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PII S0024-3205(98)00075-7

Life Sciences, Vol. 62, No. 16, pp. 1371-1378, 1998

0024-3205/98 $19.00 + .00

# IDENTIFICATION OF OPIOID RECEPTOR SUBTYPES IN ANTINOCICEPTIVE ACTIONS OF SUPRASPINALLY-ADMINISTERED MITRAGYNINE IN MICE

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**(Received in final form January 28, 1998)**

## Summary

Mitragynine (MG), a major alkaloidal constituent extracted from the plant *Mitragyna speciosa* Korth, is known to exert an opioid-like activity. Our previous study showed the involvement of opioid systems in the antinociceptive activity of MG in the tail-pinch and hot-plate tests in mice. In the present study, to clarify the opioid receptor subtypes involved in the antinociceptive action of MG, we investigated the effects of selective antagonists for μ-, δ- and κ- opioid receptors on antinociception caused by the intracerebroventricular (i.c.v.) injection of MG in the tail-pinch and hot-plate tests in mice. The coadministration of a selective μ-opioid antagonist, cyprodime (1-10 μg, i.c.v.) and the pretreatment with a selective μ1-opioid antagonist naloxonazine (1-3 μg, i.c.v.) significantly antagonized the antinociceptive activities of MG (10 μg, i.c.v.) and morphine (MOR, 3 μg, i.c.v.) in the tail-pinch and hot-plate tests. Naltrindole (1-5 ng, i.c.v.), a selective δ-opioid antagonist, also blocked the effects of MG (10 μg, i.c.v.) without affecting MOR (3 μg, i.c.v.) antinociception. Nor-binaltorphimine, a selective κ-opioid antagonist, significantly attenuated MG (10 μg, i.c.v.) antinociception in the tail-pinch test but not in the hot-plate test at the dose (1 μg, i.c.v.) that antagonized the antinociceptive effects of the selective κ-opioid agonist U50,488H in both tests, while it had no effect on MOR antinociception in either tests. These results suggest that antinociception caused by i.c.v. MG is dominantly mediated by μ- and δ-opioid receptor subtypes, and that the selectivity of MG for the supraspinal opioid receptor subtypes differs from that of MOR in mice.

*Key Words:* **mitragynine, antinociception, opioid receptor subtypes, mice**

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Mitragynine (MG, Fig. 1) is a major constituent (about 66%) of the total alkaloids extracted from the young leaves of the plant *Mitragyna speciosa* Korth. (1, 2). The leaves of this plant are known to produce opioid-like actions (3). We previously reported that i.c.v.- and intraperitoneally (i.p.)-administered MG exerted an antinociceptive effect on the mechanical and thermal noxious stimuli-induced nociceptive responses of mice (4). The effects of MG were antagonized by i.c.v. naloxone, suggesting that supraspinal opioid systems played important roles in the action of MG (4). Although the leaves of *Mitragyna speciosa* are used clinically to replace opiates in addiction treatment in Thailand (3), MG did not cause MOR-like behavioral changes such as excitation and Straub's tail reaction in mice (4). In a previous study, the antinociceptive activity of MG and MOR exhibited different sensitivities to 5-HT depletion in the tail-pinch test (5). In addition, descending noradrenergic and serotonergic systems were found to be involved in the antinociceptive activity of MG in the tail-pinch test, while the descending noradrenergic system was shown to play an important role in the action of MG in the hot-plate test (5). Whereas MOR antinociception in the tail-pinch test was mediated by the descending noradrenergic system (5), the antinociceptive action of MOR in the hot-plate test was mediated by both descending noradrenergic and serotonergic systems in mice (6). Based on these findings, it could be expected that MG and MOR may interact with different opioid receptor subtypes which modulate the function of descending noradrenergic and serotonergic systems.

