazepam without affecting the shock threshold. Recently, Agmo et al. (1995) demonstrated that naloxone could block the anxiolytic effects of benzodiazepines and pentobarbital in the rat elevated plus maze test. In humans, naloxone attenuates the anxiolytic effect of diazepam (Duka et al., 1982). In contrast, the anxiogenic effect of the benzodiazepine inverse agonist DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate), but not its convulsant action, is potentiated by pretreatment with naloxone (Duka and Stephens, 1986). These data suggest that an endogenous opioid system may be involved in the control of emotion, anxiety, and anxiolytic effect of benzodiazepines.

Several pharmacological, behavioral and biochemical studies have suggested that there are at least three major types of opioid receptors; μ, δ and κ. In addition, several endogenous opioid peptides have been found, and it has been postulated that β-endorphin and enkephalin bind to μ- and δ-opioid receptors, whereas dynorphin binds preferentially to κ-opioid receptors (Chavkin and Goldstein, 1981; Chavkin et al., 1982; Goldstein and Naidu, 1989; Lord et al., 1977; Martin et al., 1976; Wüster et al., 1979).

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Recently, these opioid receptors were cloned (Evans et al., 1992; Chen et al., 1993; Yasuda et al., 1993), and many investigators have attempted to characterize specific pharmacological properties of each opioid receptor type. These findings prompted us to investigate which opioid receptor types ($\mu$, $\delta$ and $\kappa$) are involved in regulating the anxiolytic effects of benzodiazepines.

To characterize the involvement of the opioid system in the anxiolytic effects of benzodiazepines, we investigated whether the anticonflict effect of diazepam is modulated by selective $\mu$-, $\delta$- and $\kappa$-opioid receptor antagonists in mice using the Vogel conflict model (Vogel et al., 1971) with minor modifications.

## 2. Materials and methods

### 2.1. Animals

Male ddY mice (20–23 g) were obtained from Tokyo Animal Laboratories (Tokyo, Japan). The animals were housed at a temperature of 22 ± 1°C with a 12 h light-dark cycle (light on 8:30 a.m. to 8:30 p.m.). Food and water were available ad libitum.

### 2.2. Vogel-type conflict test

#### 2.2.1. Apparatus

The test chamber consisted of a Plexiglas box (10 × 12 × 18 cm) equipped with a grid floor of stainless steel bars and a drinking bottle containing water. This chamber was enclosed in a sound-attenuated and ventilated box. The drinking spout and the grid floor were connected to a shock generator and drinkometer (VC-2050L; O'hara, Tokyo, Japan). An electric shock (a 200 ms pulse every 20 licks or a constant licking time equivalent) could be applied between the drinking spout and the grid floor. The applied currents were 0.35 mA (anticonflict test) and 0.23 mA (proconflict test). When spontaneous drinking behavior was measured, the shock was omitted (non-shock session).

#### 2.2.2. Procedure

All experiments were carried out between 10:00 a.m. and 4:00 p.m. Mice were deprived of water for 24 h prior to a training session. In the training session, mice were individually placed in the test chamber and allowed to drink water for 10 min without an electric shock. At the end of this session, mice were returned to their home cage and housed for 72 h. Afterwards, they were again deprived of water for 24 h and a test session was performed. In the test session, mice were individually placed in the test chamber, and received an electric shock every 20 licks (or a constant licking time equivalent) for 10 min. If the first shock was not delivered within 3 min, mice were excluded from the experiment. Furthermore, a non-shock session was performed using the same procedure as in the test session, for 10 min without an electric shock to examine the effects of the drugs used on spontaneous drinking behavior. A test session was performed 30 min after treatment with diazepam (i.p.). In an antagonism study, mice were injected i.p. with flumazenil 10 min prior to the test session. Mice were treated s.c. with naloxone, $\beta$-funaltrexamine, naltrindole and nor-binaltorphimine at 15 min, 24 h, 30 min and 4 h prior to the test session, respectively. E2078 and U50,488H were injected s.c. 30 min prior to the test session. The doses of each opioid receptor antagonist used in the present study were based on previous studies (Suzuki et al., 1993, 1995; Gacel et al., 1990; Noble et al., 1992, 1995; Comer et al., 1993; Ukai et al., 1994; Endoh et al., 1992; Funada et al., 1993)

### 2.3. Shock threshold test

A mouse was placed on the stainless steel plate and an electric shock was delivered from its tail, around which an electrode was wound, to the stainless steel plate. The shock threshold was determined by the latency until the mouse showed an avoidance reaction to the electric shock (jumping, pawtap or pawlick). The electric shock was 0.1 mA, and mice that showed no response within 30 s (cut-off time) were removed from the stainless steel plate. The shock threshold test was performed 30 min after diazepam injection. Mice were also treated s.c. with naloxone (3.0 mg/kg) 15 min prior to the test.

