Physical Dependence Potential of an Enkephalin Analog, EK-399, in Rats

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Accepted April 19, 1990

Abstract—The physical dependence potential of Tyr-D-Met(O)-Gly-EtPhe-$NHNHCOCH_3$·AcOH (EK-399), a novel enkephalin analog with a potent analgesic effect, was assessed in rats. The animals were given EK-399 (0.008, 0.032, 0.125, or 0.5 mg/kg), morphine (0.125, 0.5, or 2 mg/kg), pethidine (2 or 8 mg/kg), or pentazocine (2 or 8 mg/kg) every hour through an implanted intravenous cannula. After 3 days of treatment, precipitated withdrawal tests were conducted: naloxone (5 mg/kg) was administered subcutaneously. Rats treated with morphine showed withdrawal signs such as hyperirritability, salivation, diarrhea, and weight loss. Rats treated with pethidine, pentazocine, or EK-399 showed similar signs, but they were less evident than those in morphine-treated rats. In abrupt withdrawal tests after 7 days of treatment, rats treated with morphine, pethidine, or pentazocine showed weight loss, whereas rats treated with EK-399 showed little or no weight loss. In substitution tests, EK-399 suppressed the withdrawal signs in morphine-dependent rats, and vice versa. These results show that EK-399 has a morphine-like physical dependence potential that is weaker than that of morphine, pethidine, or pentazocine in rats.

The novel enkephalin analog Tyr-D-Met (O) - Gly - EtPhe - $NHNHCOCH_3$ · AcOH (EK-399) has potent analgesic activity; subcutaneously administered EK-399 was 10 times as potent as morphine in the rat tail flick test (1). The abuse potential of EK-399 has been studied using a self-administration experiment (2), and the results of this experiment showed that EK-399, like the narcotic antagonist levallorphan, has a weak reinforcing effect on drug-taking behavior in rats, suggesting that it possesses a low abuse potential (2).

The self-administration of a morphine-like drug is influenced by physical dependence. In rhesus monkeys, morphine and codeine drug-seeking behavior is intensified by the development of physical dependence on these drugs (3). If the synthetic opioid peptide EK-399 has a physical dependence potential, this may strengthen EK-399 drug-taking behavior. It should therefore be worthwhile to assess the physical dependence potential of EK-399 in order to better predict its abuse potential.

The purpose of the present study was to assess the physical dependence potential of EK-399. Animals can be made drug dependent by chronic administration of morphine-like drugs. Shortening of the intervals between each administration seems to accelerate the development of dependence (4), and physical dependence on drugs with low physical dependency, such as pentazocine and pethidine, can be detected by experiments using an administration schedule with a short interval between each dose (4–6). I attempted to produce physical dependence on EK-399 in rats using such an administration schedule.

Materials and Methods

1. Animals and housing conditions: One hundred male Jcl:Wistar rats (CLEA Japan, Inc.), weighing 135–187 g at the beginning of the experiment, were used. Each animal was subjected to a procedure in which an

indwelling siliconized rubber cannula was implanted under ketamine HCl (100 mg/kg, i.m.) and droperidol (0.8 mg/kg, i.m.) anesthesia. The cannula was implanted in the jugular vein and passed subcutaneously to an exit on the animal's back. A harness was put on the animal 2 or 3 days after surgery to protect the portion of the cannula outside of the body. Each animal was kept in an individual cage (900 $cm^2 \times 75$ cm); a laboratory rat diet (CE-2, CLEA Japan, Inc.) and tap water were continuously available in this cage. Housing and experiments were conducted in a clean cabinet (200×150×180 cm, KCR-4SS, Hitachi, Ltd.) under the following conditions: temperature, 23±3°C; relative humidity, 55±15%; air exchange, 8–12 cycles/hr; lighting, 12-hr light/dark cycle.

2. Infusion of drug: Drug infusion was started one week after surgery. The cannula was joined to a tube from the drug supplying system including a syringe pump (A-99, Razel Sci. Instru.) and a cannula swivel (model 375/22, Instech Co., Ltd.). Drugs were infused as follows: the pump was operated for 1 min every hour by an electric timer (Omron Mini Timer, SYS-C, Omron Tateisi Electronics Co.). In each infusion period, 1 ml/kg of drug solution was infused into the rat. Using this procedure, the rat received drug 24 times a day.

