Chronopharmacology of Analgesic Effect and Its Tolerance Induced by Morphine in Mice

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ABSTRACT
The influence of morphine dosing time on analgesic effect after acute or chronic treatment, recovery of analgesic effect after once developed tolerance, and their pharmacological mechanisms were investigated in ICR male mice under a 12-h light/dark cycle (light on from 7:00 AM to 7:00 PM). There was a significant 24-h rhythm in the latency of thermal response at 30 min after morphine injection. The analgesic effect was significantly greater at the dark phase than at the light phase. The rhythmic pattern resembled overall the rhythm occurring in the latency of thermal response under nondrugged state. The absolute value of morphine analgesic effect (the real time spent on the hot-plate) on days 1 and 2 after morphine daily injection was significantly larger after morphine injection at 9:00 PM than after saline injection at 9:00 PM or after morphine injection at 9:00 AM. The recovery from tolerance of analgesic effect was significantly faster at the dark phase than at the light phase. The time-dependent difference in the analgesic effect after chronic treatment or recovery from tolerance is closely related to that in the expression of μ-opioid receptor. The present study suggests that 24-h rhythm of morphine analgesic effect is consistent with 24-h rhythm of μ-opioid receptor expression.

A large number of physiological rhythms are controlled by the central nervous system, hormone secretion, and so on (Thomson et al., 1980; Naber et al., 1981). Also, many drugs vary in potency and/or toxicity according to the time in the 24-h cycle when they have been administered (Walker and Owasoyo, 1974; Ohdo et al., 1988, 1991, 1996, 1997, 1998, 2001; Koyanagi and Ohdo, 2002).

Biological rhythms in pain sensitivity have been studied in mice (Frederickson et al., 1977). Not only in rodent but also in human, biological rhythms in pain sensitivity have been studied with respect to diseases and drug action (Davis et al., 1978; Labrecque and Vanier, 1995).

Morphine exerts a broad range of other pharmacological effects in addition to its potent analgesic properties. In human, there is reduction in gastrointestinal motility, sedation, inhibition of the micturition reflex, and miosis (Clifford et al., 1982). In rodent, some of them are the same reaction, but some are different from human. Mydriasis is the opposite reaction from human (Klemfuss et al., 1979).

Pharmacological tolerance to the analgesic effect of morphine is readily produced after chronic administration of morphine particularly in experimental animals. In humans, tolerance develops very slowly with clinical pain. In dealing with tolerance, there are both associative (learned) and non-associative components. Although the factors contributing to the development of pharmacological tolerance have been the subject of many studies over past few decades, definitive answers remain unclear due to the complex nature of the problem. And chronopharmacological research of development of tolerance to the analgesic effect induced by morphine has not been done.

In the present study, we investigated the influence of morphine dosing time on the analgesic effect after acute and chronic treatment and recovery of analgesic effect from tolerance in mice. The mechanism underlying this phenomenon was investigated in terms of chronopharmacodynamics, the rhythmicity of μ-opioid receptor function.

Materials and Methods

Animals and Treatments. Male ICR mice (weighing 25–35 g, about 4 weeks old) were purchased from Charles River Japan Inc. (Kanagawa, Japan). They were housed 5 to 10 per cage in a light-
controlled room (lights on from 7:00 AM to 7:00 PM) at a room temperature of 24 ± 1°C and a humidity of 60 ± 10% with food and water ad libitum. In this case, the cage size was changed to be the same density per mouse. All mice were adapted to their light/dark cycle for 10 days before the experiments. During periods referred to as darkness, dim red light was used to aid treatment of mice. First, groups of 10 mice each were given a single i.p. dose of morphine hydrochloride (Sankyo, Tokyo, Japan) at 15 mg/kg, 30 mg/kg, or saline at the same 24-h phase (9:00 AM) for dose finding. At 30 min after morphine injection, the latency time was significantly longer in mice given morphine (15 mg/kg) than in mice given saline. Moreover, the latency time in mice given morphine (15 mg/kg) had smaller interindividual difference than that in the mice given morphine (30 mg/kg). The dose of morphine hydrochloride at 15 mg/kg was accepted as the proper dose in our experiment.