### 2.4. Rotarod performance test

Each mouse was trained to run in a rotarod (3 cm in diameter, 8 rpm; Natsume Seisakusho, Tokyo, Japan) until it could remain there for 60 s without falling. The mice were then evaluated in a rotarod performance test for 60 s 15 min after diazepam injection. Nor-binaltorphimine was injected s.c. 4 h prior to the test. The doses of nor-binaltorphimine used in the present study were decided basing on the previous studies (Endoh et al., 1992; Funada et al., 1993)

### 2.5. Measurement of body temperature

Rectal temperature was measured with a thermistor probe (Shibaura Electronics, Tokyo, Japan) inserted into the rectum and monitored on a thermometer (3-A2078; Natsume Seisakusho, Tokyo, Japan). Pre-drug rectal temperature was recorded at room temperature 30 min before vehicle treatment. Mice were treated with nor-binaltorphimine (1.0–5.0 mg/kg, s.c.) or saline (s.c.) at 4 h prior to diazepam (8.0 mg/kg, i.p.) or vehicle (i.p.). Post-drug rectal temperature was recorded at 60 min after diazepam or vehicle injection. The doses of nor-binaltorphimine used in the present study were decided

basing on the previous studies (Endoh et al., 1992; Funada et al., 1993)

### 2.6. Electric shock seizure test

Electric shock seizure was induced by passing an electric current (50 mA, 100 ms, 100 Hz, square waves) through ear-clip electrodes. Clonic and/or tonic convulsion was then observed with an electric shock. Mice were injected i.p. and s.c. with diazepam and nor-binaltorphimine 30 min and 4 h before the test, respectively. The doses of nor-binaltorphimine used in the present study were decided basing on the previous studies (Endoh et al., 1992; Funada et al., 1993)

### 2.7. Drugs

Diazepam (Profarma, Milan, Italy) and flumazenil (Yamanouchi Pharmaceutical Co., Tokyo, Japan) were suspended in vehicle consisting of 9% Tween 80 (Kishida Chemical Co., Osaka, Japan) in saline. Naloxone hydrochloride was purchased from Research Biochemical (Wayland, MA, USA). β-Funaltrexamine hydrochloride, naltrindole methanesulfonate, nor-binaltorphimine hydrochloride and U50,488H (trans-3,4-dichloro-*N*-(2-(1-pyrrolidinyl)cyclohexyl)benzenacetamide methanesulfonate hydrochloride) were synthesized by us. E2078 ([*N*-methyl-Tyr[1],*N*-α-methyl-Arg[7],D-Leu[8]]dynorphin A-(1–8) ethylamide) was supplied by Eizai (Tsukuba, Japan). These agents were dissolved in saline.

### 2.8. Data analysis

The mean number of shocks, the number of drinks (conflict test), latency (shock threshold test) and percent impairment of rotarod performance (rotarod performance test) were statistically evaluated with the nonparametric Wilcoxon test. The incidence of seizure (electric shock seizure) and changes in rectal temperature were statistically analyzed using Fischer's exact probability test and a one-way repeated analysis of variance (ANOVA) followed by Dunnett's test, respectively.

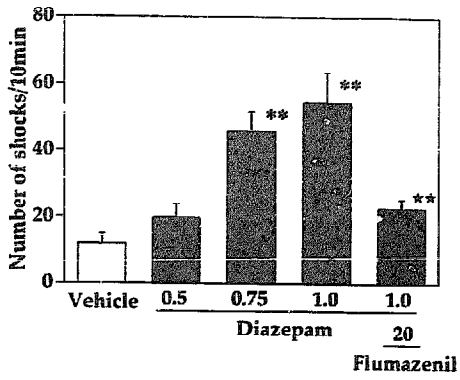


Fig. 1. Anticonflict effect of diazepam and effect of flumazenil on the anticonflict effect of diazepam in mice. Mice were treated i.p. with diazepam (0.5–1.0 mg/kg) and flumazenil (20 mg/kg) 30 min and 10 min before test session, respectively. Ordinate: number of shocks during 10 min. Each column represents the mean with S.E.M. of 10–16 mice. ** $P < 0.01$ vs. vehicle control. ** $P < 0.01$ vs. diazepam (1.0 mg/kg)-treated group.

## 3. Results

### 3.1. Effect of opioid antagonist on the anticonflict effect of diazepam

Diazepam (0.75 and 1.0 mg/kg) significantly increased the number of shocks given over 10 min ($P < 0.01$). This anticonflict effect of diazepam was completely antagonized by flumazenil (20 mg/kg; $P < 0.01$) (Fig. 1).

The effect of each opioid receptor antagonist on the anticonflict effect of diazepam is shown in Fig. 2. Naloxone blocked the anticonflict effect of diazepam; this effect of naloxone was significant at doses of 0.5, 1.0 ($P < 0.05$) and 3.0 mg/kg ($P < 0.01$) (Fig. 2A). In addition, 3 mg/kg of naloxone did not affect the shock threshold (data not