If the interval of repeated administration is shorter than the plasma half-life of a drug, the drug will rapidly accumulate in the plasma. In rats, the terminal plasma half-lives of morphine and pethidine are 97–116 min (7) and 64.5 min (8), respectively, when administered intravenously. Pentazocine disappears from the plasma with a half-life of 1–2 hr in rats after subcutaneous administration (9). The terminal plasma half-life of EK-399 is not known, but it could be estimated to be 24–48 hr from the results of the preliminary pharmacokinetic study in rats (L.F. Chasseaud et al., unpublished data). In the present experiment, the inter-infusion interval (59 min) was shorter than the terminal half-lives of these drugs. Therefore, the plasma level of the drugs might extremely increase after several infusions. However, the dosing schedule is considered to be suitable for assessing the physical dependence of these drugs, because tolerance develops rapidly to the effects of morphine, pethidine, and pentazocine (10), and to those of EK-399 (1).

The rats were divided into groups consisting of 5–15 animals each; and each rat was given 0.008, 0.032, 0.125, or 0.5 mg/kg/hr of EK-399; 0.125, 0.5, or 2 mg/kg/hr of morphine HCl; 2 or 8 mg/kg/hr of pethidine HCl; or 2 or 8 mg/kg/hr of pentazocine. The dose of EK-399, 0.125 mg/kg/hr (3 mg/kg/day), was determined on the basis of the results of a 4-week intraperitoneal toxicity study in rats in which death occurred at 4 mg/kg/day, whereas no adverse effects were detected at 2 mg/kg/day (S. Chiba et al., unpublished data). A higher dose of EK-399, 0.5 mg/kg/hr (12 mg/kg/day), was also tested because the dosing schedule in the present study (once hourly, 24 times a day for 7 days) was different from that in the 4-week toxicity study (once daily for 29 or 30 days). The low dose of EK-399, 0.008 mg/kg/hr (0.192 mg/kg/day), was determined on the basis of the ED50 value (0.16 mg/kg, s.c.) of EK-399 in the tail flick test for the assessment of the analgesic effect (1). The doses of morphine, pethidine, and pentazocine were chosen on the basis of results of other physical dependence producing experiments (5, 6), and analgesic potencies of these opioids: Severe or moderate withdrawal signs were observed in rats after naloxone injection following hourly administration of morphine at 2 mg/kg, pethidine at 8 mg/kg, or pentazocine at 4 mg/kg for 3 days (5, 6). The ED50 values of morphine and pentazocine in the rat tail flick test are 1.7 and 7.2 mg/kg (i.m.), respectively (11). The analgesic effect of pethidine is 0.01–0.25 times as potent as that of morphine in rats (12).

As the control, a group of rats was given 1 ml/kg of physiological saline every hour. Rats were weighed once daily between 1:30 and 2:00 p.m. throughout the experiment.

3. Precipitated withdrawal test: Five to 15 rats treated with each drug were used. After 3 days (72 hr) of treatment, the infusion of the drug was interrupted sometime between 12:30 and 1:00 p.m., and 5 mg/kg of naloxone HCl was given to these animals subcutaneously one hour later (sometime between 1:30 and 2:00 p.m.). Immediately after naloxone

injection, each animal was placed individually in its own cage, and the following withdrawal signs were checked for 2 hr: hyperirritability, teeth chattering, decrease in spontaneous activity, jumping, ptosis, salivation, and diarrhea. The rats were weighed at 30, 60, and 120 min after naloxone injection. Infusion of the drug was started again sometime between 4:00 and 4:30 p.m.

4. **Abrupt withdrawal test:** Four to 7 rats treated with each drug were used. After 7 days of treatment, the infusion of the drug was stopped sometime between 12:30 and 1:00 p.m., and physiological saline was infused once an hour in place of the drug solution. Twenty-four, 48, and 72 hr after drug infusion was stopped, the rats were weighed and withdrawal signs were checked.

5. **Substitution test:** From the tests described above, it was found that physical dependence could be produced by treatment with morphine, 2 mg/kg/hr, or EK-399, 0.125 mg/kg/hr, for 3 days. Therefore, seven rats treated with morphine at 2 mg/kg/hr (morphine-dependent rats) and 10 rats treated with EK-399 at 0.125 mg/kg/hr (EK-399-dependent rats) were used. After 7 days of treatment, EK-399 was substituted for morphine at a dose of 0.032 or 0.125 mg/kg/hr, while morphine was substituted for EK-399 at a dose of 0.5 or 2 mg/kg/hr. Body weight changes and withdrawal signs were checked at 24, 48, and 72 hr after the substitution.