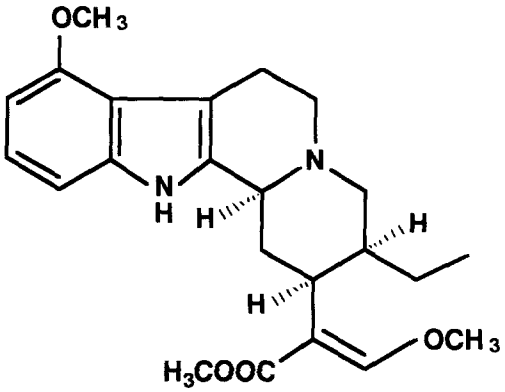


Fig. 1
The chemical structure of mitragynine, a major alkaloidal component extracted from the leaves of *Mitragyna speciosa* Korth.

Four opioid receptor subtypes, μ-, δ-, κ- and ε-, have been found in the central nervous system (CNS). Antinociception is known to be mainly mediated by the stimulation of the μ-, δ- and κ- receptors (7, 8). A previous report (9) implicated a μ1-opioid receptor subtype in supraspinal analgesia in a mouse spinal transection model. In the present study, to further clarify the opioid receptor subtypes involved in the antinociceptive action of supraspinally-administered MG, we investigated the effects of the μ-opioid antagonist cyprodime (10), the irreversible μ1-opioid antagonist naloxonazine (11), the δ-opioid antagonist naltrindole (12) and the selective κ-opioid antagonist nor-binaltorphimine (Nor-BNI) (13, 14) on the antinociceptive action of MG in the tail-pinch and hot-plate tests in mice.

## Methods

### *Animals*

Male ddY mice (Japan SLC, Shizoka, Japan) were obtained at the age of 4 weeks. The animals were housed in groups of 15 per cage (35 x 30 x 16 cm), on a 12 hr light/dark cycle (lights on: 07:30-19:30) at 25 ± 1 °C for at least 1 week before the experiments. Food and water were given ad libitum. Each animal was used only once in the experiments. These studies were conducted in accordance with the standard established by the Guide for the Care and Use of Laboratory Animals, of Toyama Medical and Pharmaceutical University.

***Measurement of nociception in the tail-pinch and hot-plate tests***

***1) Tail-pinch test***

The nociceptive response in the tail-pinch test was measured according to the modified Haffner's method as previously reported (4, 5, 15). Briefly, mice were pretested by pinching their tails with hemostatic forceps (3 mm width, 500 g constant pressure), and only the mice that showed a nociceptive response such as biting the forceps within 2 sec were used for the experiments. The latency of nociceptive responses in these animals was expressed as the tail-pinch latency. To minimize tissue damage, a cut-off time of 6 sec was selected.

***2) Hot-plate test***

In the hot-plate test, an a nimal was placed on a metal plate maintained at 55 ± 0.5 °C. The latency to show nociceptive responses such as hind paw licking, hind paw flicking, or jumping was measured according to the method of Eddy and Leimbach (16) and Hunskaar et al. (17), and expressed as the hot-plate latency. Only the mice that showed the nociceptive response within 18 sec were used for the experiments. To minimize tissue damage, a cut-off time of 45 sec was selected.

***3) Determination of the supraspinal opioid receptor subtypes involved in i.c.v. mitragynine-induced antinociception***

To determine the roles of the supraspinal μ- and δ-opioid receptors on the MG-induced antinociception, MG (10 μg, i.c.v.) was coinjected with the selective μ-opioid antagonist cyprodime (1-10 μg, i.c.v.) or the selective δ-opioid antagonist naltrindole (1-5 ng, i.c.v.) in a total volume of 3.0 μl. MOR (3 μg i.c.v.), a reference opioid agonist, was also coadministered with cyprodime (1-10 μg, i.c.v.) or naltrindole (1-5 ng, i.c.v.). To test the possibility that the supraspinal κ- and μ1-receptors are involved in the antinociception caused by MG and MOR, a selective κ-opioid antagonist, nor-binaltorphimine (Nor-BNI; 0.2-5 μg, i.c.v.) and an irreversible μ1-opioid antagonist, naloxonazine (1-3 μg, i.c.v.) were administered 30 min and 24 h, respectively, before the MG or MOR treatment. U50,488H (50 μg) was also used as a reference κ-opioid receptor agonist, and was injected i.c.v. 30 min after the Nor-BNI treatment. MG, MOR, U50,488H or vehicle was injected i.c.v. 15 min before the start of the experiments. The doses of these antagonists and time-courses of opioid antagonist administration were chosen according to the previous reports (18-20) and our preliminary study. Our previous report (4) and preliminary study showed that the effects of MG, MOR and U50,488H administered i.c.v. in the tail-pinch and hot-plate tests were maximal at about 15 min after administration. In addition, MG (1-10 μg, i.c.v.) produced a dose-dependent antinociceptive activity in the tail-pinch and hot-plate tests and the antinociceptive response of 10 μg (i.c.v.) MG was almost the same as that of 3 μg (i.c.v.) MOR in both tests (4). Thus, in the present study we compared the nociceptive responses among different groups at 15 min after i.c.v. administration of 10 μg MG, 3 μg MOR, or 50 μg U50,488H.