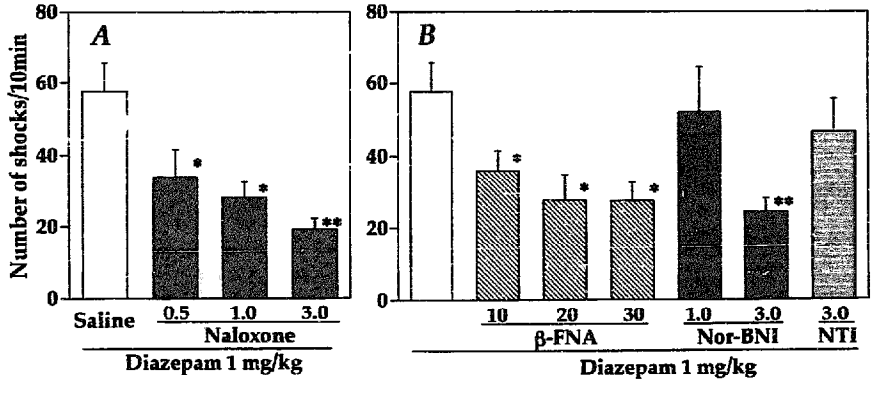


Fig. 2. Effect of each opioid receptor antagonist on the anticonflict effect of diazepam in mice. Mice were injected s.c. with naloxone (0.5–3.0 mg/kg) 15 min before test session (A). β-Funaltrexamine (β-FNA: 10–30 mg/kg, s.c.), nor-binaltorphimine (nor-BNI: 1.0 and 3.0 mg/kg, s.c.) and naltrindole (NTI: 3.0 mg/kg, s.c.) were injected 24 h, 4 h and 30 min before test session, respectively. Ordinate: number of shocks during 10 min. Each column represent the mean with S.E.M. of 10–22 mice. * $P < 0.05$, ** $P < 0.01$ vs. saline-treated diazepam group.

Table 1
Effect of each opioid antagonist with diazepam on spontaneous drinking behavior in mice

| Drug | Drinking counts/10 min |
|---|---|
| Vehicle | 52.80 ± 3.86 |
| Diazepam 0.5 mg/kg | 53.44 ± 3.10 |
| + Naloxone 3 mg/kg | 51.50 ± 5.58 |
| + β-FNA 30 mg/kg | 64.78 ± 6.82 |
| + Nor-BNI 3 mg/kg | 66.13 ± 10.34 |

Naloxone, β-funaltrexamine (β-FNA) and nor-binaltorphimine (nor-BNI) were injected s.c. 15 min, 24 h and 4 h before test session, respectively. Each value represents the mean of the drinking counts during 10 min with S.E.M. of ten mice.

shown). Furthermore, the anticonflict effect of diazepam was significantly attenuated by pretreatment with β-funaltrexamine (10, 20 and 30 mg/kg; $P < 0.05$) or nor-binaltorphimine (3.0 mg/kg; $P < 0.01$), but not with naltrindole (3.0 mg/kg; $P > 0.05$) (Fig. 2B). Neither naloxone, β-funaltrexamine nor nor-binaltorphimine affected the number of drinks with diazepam (Table 1). However, 3.0 mg/kg of naloxone produced a significant proconflict effect, and 1.0 mg/kg of naloxone and β-funaltrexamine alone tended to produce a proconflict effect (Table 2).

### 3.2. Effect of nor-binaltorphimine on diazepam-induced motor incoordination

Diazepam produced significant motor incoordination versus the vehicle control ($P < 0.01$). This diazepam (4.0 mg/kg)-induced motor incoordination was not inhibited by pretreatment with nor-binaltorphimine (1.0, 3.0 or 5.0 mg/kg) (Table 3). In addition, nor-binaltorphimine alone did not affect rotarod performance (data not shown).

### 3.3. Effect of nor-binaltorphimine on diazepam-induced hypothermia

Diazepam (8.0 mg/kg) produced significant hypothermia ($P < 0.01$) versus the vehicle control. Nor-binaltorphimine (5.0 mg/kg) alone did not affect rectal temperature versus the control group (data not shown).

Table 2
Effect of each opioid receptor antagonist on proconflict response in mice

| Drug (mg/kg) | | Intensity of shock: 0.23 mA | |
|---|---|---|---|
| | | No. shocks (% inhibition) | |
| Vehicle control | | 39.8 ± 5.3 | – |
| Naloxone | 1.0 | 28.7 ± 6.5 | (27.8%) |
| | 3.0 | 25.3 ± 3.8 | (36.5%) |
| β-FNA | 20 | 30.4 ± 6.6 | (23.6%) |
| | 30 | 25.2 ± 5.0 | (36.6%) |
| NTI | 3.0 | 35.4 ± 5.6 | (11.1%) |
| Nor-BNI | 3.0 | 37.8 ± 8.1 | (4.9%) |

Naloxone was injected s.c. 15 min before test session. β-FNA, NTI and nor-BNI were injected s.c. 24 h, 30 min and 4 h before test session, respectively. Diazepam was injected i.p. 30 min before test session. Each value represents the mean of the drinking counts/10 min with S.E.M. of 10–19 mice and % inhibition for vehicle control. * $P < 0.05$ vs. vehicle control.

Furthermore, pretreatment with nor-binaltorphimine (1.0, 3.0 or 5.0 mg/kg) did not suppress diazepam-induced hypothermia (Table 3).