In the study observing the 24-h rhythm in morphine analgesic effect, groups of 10 mice each were injected a single i.p. dose of morphine (15 mg/kg) or saline at one of six time points: 9:00 AM, 1:00 PM, 5:00 PM, 9:00 PM, 1:00 AM, or 5:00 AM. The brainstem for μ-opioid receptor binding assay was prepared from groups of six mice each. The brain of intact mice was excised quickly, cerebral cortex and cerebellum were removed, and the brainstem was isolated on the ice-cold petri-dish using the brain atlas of Franklin and Paxinos (1997) at 9:00 AM or 9:00 PM.

To observe influence of dosing time on analgesic effect during morphine daily injection, the analgesic effect in groups of 10 mice each was determined every day during morphine (15 mg/kg i.p.) or saline daily injection at 9:00 AM or 9:00 PM for 5 days.

The brainstem for μ-opioid receptor binding assay was prepared from groups of five mice each before morphine or saline injection on day 1 to 5 during morphine (15 mg/kg i.p.) or saline daily injection at 9:00 AM or 9:00 PM for 5 days. Namely, the brainstem for the receptor-binding assay both at 9:00 AM and 9:00 PM on day 1 was prepared from nontreated mice, and the brainstem on day 2 was prepared from the mice before morphine injection on day 2 after morphine injection at 9:00 AM or 9:00 PM on day 1. The brainstem on days 3 to 5 was prepared by a similar schedule to that of the brainstem on day 2.

To observe the recovery from tolerance of analgesic effect (Fig. 1, time schedule for the experiment on daily injection of morphine at 9:00 AM or 9:00 PM for 5 days and then 2 days washout period), the analgesic effect in groups of five mice each was determined after a single injection of morphine (15 mg/kg i.p.) or saline at 3:00 PM on the following day with 2 days washout period after morphine (15 mg/kg i.p.) or saline daily injection at 9:00 AM or 9:00 PM for 5 days.

**Determination of Analgesic Effect.** A thermal technique (hot-plate analgesia meter MK-350; Muromachi Kikai Co., Ltd., Tokyo, Japan) was used to evaluate analgesic effect after the injection of morphine or saline (Kavaliers and Hirst, 1983). The surface of plate was maintained at a temperature of 55 ± 0.5°C. Morphine analgesic effect was determined at 30 min after morphine injection. Time (in seconds) to either hind paw licking or jumping was recorded as pain response latency. To avoid heat injury, mice not responding 120 s were removed from the hot-plate in the present study. Latency of those mice was as 120 s. Mice were used only once, not repetitively. There were three reasons why we selected 120 s as cut-off latency. First, the time of 120 s was referred to in a previous article (Bansinath et al., 1990). Second, there was no behavioral and pathological change after hot-plate test for 120 s. Third, the time length of 120 s as cut-off latency was needed to observe the dosing time-dependent difference of latency to the hot-plate, because both latencies in the saline and the morphine group were remarkably longer during the dark phase than the light phase. Also, to avoid the likelihood of habituation or tolerance of mice to the hot-plate, all mice were exposed to the hot-plate only once throughout the experiment. Namely, mice were exposed to hot-plate only once either before or after injection of morphine. For example, latency on day 1 to observe influence of dosing time on analgesic effect during morphine daily injection was determined only once at 30 min after first injection of morphine.