6. **Drugs:** Morphine HCl (Takeda Chemical Industries, Ltd.), EK-399 (synthesized in our Pharmaceutical Production Research Laboratories), and naloxone HCl (Sigma Chemical Co.) were dissolved in physiological saline. Doses of pethidine HCl (Pethidine Hydrochloride Injection, 50 mg/ml; Takeda Chemical Industries, Ltd.) and pentazocine (Peltazon® Injection 30, 30 mg/ml; Grelan Pharmaceutical, Co., Ltd.) were prepared by diluting the solutions for injection in the commercially available ampoules with physiological saline. Doses of pentazocine are expressed in terms of the free base, and those of other drugs are given in terms of the respective salt.

Results

1. **General condition during drug infusion:** The rats treated with 0.5 mg/kg/hr of EK-399 showed catalepsy, stained fur by urine, cyanosis, and weight loss (Fig. 1) for the first 2 or 3 days of the dosing period. Slight cyanosis and stained fur by urine were also observed in rats treated with EK-399 at 0.125 mg/kg/hr. Decreases of body weight gain were noted in rats treated with EK-399 at 0.125 mg/kg/hr, morphine at 2 mg/kg/hr, pethidine at 2 and 8 mg/kg/hr, and pentazocine at 8 mg/kg/hr (Fig. 1).

2. **Precipitated withdrawal test:** Withdrawal signs observed in each group of rats after naloxone injection are summarized in Table 1. Body weight changes in each group are

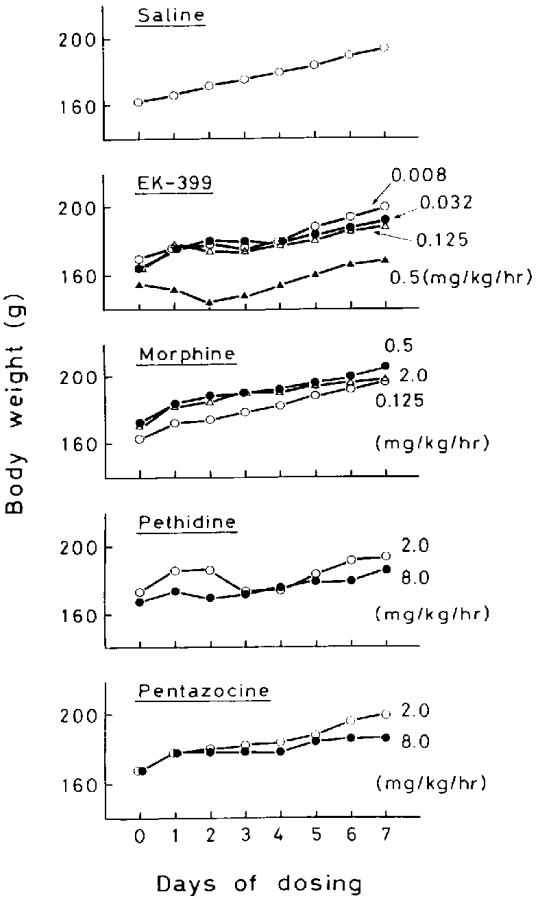

Fig. 1. Body weights of rats treated intravenously with saline, EK-399, morphine, pethidine, or pentazocine. The drugs were given to the rats hourly for 7 days. Each plot represents the mean of 5–15 animals.

Table 1. Incidence of individual withdrawal signs in rats after naloxone (5 mg/kg, s.c.) injection[a]

Drug	Saline	EK-399				Morphine			Pethidine		Pentazocine	
Dose (mg/kg/hr)	1 ml/kg/hr	0.008	0.032	0.125	0.5	0.125	0.5	2.0	2.0	8.0	2.0	8.0
No. of animals	13	6	6	14	6	6	5	15	6	6	5	5
Hyperirritability	+	−	−	+	−	+	++	++	−	−	−	−
Teeth chattering	−	−	−	+	++	−	++	++	+	−	+	+++
Decrease in activity	+	−	+	+++	++	−	+	+	−	−	+	+
Jumping	−	−	−	−	−	−	−	−	+	+	−	−
Ptosis	+	−	−	++	+	−	−	+	+	++	−	+
Salivation	−	−	−	−	−	−	+	+++	++	+++	++	++
Diarrhea	−	−	+	+	−	−	++	+++	++	+	−	−