### 3.4. Effect of nor-binaltorphimine on the anticonvulsant effect of diazepam

The incidence of convulsions induced by electric shock was significantly decreased by diazepam versus the vehicle control group ($P < 0.01$). This anticonvulsant effect of diazepam was not affected by pretreatment with nor-binaltorphimine (1.0, 3.0 or 5.0 mg/kg) (Table 3).

### 3.5. The anticonflict effect of κ-opioid receptor agonists

The stable dynorphin analog E2078, at dose of 0.5 ($P < 0.01$) and 1.0 mg/kg ($P < 0.05$), significantly increased the number of shocks during the test session, and 0.5 mg/kg of E2078 did not affect spontaneous drinking behavior. This anticonflict effect of E2078 was significantly antagonized by pretreatment of nor-binaltorphimine (Fig. 3). Furthermore, the selective κ-opioid agonist U50,488H (1.0 mg/kg) also produced a significant increase in the number of shocks ($P < 0.01$) without affect-

Table 3
Effect of nor-BNI on the diazepam-induced motor incoordination, hypothermia and anticonvulsant action in mice

| | % Rotarod impairment [a] | ΔT rectal temperature [b] | Electric shock seizure [c] |
|---|---|---|---|
| Control | 0 ± 0 | −0.15 ± 0.13 | 10/11 |
| Diazepam | 82.4 ± 6.9 * | −1.52 ± 0.24 * | 1/10 * |
| + Nor-BNI 1.0 | 77.2 ± 13.1 * | −1.64 ± 0.28 * | 2/10 * |
| + Nor-BNI 3.0 | 82.7 ± 9.7 * | −1.26 ± 0.21 * | 0/10 * |
| + Nor-BNI 5.0 | 80.8 ± 12.2 * | −1.72 ± 0.27 * | 0/10 * |

[a] Rotrarod performance: mice were treated i.p. with diazepam (4.0 mg/kg) 15 min prior to test. Each value represents the mean with S.E.M. of 8–13 mice. * $P < 0.01$ vs. control group. [b] Body temperature: mice were treated i.p. with diazepam (8.0 mg/kg) 60 min prior to test. Each value represents the mean of rectal temperature changes (ΔT) for the control (vehicle + saline-treated group) with S.E.M. of 8–13 mice. * $P < 0.01$ vs. control group. [c] Electricshock seizure: diazepam (1.0 mg/kg) was injected i.p. 30 min prior to test. * $P < 0.01$ vs. control group. Nor-binaltorphimine (nor-BNI) was injected s.c. 4 h before test.

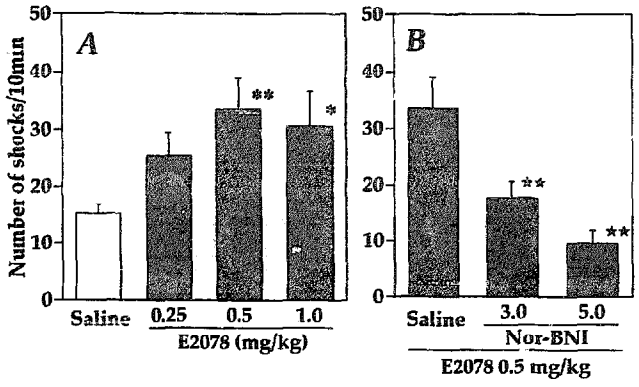


Fig. 3. Anticonflict effect of E2078 (A) and effect of nor-binaltorphimine (nor-BNI) on the anticonflict effect of E2078 (B) in mice. Mice were treated s.c. with E2078 (0.25–1.0 mg/kg) 30 min before test session. Ordinate: number of shocks during 10 min. Each column represents the mean with S.E.M. of 10–18 mice. * $P < 0.05$, ** $P < 0.01$ vs. saline control. ** $P < 0.01$ vs. saline-treated E2078 (0.5 mg/kg) group.

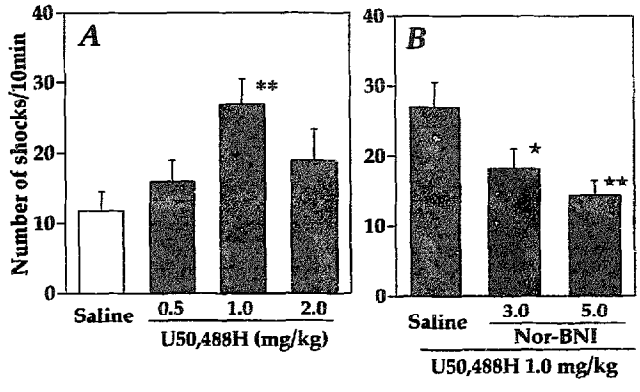


Fig. 4. Anticonflict effect of U50,488H (A) and effect of nor-binaltorphimine (nor-BNI) on the anticonflict effect of U50,488H (B) in mice. Mice were treated s.c. with U50,488H (0.5–2.0 mg/kg) 30 min before test session. Ordinate: number of shocks during 10 min. Each column represents the mean with S.E.M. of 11–23 mice. ** $P < 0.01$ vs. saline control. * $P < 0.05$. ** $P < 0.01$ vs. saline-treated U50,488H (1.0 mg/kg) group.

ing spontaneous drinking behavior. This effect of U50,488H was significantly antagonized by nor-binaltorphimine (Fig. 4). However, three of ten animals treated with 2.0 mg/kg U50,488H did not drink water even without electric shock. The shock threshold to electric shock was not affected by treatment with E2078 or U50,488H at doses of 0.5 and 1.0 mg/kg, respectively (Table 4).