**Specific μ-Opioid Receptor Binding Assay.** Brainstem was homogenized in 1 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4). Homogenate was then centrifuged at 15,000 rpm for 15 min at 4°C. The obtained pellet was resuspended in 1 ml of Tris-HCl buffer (pH 7.4) and incubated at 37°C for 15 min. Then, homogenate was centrifuged again. The pellet was resuspended in 3 ml of Tris-HCl buffer. The protein concentration was approximately 2 mg/ml using Lowry’s method (DC protein assay; Bio-Rad, Hercules, CA). The binding assay was performed with a reaction mixture (total volume, 200 μl) containing 100 μl aliquot of brainstem homogenate, 0.156 to 5 nM [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin ([3H]DAMGO; Amsterdam Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). Non-specific binding was determined in the presence of 10 nM naloxone (Sigma-Aldrich, St. Louis, MO). After incubation at 37°C for 30 min, the reaction mixture together with 100 μl of Tris-HCl buffer was laid over the 300 μl of ice-cold fetal bovine serum, and centrifuged at 10,000 rpm for 1 min at 4°C. The supernatant was removed and then the pellet transferred to scintillation vials with 10 ml of scintillation cocktail and counted by a liquid scintillation counter (LSC-1000; Aloka Co., Tokyo, Japan) after keeping 6 h. Specific binding is the difference between binding determined in the presence of ligand and in the presence of ligand and calculated as follows: specific binding (femtomoles per milligram of protein) = [total binding (femtomoles per milligram of protein)] – [non-specific binding (femtomoles per milligram of protein)]. The data were plotted according to the method of Scatchard (1949). The number of μ-opioid receptor and the dissociation constant (Kd) were calculated.

**Statistical Analysis.** Analysis of variance was used for the comparison among six different dosing times, and Bonferroni’s test was

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**Figure 1.** Time-schedule for the experiment on daily injection of morphine at 9:00 AM or 9:00 PM for 5 days and then 2-day washout period.
applied for the multiple comparison. Student's t test was used for two independent groups, namely, for comparison of binding assay between saline group at 9:00 AM and at 9:00 PM and for comparison of binding assay between morphine group and saline group. The 5% level of probability was considered significant.

Results

Twenty-Four-Hour Rhythm of Analgesic Effect. The mice given saline showed a significant 24-h rhythm in the latency of their response to the thermal stimulus, spending significantly longer latency time during the dark phase than during the light phase \([F(5,54) = 6.365, P < 0.01; \text{Fig. 2}]\). The mice given morphine (15 mg/kg i.p.) also showed a significant 24-h rhythm in the time spent on the hot-plate with the shortest latency at early light phase and the longest latency at early dark phase \([F(5,54) = 3.537, P < 0.01]\). At the dark phase, the time spent on the hot-plate after morphine injection was significantly longer compared with that after saline injection \((P < 0.01)\). The rhythmic pattern of analgesia induced by morphine resembled overall the rhythm occurring after saline injection.

Time Dependence of \(\mu\)-Opioid Receptor Function. The specific binding data were analyzed by the method of Scatchard. \(B_{\text{max}}\) for \([3H]\)DAMGO calculated from the intercept of the Scatchard plot on the abscissa was significantly larger in brainstem prepared at 9:00 PM than at 9:00 AM \((9:00\text{ AM}, 8.26 \pm 0.26 \text{ fmol/mg protein} (n = 6, \text{mean } \pm \text{S.E.}); 9:00\text{ PM}, 10.22 \pm 0.21 \text{ fmol/mg protein}, P < 0.01)\). The apparent \(K_d\) value did not differ significantly between brainstems prepared at 9:00 AM and 9:00 PM \((9:00\text{ AM}, 1.35 \pm 0.10 \text{ nM}; 9:00\text{ PM}, 1.55 \pm 0.12 \text{ nM})\).

Influence of Dosing Time on Analgesic Effect during Morphine Daily Injection. Fig. 3 shows the time spent on the hot-plate during morphine (15 mg/kg i.p.) daily injection at 9:00 AM or 9:00 PM for 5 days. On day 1, the time spent on the hot-plate was significantly longer after morphine daily injection at 9:00 AM than saline daily injection at 9:00 AM \((P < 0.05)\). On days 1 and 2, the time spent on the hot-plate was significantly longer after morphine daily injection at 9:00 PM than saline daily injection at 9:00 PM \((P < 0.05)\). On days 1 and 2, the time spent on the hot-plate was significantly longer after morphine daily injection at 9:00 PM than at 9:00 AM \((P < 0.01)\).