***Drugs***

MG (molecular weight: 398.5) was purified from the alkaloidal fraction extracted from the young leaves of *Mitragyna speciosa* Korth. as previously described and the purity (>99%) of MG was confirmed by $^1$H-NMR analysis (500 MHz, $CDCl_3$) (1). The following drugs were used: cyprodime HBr and nor-binaltorphimine 2HCl (Research Biochemicals Inc., Natick, MA, USA), U50,488H and naltrindole HCl (Sigma Chemical Co., St. Louis, MO, USA), naloxonazine 2HCl (Wako Pure Chemicals, Osaka, Japan) and morphine HCl (Dainippon Pharmaceutical Co., Tokyo, Japan).

MG was dissolved in 1% acetic acid, and the pH was adjusted up to 4.7 with 1N-NaOH. Cyprodime and naloxonazine were each dissolved in 0.2% acetic acid. The other test drugs were

dissolved in saline. All drug solutions were freshly prepared just before the start of the experiments. The i.c.v. injection was performed in a total volume of 3 µl according to the methods of Haley and McCormick (21).

### *Statistical analysis*

The effects of the drugs on the nociceptive response were analyzed by the Kruskal-Wallis analysis of variance followed by the Mann-Whitney U-test for multiple comparisons. Differences of $P<0.05$ were considered statistically significant.