Table 4
Effects of E2078 and U50,488H on the spontaneous drinking behavior and the shock threshold in mice

| | Drinking count | Latency (s) |
|---|---|---|
| Saline | 52.8±3.9 | 6.25±1.46 |
| E2078 | 44.2±4.4 | 5.56±0.99 |
| U50,488H | 63.8±7.9 | 6.84±0.91 |

Mice were treated s.c. with saline, E2078 (0.5 mg/kg) and U50,488H (1.0 mg/kg) 30 min prior to test session. Each value represents the mean of drinking counts and avoidance latency to electric shock with S.E.M. of 7–11 mice.

## 4. Discussion

The present study demonstrated that diazepam produces a significant anticonflict effect which is completely antagonized by benzodiazepine binding site antagonist flumazenil (Hunkeler et al., 1981). This suggests that the anticonflict effect of diazepam may be mediated via benzodiazepine binding sites. We first confirmed that the endogenous opioid system was involved in the anxiolytic effect of diazepam based on the result that the nonselective opioid receptor antagonist naloxone blocked the anticonflict effect of diazepam without affecting either the nociceptive threshold to shock or spontaneous drinking behavior. These findings are consistent with the result of other previous studies (Agmo et al., 1995; Duka et al., 1981; Soubrie et al., 1980). Furthermore, we demonstrated that the anticonflict effect of diazepam was significantly abolished by pretreatment with the selective $\mu$-opioid receptor antagonist $\beta$-funaltrexamine or the selective $\kappa$-opioid receptor antagonist nor-binaltorphimine, but not with the $\delta$-opioid receptor antagonist naltrindole. In addition, pretreatment with these antagonists did not influence spontaneous drinking behavior. These results suggest that the anticonflict effect of diazepam may be partially mediated by stimulation of $\mu$- and/or $\kappa$-opioid receptors by endogenous opioid peptides. However, since naloxone and $\beta$-funaltrexamine alone both produced a significant and a tendency towards a proconflict effect, respectively, the suppression of diazepam's anticonflict effect by naloxone and $\beta$-funaltrexamine might be due to functional antagonism between 'anti'- and 'pro'-conflict effects. In contrast, 3.0 mg/kg of nor-binaltorphimine, which abolished the anticonflict effect of diazepam, did not produce a proconflict response. Therefore, although the role of $\mu$-opioid receptors in the anticonflict effect of diazepam cannot be excluded, the anticonflict effect of diazepam may be mediated via the activation of $\kappa$-opioid receptors rather than $\mu$-opioid receptors by endogenous opioids.

It is well known that benzodiazepines have sedative, anticonvulsant, anxiolytic and hypothermic effect. $\kappa$-Opioid receptor agonists also have sedative, anticonvulsant and hypothermic effect (Phillai and Ross, 1986; Tortella et al., 1985; Von Voigtlander et al., 1987). Thus it was interesting to determine whether diazepam-induced motor incoordination, hypothermia and anticonvulsion are also suppressed by pretreatment with nor-binaltorphimine. The present data showed that nor-binaltorphimine did not suppress these three pharmacological effects of diazepam. These results are consistent with previous reports that naloxone has no effect on DMCM-induced convulsion and diazepam-induced motor incoordination (Agmo et al., 1995; Duka and Stephens, 1986), while U50,488H does not

suppress the bicuculline- and pentylenetetrazole-induced seizure (Von Voigtlander et al., 1987). Considering these findings, the endogenous κ-opioid system (dynorphinergic) may specially regulate the neural pathway involved in the anxiolytic effect of diazepam.

Many investigators have demonstrated that a marked anxiolytic effect is produced by microinjection of benzodiazepines into the amygdala and mammillary body (Kataoka et al., 1987; Nagy et al., 1979; Scheel-Krüger and Petersen, 1982; Shibata et al., 1982, 1986, 1989). Yamashita et al. (1989) reported that electrical lesion of the amygdala and mammillary body produces an anticonflict effect. Interestingly, κ-opioid receptors are densely distributed in the these areas as compared to μ- and δ-opioid receptor (Mansour et al., 1987, 1988, 1994). Therefore, an endogenous dynorphin system in these brain areas may play an important role in the anxiolytic effect of diazepam.