Influence of Dosing Time on \(\mu\)-Opioid Receptor Function during Morphine Daily Injection. Fig. 4 shows the \(\mu\)-opioid receptor function during morphine (15 mg/kg daily injection for 5 days. Each value is the mean with S.E.M of five mice. \(*, P < 0.05; **, P < 0.01\) compared between the two dosing times using Bonferroni's test.
i.p.) daily injection at 9:00 AM or 9:00 PM for 5 days. On days 1 and 2, the specific binding of [3H]DAMGO in brainstem was significantly larger after morphine daily injection at 9:00 PM than at 9:00 AM (day 1, \( P < 0.05 \); day 2, \( P < 0.01 \)). On days 3 to 5, the specific binding of [3H]DAMGO did not differ significantly between brainstem prepared after morphine daily injection at 9:00 AM and 9:00 PM. The specific binding data in brainstem after morphine or saline daily injection at 9:00 AM for 5 days were analyzed by the method of Scatchard. The \( B_{\text{max}} \) value for [3H]DAMGO was significantly smaller after morphine daily injection at 9:00 AM than saline daily injection at 9:00 AM (saline, 8.26 ± 0.26 fmol/mg protein; morphine, 4.46 ± 0.33 fmol/mg protein, \( P < 0.01 \)). The apparent \( K_d \) value did not differ significantly between brainstem prepared after morphine daily injection at 9:00 AM and after saline daily injection at 9:00 AM (saline, 1.35 ± 0.10 nM; morphine, 1.19 ± 0.09 nM).

**Influence of Dosing Time on Recovery of Analgesic Effect from Tolerance.** Fig. 5 shows the time spent on the hot-plate after a single injection of morphine (15 mg/kg i.p.) or saline at 3:00 PM on the following day with 2-day washout period after morphine (15 mg/kg, i.p.) or saline daily injection at 9:00 AM or at 9:00 PM for 5 days (Fig. 1). There was no significant difference between the time spent on the hot-plate in mice after saline injection at 3:00 PM on the following day with 2-day washout period after saline daily injection at 9:00 AM for 5 days and after saline daily injection at 9:00 PM for 5 days (data not shown). Also, there was no significant difference between the time spent on the hot-plate in mice after a single injection of morphine at 3:00 PM on the following day with 2-day washout period after saline daily injection at 9:00 AM for 5 days and saline daily injection at 9:00 PM (data not shown). Therefore, we combined the values between both saline groups injected at 9:00 AM and 9:00 PM.

The time spent on the hot-plate in mice after a single injection of morphine at 3:00 PM on the following day with 2-day washout period after morphine daily injection at 9:00 AM for 5 days was not significantly different from that in mice after a single injection of saline at 3:00 PM on the following day with 2-day washout period after saline daily injection for 5 days. Namely, recovery from tolerance of analgesic effect was not observed in mice after a single injection of morphine at 3:00 PM on the following day with 2-day washout period after morphine daily injection at 9:00 AM for 5 days.

The time spent on the hot-plate in mice after a single injection of morphine at 3:00 PM on the following day with 2-day washout period after morphine daily injection at 9:00 PM for 5 days was not significantly different from that in mice after a single injection of morphine at 3:00 PM on the following day with 2-day washout period after saline daily injection for 5 days, but was significantly shorter than that in mice after a single injection of morphine at 3:00 PM on the following day with 2-day washout period after saline daily injection for 5 days (\( P < 0.01 \); Fig. 5). Namely, almost complete recovery from tolerance of analgesic effect was shown in mice after a single injection of morphine at 3:00 PM on the following day with 2-day washout period after morphine daily injection at 9:00 PM for 5 days.