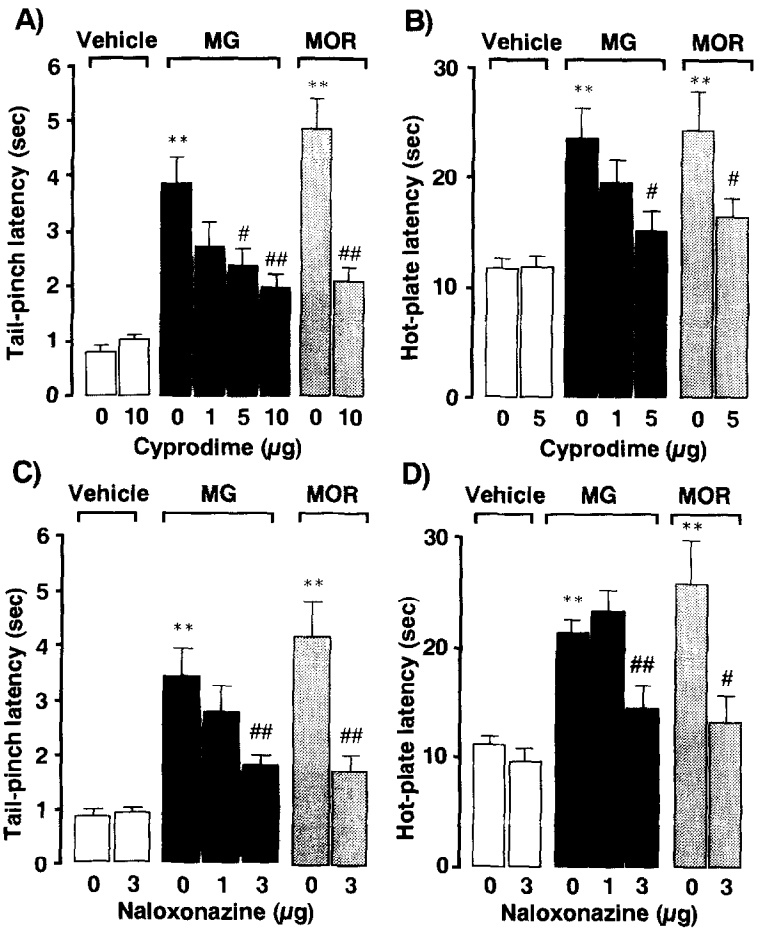


Fig. 2

Effects of the µ-opioid antagonist cyprodime (A, B) and the µ1-opioid antagonist naloxonazine (C, D) on i.c.v.-administered mitragynine- and morphine-induced antinociception in the tail-pinch and hot-plate tests in mice. In A and B: Mitragynine (MG: 10 µg, i.c.v.), morphine (MOR: 3 µg, i.c.v.) or vehicle was coadministered with cyprodime (1-10 µg, i.c.v.). In C and D: Naloxonazine (1-3 µg) was i.c.v. injected 24 hr before the start of the experiments. Fifteen min after the injections of MG and MOR, the latency of the nociceptive response in the tail-pinch (A, C) and hot-plate (B, D) test was measured. Each data column represents the mean ± S.E.M. of 7-9 mice. **P< 0.01 compared with the animals treated with vehicle alone. #P<0.05 and ##P<0.01 compared with the group treated with MG or MOR alone (Mann-Whitney U-test).

## Results

### *Effects of μ- and μ1-opioid antagonists on MG antinociception*

As shown in Fig. 2, centrally-administered MOR (3 μg i.c.v.) significantly prolonged the nociceptive latencies in the tail-pinch and hot-plate tests in the mice. The i.c.v. injection of MG (10 μg) also significantly prolonged the nociceptive latencies in the tail-pinch and hot-plate tests. The antinociceptive actions of MG (10 μg, i.c.v.), as well as those of MOR (3 μg, i.c.v.) in the tail-pinch and hot-plate tests were dose-dependently attenuated by the coadministration of cyprodime (1-10 μg, i.c.v.), a μ-opioid antagonist and by pretreatment with i.c.v. naloxonazine (1-3 μg), an irreversible μ1-opioid antagonist.

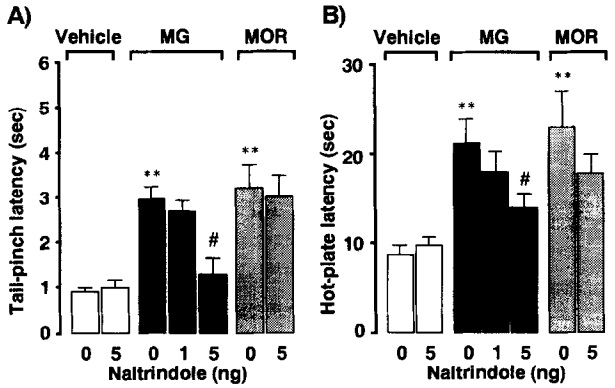


Fig. 3

Effects of the δ-opioid antagonist naltrindole on i.c.v.-administered mitragynine- and morphine-induced antinociception in the tail-pinch (A) and hot-plate (B) tests in mice. Mitragynine (MG: 10 μg, i.c.v.) or morphine (MOR: 3 μg, i.c.v.) or vehicle was coadministered with naltrindole (1-5 ng). After 15 min, the latency of the nociceptive responses was measured. Each data column represents the mean ± S.E.M. of 7-9 mice. **P< 0.01 compared with the animals administered vehicle alone. #P<0.05 compared with MG or MOR alone (Mann-Whitney U-test).