To further characterize the involvement of opioid systems in the regulation of anxiety, the anticonflict effect of two exogenous κ-opioid receptor agonists was examined. The stable dynorphin analog E2078 produced an anticonflict effect which was antagonized by pretreatment with nor-binaltorphimine. These results support our findings that an endogenous κ-opioid system may be associated with the anticonflict effect of diazepam. Furthermore, the highly selective κ-opioid receptor agonist U50,488H also induced a significant anticonflict effect which was completely blocked by nor-binaltorphimine. Moreover, each dose of these κ-opioid receptor agonists that produced a significant anticonflict effect did not affect either spontaneous drinking behavior or the sensitivity to electric shock. Therefore, the anticonflict effect of κ-opioid receptor agonists may result not from nonspecific effects, such as increasing water intake or the shock threshold, but rather to inhibition of the conflict (anxiety) situation. The present experimental findings suggested that κ-opioid receptor agonists have an anxiolytic effect, which is low to moderate compared with that of diazepam.

The highest dose of the κ-opioid agonist U50,488H (2.0 mg/kg) did not produce a significant anticonflict effect. This may be due to the sedative effect of U50,488H. In fact, 2.0 mg/kg of U50,488H decreased the number of drinks. In addition, 2.0 mg/kg of U50,488H produced a significant place aversion in mice (Funada et al., 1993). Therefore, reduction of the anticonflict effect at the highest dose of U50,488H may result from the sedative and/or strong dysphoric effects of U50,488H.

Recently, Privette and Terrian (1995) showed that low doses of the κ-opioid receptor agonists U50,488H and U69,593 produce anxiolytic effects in the rat elevated plus-maze test. This report supports our findings using a Vogel-type conflict test in mice. In addition, we demonstrated that the anticonflict effect of κ-opioid receptor agonists was completely antagonized by pretreatment with nor-binaltorphimine. Therefore, the anxiolytic effects of E2078 and U50,488H may be mediated by κ-opioid receptors. It have been demonstrated that κ-(especially $\kappa_1$) opioid receptors are densely distributed in the amygdala and mammillary body, which are important sites for the anxiolytic effects of benzodiazepines. Moreover, κ-opioid receptor agonists inhibit voltage-dependent $Ca^{2+}$ current through N-type $Ca^{2+}$ channels (Adamson et al., 1989; Xiang et al., 1990) and inhibit neurotransmitter release (Cui et al., 1994; Mulder et al., 1989; Werling et al., 1989). Taken together, these findings suggest that the anticonflict effects of κ-opioid receptor agonists may be due to the inhibition of neurotransmitter release, which is potentiated by anxiogenic situations, by blocking $Ca^{2+}$ influx through the closure of N-type $Ca^{2+}$ channels in these brain areas. Although the mechanism of the anticonflict effect of κ-opioid receptor agonist is still unclear, clarification of this point by further studies may help us to elucidate the mechanism of the anxiolytic effect of benzodiazepines and/or anxiety.

In summary, the anticonflict effect of diazepam was abolished by pretreatment with naloxone, β-funaltrexamine or nor-binaltorphimine, but not with naltrindole. However, naloxone and β-funaltrexamine alone both tended to produce a proconflict effect. In addition, nor-binaltorphimine had no effect on diazepam-induced motor incoordination, hypothermia and anticonvulsion. These results suggest that the endogenous κ-opioid system may be involved in the anxiolytic effect of diazepam. Furthermore, we demonstrated that the stable dynorphin analog E2078 and the selective κ-opioid receptor agonist U50,488H possess anxiolytic effect may play an important role in regulating anxiolytic effect benzodiazepines and anxiety itself.

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## References

Adamson, P., J.-Z. Xiang, T. Mantzourides, M.J. Brammer and I.C. Campbell, 1989, Presynaptic $\alpha_2$-adrenoceptor and κ-opioid receptor occupancy promotes closure of neuronal (N-type) calcium channels, Eur. J. Pharmacol. 174, 63.

Agmo, A., A. Galvan, A. Heredia and M. Morales, 1995, Naloxone blocks the antianxiety but not the motor effects of benzodiazepines and pentobarbital: experimental studies and literature review, Psychopharmacology 120, 186.

Billingsley, M.L. and R.K. Kubena, 1978, The effects of naloxone and picrotoxin on the sedative and anticonflict effects of benzodiazepines, Life Sci. 22, 897.

Britton, K.T., J. Morgan, J. Rivier, W. Vale and G.F. Koob, 1985, Chlordiazepoxide attenuates response suppression induced by corticotropin-releasing factor in the conflict test, Psychopharmacology 86, 170.

Britton, K.T., G. Lee, W. Vale, J. Rivier and G.F. Koob, 1986, Corticotropin releasing factor (CRF) receptor antagonist blocks activating and 'anxiogenic' action of CRF in the rat, Brain Res. 369, 303.

Britton, K.T., G. Lee and G.F. Koob, 1988, Corticotropin releasing factor and amphetamine exaggerate partial agonist properties of benzodiazepine antagonist Ro 15-1788 in the conflict test, Psychopharmacology 94, 306.

Chavkin, C. and A. Goldstein, 1981, Specific receptor for the opioid peptide dynorphin: structure-activity relationships, Proc. Natl. Acad. Sci. USA 78, 6543.

Chavkin, C., I.F. James and A. Goldstein, 1982, Dynorphin is a specific endogenous ligand of the κ-opioid receptor, Science 215, 413.

Chen, Y., A. Mestek, J. Liu, J.A. Hurley and L. Yu, 1993, Molecular cloning and functional expression of a μ-opioid receptor from rat brain, Mol. Pharmacol. 44, 8.