**Influence of Dosing Time on Recovery of \( \mu \)-Opioid Receptor Function from Tolerance.** Fig. 6 shows the specific binding of [3H]DAMGO in brainstem prepared before a single injection of morphine (15 mg/kg i.p.) or saline at 3:00 PM on the following day with 2-day washout period after.
morphine (15 mg/kg i.p.) daily injection at 9:00 AM or at 9:00 PM for 5 days, or saline daily injection for 5 days, respectively. There was no significant difference between the specific binding of \(^{[3]H}\)DAMGO in brainstem prepared at 3:00 PM on the following day with 2-day washout period after saline daily injection at 9:00 AM for 5 days and saline daily injection at 9:00 PM for 5 days (data not shown). Therefore, we combined the values between both saline groups injected at 9:00 AM and 9:00 PM. Similar results should be observed between saline (first column) and single injection (before injection) (second column) in Fig. 6. However, we set the different group based on the experimental design of Fig. 5. The specific binding of \(^{[3]H}\)DAMGO was significantly smaller in brainstem prepared before a single injection of morphine or saline at 3:00 PM on the following day with 2-day washout period after morphine daily injection at 9:00 AM for 5 days than after saline daily injection for 5 days \( (P < 0.01, \) respectively; Fig. 6). On the other hand, the specific binding of \(^{[3]H}\)DAMGO showed no significant difference between brainstems prepared before a single injection of morphine or saline at 3:00 PM on the following day with 2-day washout period after saline daily injection at 9:00 PM for 5 days and saline daily injection for 5 days.

**Discussion**

In the present study, there was a significant 24-h rhythm in the latency of thermal response after morphine injection with a trough at the light phase and a peak at the dark phase. Especially, at the dark phase, the time spent on the hot-plate after morphine injection was significantly longer compared with nondrugged state. The rhythmic pattern of analgesic effect induced by morphine was similar to that of the sensitivity of mice to painful stimuli in nondrugged state. The result is consistent with the previous findings (Fred-erickson et al., 1977; Kavaliers and Hirst, 1983).

There are 24-h rhythms for locomotor activity and body temperature with a trough at the light phase and a peak at the dark phase (Ohdo et al., 2001). These rhythms are similar to 24-h rhythm in the latency of thermal response after morphine or saline injection. Certainly, hot-plate method may be the test related to locomotor activity, which is also influenced by light/dark cycle (Kavaliers and Hirst, 1983). However, even if using another test, for example, tail-flick test, which can reduce the influence of locomotor activity and body temperature on latency response in nondrugged state, a similar rhythm is observed (Bar-Or and Brown, 1989). Although tail-flick test may be a better method, it may have some limitations such as restricted behavior and stress induced by holding. Regardless, the rhythmic pattern of pain response in both methods is supported by the nocturnal increase in endorphin, enkephalin (Weschel and Frederickson, 1981), and opioid receptor. On the other hand, the latency time after saline injection at 1:00 AM was shorter than the latency after saline injection at other time during the dark phase in the present study. Although the reason is not clear, increase of activity at this time may reduce the latency time.

The potent opioid peptide dynorphin shows a marked nocturnal increase in the rat hypothalamus, whereas the pituitary displays a corresponding nocturnal depression (Przewlocki et al., 1983). Brain distribution of \(^{[3]H}\)DAMGO binding sites has been studied by saturation binding in rat brain (Bhargava and Gulati, 1990). In contrast to the mice in the present study, \(^{[3]H}\)DAMGO binding to membranes of pons and medulla in rat had a much higher \(B_{max}\) value (about 38 fmol/mg protein compared with 8.26 fmol/mg protein) and \(K_d\) value (about 10 nM compared with 1.35 nM). In the present study, the number of \(\mu\)-opioid receptors was significantly larger in brainstem prepared at 9:00 PM than at 9:00 AM. This result is supported by a previous chronopharmacological finding on \(\mu\)-opioid receptor (Naber et al., 1981). Thus, the 24-h rhythms in endogenous opioid activity and \(\mu\)-opioid receptor expression seem to be mainly responsible for the rhythm in the basal pain sensitivity of mice in nondrugged state.