[a]: +++, sign present in 100% of the animals; ++, sign present in 50–99% of the animals; +, sign present in 1–49% of the animals; −, sign absent. Animals were treated with the drug for 72 hr before naloxone injection.

shown in Fig. 2. In the saline-treated group, slight irritability, a decrease in spontaneous activity, and ptosis were observed in each one of the 13 rats, but most of the animals in the group showed no abnormalities. The mean body weight loss for the 2 hr after naloxone injection was 1.1% of the pre-naloxone level in the control group.

Rats treated with 0.032, 0.125, or 0.5 mg/kg/hr of EK-399 showed a decrease in spontaneous activity, teeth chattering, ptosis, diarrhea, and body weight loss after naloxone injection; the mean percentage of weight loss for the 2 hr after naloxone injection was 3.7% in the 0.032 mg/kg/hr group, 3.3% in the 0.125 mg/kg/hr group, and 2.1% in the 0.5 mg/kg/hr group. The rats treated with 0.008 mg/kg/hr of EK-399 showed no abnormalities, and the weight loss in this group was comparable to that in the control group.

In the groups treated with morphine at 0.5 and 2.0 mg/kg/hr, hyperirritability, teeth chattering, salivation, diarrhea, and body weight loss were noted; and the severity of these withdrawal signs was dose-dependent. The mean percentage of weight loss for the 2 hr after naloxone injection was 4.2% in the 0.5 mg/kg/hr group and 7.7% in the 2.0 mg/kg/hr group. The rats treated with 0.125 mg/kg/hr of morphine were slightly irritable, but the weight loss in this group was comparable to that in the control group.

Rats treated with pethidine showed salivation, teeth chattering, jumping, ptosis, diarrhea, and body weight loss; the mean percentage of weight loss was 4.4% in the 2 mg/kg/hr group and 4.3% in the 8 mg/kg/hr group. Rats treated with pentazocine showed teeth chattering, salivation, and body weight loss; the mean percentage of weight loss was 2.5% in the 2 mg/kg/hr group and 3.7% in the 8 mg/kg/hr group.

3. Abrupt withdrawal test: Body weight changes after the abrupt withdrawal of the drugs are shown in Fig. 3. Rats treated with 0.125 mg/kg/hr of EK-399 showed a slight