Comer, S.D., E.M. Hoenicke, A.I. Sable, R.W. Mcnutt, K.J. Chang, B.R.D. Costa, H.I. Mosberg and J.H. Woods, 1993, Convulsive effects of systemic administration of the delta opioid agonist BW373U86 in mice, J. Pharmacol. Exp. Ther. 267, 888.

Cui, Y., T.-F. Lee and C.H. Wang, 1994, Species difference in the modulatory effect of κ agonist on 5-HT release from ground squirrel and rat hippocampus, Neurosci. Lett. 175, 126.

Duka, T. and D.N. Stephens, 1986, Potentiation of the propunishment, but not convulsant action of the β-carboline DMCM by naltrexone, Pharmacol. Biochem. Behav. 25, 595.

Duka, T., R. Cumin, W. Haefely and A. Herz, 1981, Naloxone blocks the effect of diazepam and meprobamate on conflict behaviour in rats, Pharmacol. Biochem. Behav. 15, 115.

Duka, T., M.J. Millan, B. Ulsamer and A. Doenicke, 1982, Naloxone attenuates the anxiolytic action of diazepam in man, Life Sci. 31, 1833.

Endoh, T., H. Matsuura, C. Tanaka and H. Nagase, 1992, Nor-binaltorphimine: A potent and selective κ-opioid receptor antagonist with long-lasting activity in vivo, Arch. Int. Pharmacodyn. 316, 30.

Evans, C.J., D.E. Keith, Jr., H. Morrison, K. Magendzo and R.H. Edwards, 1992, Cloning of a delta opioid receptor by functional expression, Science 258, 1952.

Funada, M., T. Suzuki, M. Narita, M. Misawa and H. Nagase, 1993, Blockade of morphine reward through the activation of κ-opioid receptors in mice, Neuropharmacology 32, 1315.

Gacel, G.A., E. Fellion, A. Baamonde, V. Dauge and R.P. Roques, 1990, Synthesis, biochemical and Pharmacological properties of BUBUC, highly selective and systemically active agonist for in vivo studies of δ-opioid receptors, Peptides, 11, 983.

Goldstein, A. and A. Naidu, 1989, Multiple opioid receptors: ligand selectivity profiles and binding site signatures, Mol. Pharmacol. 36, 265.

Hughes J., P. Borden, B. Costall, A. Domeney, E. Kelly, D.C. Horwell, J.C. Hunter, R.D. Pinnock and G.N. Woodruff, 1990, Development of a class of selective cholecystokinin type B receptor antagonists having potent anxiolytic activity, Proc. Natl. Acad. Sci. USA 87, 6728.

Hunkeler, W., H. Mohler, L. Pieri, P. Polc, E.P. Bonetti, R. Cumin, R. Schaffner and W. Haefely, 1981, Selective antagonists of benzodiazepines, Nature 290, 514.

Kataoka, Y., K. Shibata, K. Yamashita and S. Ueki, 1987, Differential mechanisms involved in the anticonflict action of benzodiazepines injected into central amygdala and mammillary body, Brain Res. 416, 243–247.

Lord, A.H., J. Watt, S. Karll and H.W. Kosterlitz, 1977, Endogenous opioid peptides: multiple agonists and receptors, Nature 267, 495.

Martin, W.R., C.G. Eades, J.A. Thompson, R.E. Huppler and P.E. Gilbert, 1976, The effects of morphine- and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog, J. Pharmacol. Exp. Ther. 197, 517.

Mansour, A., H. Khachaturian, M.E. Lewis, H. Akil and S.J. Watoson, 1987, Autoradiographic differentiation of mu, delta and kappa opioid receptors in the rat forebrain and midbrain, J. Neurosci. 7, 2445.

Mansour, A., H. Khachaturian, M.E. Lewis, H. Akil and S.J. Watson, 1988, Anatomy of CNS opioid receptors, Trans Neurosci. 11, 308.

Mansour, A., C.A. Fox, F. Meng, H. Akil and S.J. Watson, 1994, $\kappa_1$ Receptor mRNA distribution in the rat CNS: comparison to κ receptor binding and prodynorphin mRNA, Mol. Cell. Neurosci. 5, 124.

Mulder, A.H., G. Wardeh, F. Hogenboom and L. Frankhuyzen, 1989, Selectivity of various opioid peptides towards delta-, kappa- and mu-opioid receptors mediating presynaptic inhibition of neurotransmitter release in the brain, Neuropeptides 14, 99.

Nagy, J., K. Zembo and L. Desci, 1979, Anti-anxiety action of diazepam after intra-amygdaloid application in the rat, Neuropharmacology 18, 573.

Noble, F., A. Blommaert, M.C. Fournie-Zaluski and B.P. Roques, 1995, A selective $CCK_B$ receptor antagonist potentiates μ-, but not δ-opioid receptor-mediated antinociception in the formalin test, Eur. J. Pharmacol. 275, 145.