In dealing with tolerance, there are both associative (learned) and nonassociative components. The former fluctuates on a day/night basis and can impact on a variety of behaviors. However, in the present study, we used independent mice for each study and each mouse was used for hot-plate test only once. Therefore, there was no environmental cue. Nonassociative tolerance may have been developed in this case. No significant dosing time-dependent change was observed in the degree of decrease (the difference between drug effect on each of days 2 to 5 and drug effect on day 1 compared with the difference between baseline and drug effect on day 1) on analgesic effect after morphine daily injection. Therefore, the absolute value of morphine analgesic effect (the real time spent on the hot-plate) on days 1 and 2 after morphine daily injection was significantly larger after morphine injection at 9:00 PM than after saline injection at 9:00 AM or after morphine injection at 9:00 AM. Therefore, we suggest that it is possible to keep the useful analgesic effect in the case of dosing at the dark phase compared with dosing at the light phase. The dosing time-dependent change in down-regulation of \(\mu\)-opioid receptor after morphine daily injection was closely related to that in the degree of decrease of analgesic effect. The findings were supported by the previous findings (Bhargava and Gulati, 1990). The down-regulation of brain \(\mu\)-opioid receptor in morphine tolerance is probably mediated by alterations in the rates of receptor synthesis and degradation (Bohm et al., 1997); however, chronic treatment with a variety of opioid receptor agonists in vivo has no influence on the mRNA of opioid receptor in the central nervous system (Brodsky et al., 1995; Buzas et al., 1996; Castelli et al., 1997). Therefore, degradation may have bigger contribution to down-regulation of \(\mu\)-opioid receptor in tolerance.

A significant dosing time dependence was also demonstrated for recovery from tolerance of analgesic effect after morphine daily injection. The measurement time of pain was set to the same circadian phase (3:00 PM) to eliminate the circadian stage-dependent effect of pain. As a result, the different periods of washout on recovery from tolerance of analgesic effect after morphine daily injection were designed between the two dosing times. Namely, the washout period after morphine daily injection at 9:00 AM was 78 h, whereas that after morphine daily injection at 9:00 PM was 66 h. In spite of shorter washout period, the recovery of analgesic effect from tolerance was faster by dosing at dark phase compared with light phase.

In the animal experiment, the pharmacokinetics of morphine and its metabolites change after chronic treatment and depend on dosing schedule (Gregg and Maree, 1995). Also, the induction of P-glycoprotein in brain is reported in morphine-tolerant
rat (Aquilante et al., 2000). The change of morphine and its metabolites concentration in tissue that has high density of µ-opioid receptor, for example, brainstem and spinal cord, may contribute to that of morphine analgesic effect after the chronic treatment of morphine. However, in the human studies, there is a significant dosing time dependence with respect to Cmax and area under the curve of morphine, but there is no correlation between the rhythmity of morphine concentrations and analgesic effects (Glynn and Lloyd, 1976; Sandrini et al., 1986; Strian et al., 1989; Gourlay et al., 1995). The development of tolerance to analgesic effect of morphine is related to not pharmacokinetics of morphine in serum but modification of opioid receptor systems in the central nervous system (Bhargava and Gulati, 1992). The 24-h rhythm in effectiveness of other opioids has been investigated in human (Hummel et al., 1995) as well as animal. Plasma opioid concentration may not, but pain sensitivity may contribute to the 24-h rhythm of effectiveness.

Although pain often lasts throughout a 24-h period and sensitivity to pain shows 24-h rhythm in healthy humans (Bauardulle-Badie et al., 1990) and patients (Vanier et al., 1992), there is no rhythmic marker to predict 24-h rhythm in effectiveness of opioids. Adjustment of the dosage with monitoring the 24-h rhythm of µ-opioid receptor expression may contribute to more effective chronotherapy of morphine used in several administration routes. Even in constant rate infusion, morphine concentration may vary associated with 24-h rhythm of blood flow and metabolism. The drug formulation considered with 24-h rhythm of pharmacokinetics and µ-opioid receptor may be necessary in the future. Our chronopharmacological findings are related to both activity/rest cycle and light/dark cycle. However, there is 24-h rhythm of pain sensitivity even in constant light in mice (Overio et al., 1982). The 24-h rhythm of physiological and behavioral function, including pain sensitivity, can be shifted by feeding schedule in mice (unpublished data). Also, the timing of diet intake shows a synchronizing effect on the 24-h rhythm of cortisol, body temperature, and urinary excretion in humans (Saito et al., 1989; Nishimura et al., 1992). Feeding schedule may be one of indices to establish the dosing schedule of morphine based on 24-h rhythm of µ-opioid receptor.

References

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