### *Effects of the δ-opioid antagonist naltrindole and the selective κ-opioid antagonist nor-binaltorphimine on MG antinociception*

The MOR (3 μg, i.c.v.)-induced antinociception in the tail-pinch and hot-plate tests was not significantly affected by 5 ng (i.c.v.) naltrindole, a δ-opioid antagonist (Fig. 3), whereas the MG (10 μg, i.c.v.) antinociception in both tests was significantly attenuated by this δ-opioid antagonist in a dose-dependent manner (1-5 ng, i.c.v.). When mice were pretreated with nor-binaltorphimine (1 μg, i.c.v.), a selective κ-opioid antagonist, the κ-opioid receptor agonist U50,488H-induced antinociception in the tail-pinch and hot-plate tests was almost completely abolished (Fig. 4). At the same dose (1 μg, i.c.v.), nor-binaltorphimine significantly attenuated the antinociceptive activity of MG (10 μg, i.c.v.) in the tail-pinch test but it, even at a high dose (5 μg, i.c.v.), failed to block the MG-induced antinociception in the hot-plate test. In contrast, this antagonist had no effect on the antinociceptive effects of MOR (3 μg, i.c.v.) in the tail-pinch test or hot-plate test (Fig. 4).

## Discussion

Our previous study demonstrated that the supraspinal opioid receptors are involved in the antinociceptive action of MG on the mechanical and thermal noxious stimuli-induced nociceptive

responses in mice (4). The purpose of the present study was to clarify the opioid receptor subtypes implicated in the antinociceptive activity of MG in mice. The results of this study suggest that MG antinociception is predominantly mediated by the supraspinal μ- and δ-opioid receptor subtypes.

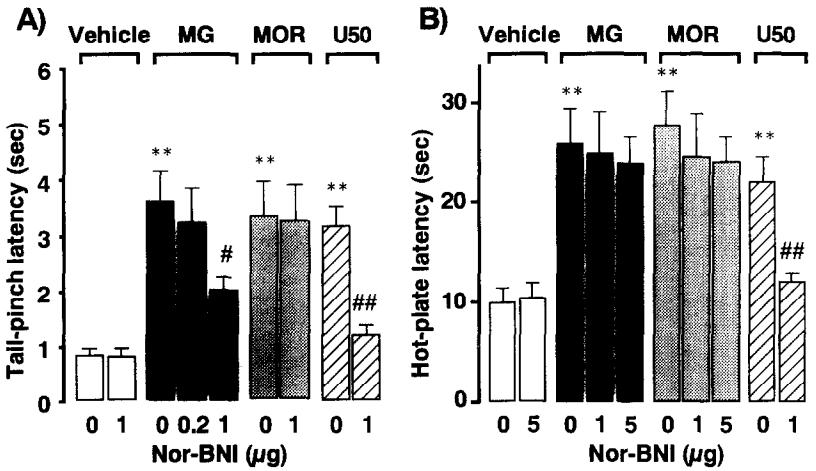


Fig. 4

Effects of the κ-opioid antagonist nor-binaltorphimine on i.c.v.-administered mitragynine-, morphine- and U50,488H-induced antinociception in the tail-pinch (A) and hot-plate (B) tests in mice. Nor-binaltorphimine (Nor-BNI: 0.2-5 μg, i.c.v.) was administered 30 min before mitragynine (MG: 10 μg, i.c.v.), morphine (MOR: 3 μg, i.c.v.) or U50,488H (U50: 50 μg, i.c.v.). After 15 min, the latency of the nociceptive responses was measured. Each data column represents the mean ± S.E.M. of 7-9 mice. **P< 0.01 compared with the animals treated with vehicle alone. #P<0.05 and ##P<0.01 compared with MG, MOR or U50 alone (Mann-Whitney U-test).