Noble, F., M. Soleilhac, E. Soroca-Lucas, S. Turcaud, M.C. Fournie-Zaluski and B.P. Roques, 1992, Inhibition of the enkephalin-metabolizing enzymes by first systematically active mixed inhibitor prodrug RB 101 induces potent analgesic responses in mice and rats, J. Pharmacol. Exp. Ther. 261, 181.

Phillai, N.P. and D.H. Ross, 1986, Interaction of κ opioid agonists with calcium channel antagonists in the modulation of hypothermia, Eur. J. Pharmacol. 132, 237.

Privette, T.H. and D.M. Terrian, 1995, Kappa opioid agonists produce anxiolytic-like behavior on the elevated plus-maze, Psychopharmacology 118, 444.

Scheel-Krüger, J. and E.N. Petersen, 1982, Anticonflict effect of the benzodiazepines mediated by a GABAergic mechanism in the amygdala, Eur. J. Pharmacol. 82, 115.

Shibata, K., Y. Kataoka, Y. Gomita and S. Ueki, 1982, Localization of the site of the anticonflict action of benzodiazepines in the amygdaloid nucleus of rats, Brain Res. 234, 442.

Shibata, K., Y. Kataoka, K. Yamashita and S. Ueki, 1986, An important role the central amygdaloid nucleus and mammillary body in the mediation of conflict behavior in rats, Brain Res. 372, 159.

Shibata, S., K. Yamashita, E. Yamamoto, T. Ozaki and S. Ueki, 1989, Effects of benzodiazepine and GABA antagonists on anticonflict effects of antianxiety drug injected into the rat amygdala in a water-lick suppression test, Psychopharmacology 98, 38.

Singh, L., A.S. Lewis, M.J. Field, J. Hughes and G.N. Woodruff, 1991, Evidence for an involvement of the brain cholecystokinin B receptor in anxiety, Proc. Natl. Acad. Sci. USA 88, 1130.

Soubrie, P., A. Jobert and M. H. Theibot, 1980, Differential effects of naloxone against the diazepam-induced release of behavior in rat in three aversive situations, Psychopharmacology 69, 101.

Suzuki, T., M. Funada, M. Narita, M. Misawa and H. Nagase, 1993, Morphine-induced place preference in the CXBK mouse: characteristics of μ opioid receptor subtypes, Brain Res. 602, 45.

Suzuki, T., M. Tsuji, T. Mori, M. Misawa, T. Endoh and H. Nagase, 1995, Effects of a highly selective nonpeptide d opioid receptor agonist, TAN-67, on morphiine-induced antinociception in mice, Life Sci. 57, 155.

Tortella, F.C., L. Robles and J.W. Holaday, 1985, U50,488, a highly selective kappa opioid: anticonvulsant profile in rats, J. Pharmacol. Exp. Ther. 237, 49.

Ukai, M., T. Toyoshi, M. Mizutani, Y. Sasaki and T. Kameyama, 1994, Differential modulation of behavioral responses by [D-Ala$^2$, NMePhe$^4$, Gly-ol]enkephalin, [Dala$^2$]deltorphin II and [D-Pen$^2$, L-Pen$^5$]enkephalin in the mouse, Jpn. J. Psychopharmacol. 14, 161.

Vogel, J.R., B. Beer and D.E. Clody, 1971, A simple reliable conflict procedure for testing anti-anxiety agents, Psychopharmacologia 21, 1.

Von Voigtlander, P.F., E.D. Hall, M.C. Ochoa, R.A. Lewis and H.J.

Triezenberg, 1987, U-54494A: a unique anticonvulsant related to kappa opioid agonists, J. Pharmacol. Exp. Ther. 243, 542.

Wahlestedt, C., E.M. Pich, G.F. Koob, F. Yee and M. Heilig, 1993, Modulation of anxiety and neuropeptide Y-Y1 receptors by antisense oligodeoxynucleotides, Science 259, 528.

Werling, L.L., P.N. McMahon, P.S. Portoghese, A.E. Takemori and B.M. Cox, 1989, Selective opioid antagonist effects on opioid-induced inhibition of release of norepinephrine in guinea pig cortex, Neuropharmacology 28, 103.

Wüster, M., R. Schulz and A. Herz, 1979, Highly specific opiate receptors for dynorphin-(1–13) in the mouse vas deferens, Eur. J. Pharmacol. 62, 235.

Xiang, J.-Z., P. Adamson, M.J. Brammer and I.C. Campbell, 1990, The κ-opiate agonist U50,488H decreases the entry of $^{45}Ca$ into rat cortical synaptosomes by inhibiting N- but not L-type calcium channels, Neuropharmacology 29, 439.

Yamashita, Y., Y. Kataoka, Y Shibata, T. Ozaki, A. Miyazaki, M. Kagoshima and S. Ueki, 1989, Neuroanatomical substrates regulating rat conflict behavior evidenced by brain lesioning, Neurosci. Lett. 104, 195.

Yasuda, K., K. Raynor, H. Kong, C.D. Breder, J. Takeda, T. Reisine and G.I. Bell, 1993, Cloning and functional comparison of κ and δ opioid receptors from mouse brain, Proc. Natl. Acad. Sci. USA 90, 6736.