When administered i.c.v., the μ-opioid antagonist cyprodime attenuated the antinociceptive effect of the i.c.v. injection of MG, as well as that of i.c.v. MOR. The involvement of μ-opioid receptors in the action of MOR is consistent with previous findings. Roerig et al. (22) and Sora et al. (23) showed that MOR exerted an antinociceptive activity against thermal stimulation through supraspinal and spinal μ-opioid receptors in mice. Considering evidence that μ-opioid receptors are involved in the transmission of nociceptive information caused by not only thermal stimulation but also mechanical noxious stimulation (24), the present results suggest that supraspinal μ-opioid receptors play important roles in the antinociception effects of MG and MOR on the responses of the mice to both mechanical and thermal noxious stimuli. In addition, it should be noted that the i.c.v. MG-induced antinociception, as well as the i.c.v. MOR-induced antinociception, was antagonized by i.c.v. naloxonazine, a selective μ1-opioid antagonist, in the tail-pinch and hot-plate tests. Autoradiographic studies of mice have demonstrated that μ-opioid receptors located in the frontal cortex, periaqueductal gray matter, and laminae I and II of the spinal cord, regions which are known to play major roles in antinociception, are dominantly μ1- and not μ2-subtype (25, 26). Moreover, it has been proposed that supraspinal opioid-induced analgesia is mediated primarily by a μ1-opioid receptor subtype (27-29), although there is a conflicting report (30). Taken together, it is likely that the i.c.v.-injected MG exerts antinociceptive activity against mechanical and thermal noxious stimuli via a supraspinal μ1-opioid receptor subtype.

A δ-opioid receptor subtype is located in various regions of the CNS in rodents (25, 31). In the present study, the coadministration of i.c.v. naltrindole, a δ-opioid antagonist, blocked the antinociceptive actions of i.c.v. MG but not i.c.v. MOR in the tail-pinch and hot-plate tests, suggesting that MG is also capable of stimulating the supraspinal δ-opioid receptor subtype. The involvement of the δ-opioid receptor subtype in the pharmacological activity of MG antinociception is consistent with the results of a recent in vitro study in this laboratory (32). In the in vitro study, we found that MG inhibited cAMP production stimulated by forskolin in NG108-15 cells via a δ-opioid receptor subtype. In addition, it was found that MG exhibited 30-fold less potent inhibitory activity than MOR in terms of the in vitro cAMP formation. The latter finding disagrees with the present finding that naltrindole failed to block the antinociceptive action of MOR. The reason for this discrepancy between the present and previous findings on the role of δ-opioid receptors in the action of MOR remains unclear, but it may be due to the difference in the type of the opioid receptor subtypes involved in the *in vivo* and *in vitro* pharmacological activity of MOR. In contrast to NG108-15 cells, which express only the δ-type of opioid receptors, multiple opioid receptor subtypes with different distribution patterns have been found throughout the CNS of animals (33, 34).

The selective κ-opioid antagonist Nor-BNI antagonized the antinociceptive effects in the tail-pinch and hot-plate tests of i.c.v. U50,588H, a selective κ-opioid receptor agonist, whereas the i.c.v. MG-induced antinociception in the tail-pinch test but not in the hot-plate test was antagonized by Nor-BNI. The finding that MG exhibited different sensitivity to the κ-opioid antagonist depending on the type of noxious stimulation suggests that the antinociceptive action of i.c.v. MG on the mechanical noxious stimulation is not due to direct stimulation of κ-opioid receptors by MG. It has been reported that the antinociception mediated by κ-opioid receptor stimulation depends on a descending serotonergic system in mice (35, 36). Moreover, U50,488H appears to enhance the release of $^{3}$H-5-HT from mouse brain slices (37). In our previous study, both descending noradrenergic and serotonergic systems were involved in the antinociceptive action of i.c.v. MG on the mechanical noxious stimulation and the effect of i.c.v. MG was attenuated by intrathecal treatments with the α2-adrenoceptor antagonist idazoxan and the 5-HT receptor antagonist cyproheptadine. On the other hand, the descending noradrenergic system was the predominant contributor to the effect of supraspinal MG on the nociceptive response caused by thermal noxious stimulation (5). Thus, it would be expected that i.c.v. MG may indirectly interact with supraspinal κ-opioid receptors which are involved in modulation of the descending serotonergic system.

In conclusion, the present results suggest that MG is capable of directly interacting with supraspinal μ- or δ- opioid receptor subtypes, and exhibits a suppressive action on the mechanical and thermal stimuli-induced nociceptive responses, and that the selectivity of MG for the supraspinal and spinal opioid receptor subtypes differs from that of MOR in mice.

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