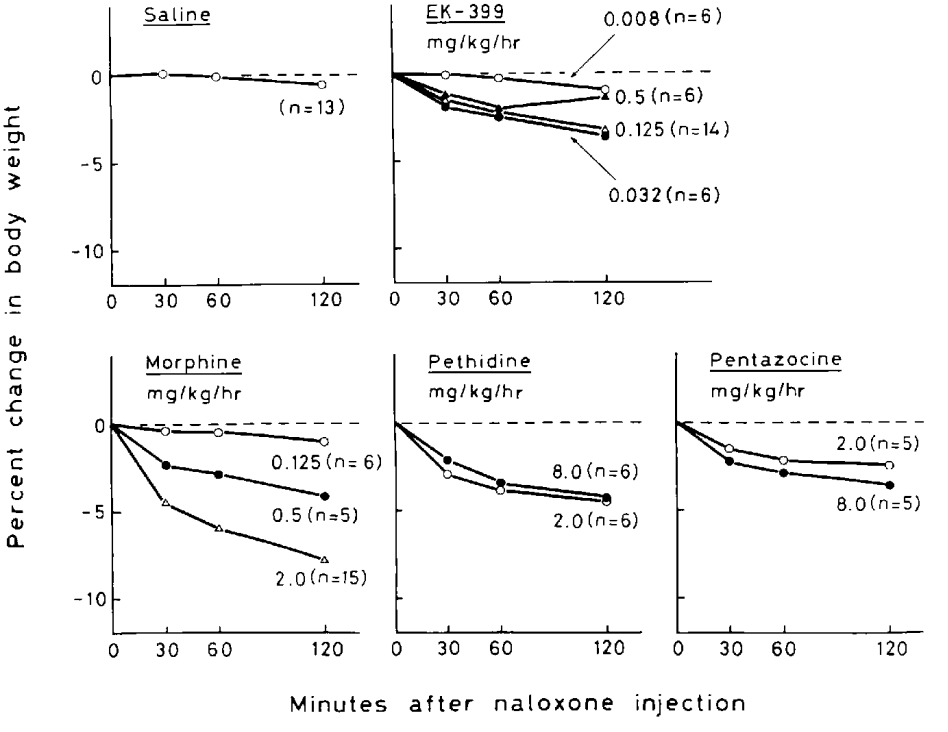

Fig. 2. Body weight changes in rats after naloxone injection. Rats were treated intravenously with saline, EK-399, morphine, pethidine, or pentazocine hourly for 3 days; and on the 4th day, they were given naloxone at 5 mg/kg, s.c.

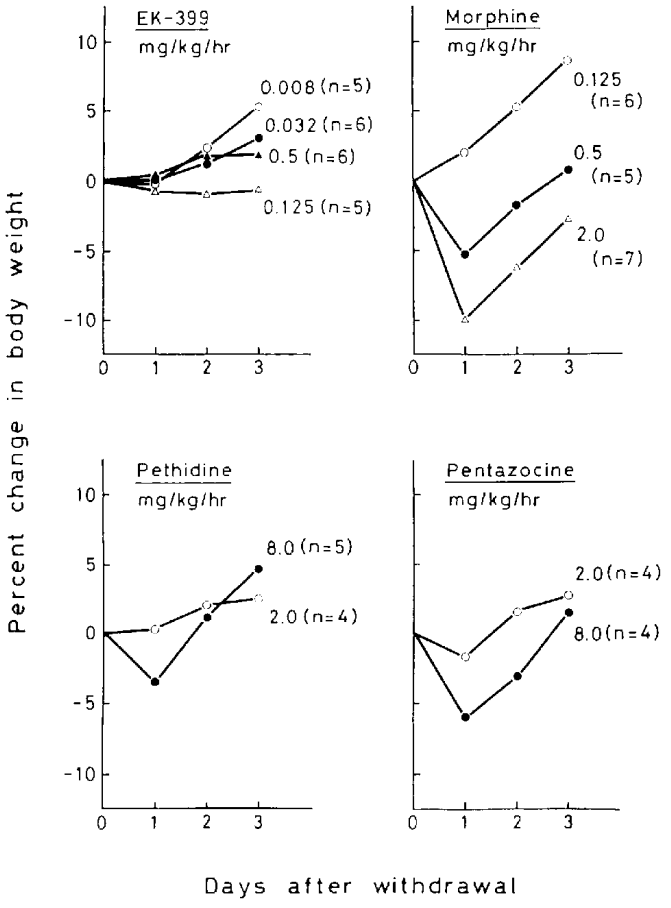

Fig. 3. Body weight changes in rats following the abrupt withdrawal of the drug. Rats were treated intravenously for 7 days with EK-399, morphine, pethidine, or pentazocine; and then the drug was abruptly withdrawn.

weight loss (0.9% of the pre-withdrawal level) for the first 24 hr of the withdrawal period and no weight gain for the next 48 hr. No weight loss was detected in the 0.008, 0.032, or 0.5 mg/kg/hr group. No abnormalities in general condition were seen in the EK-399-treated animals.

The rats in the groups treated with morphine at 0.5 and 2.0 mg/kg/hr showed dose-dependent decreases in body weight; the mean percentage of weight loss for the 24 hr after the withdrawal was 5.2% in the 0.5 mg/kg/hr group and 10.1% in the 2.0 mg/kg/hr group. No weight loss was detected in the 0.125 mg/kg/hr group. Diarrhea was seen in the 2.0 mg/kg/hr group.

In the pethidine-treated groups, rats given 8 mg/kg/hr showed a weight loss of 3.4% for the 24 hr after the withdrawal, whereas rats given 2 mg/kg/hr showed no weight loss. Rats in the groups treated with pentazocine at 2 and 8 mg/kg/hr showed weight loss; the mean percentage of weight loss was 1.8% in the 2 mg/kg/hr group and 5.9% in the 8 mg/kg/hr group. Diarrhea was noted in the 8 mg/kg/hr group.

4. Substitution test: Body weight changes during the substitution period are shown in Fig. 4. The morphine-dependent rats were given 0.032 or 0.125 mg/kg/hr of EK-399 in place of morphine. EK-399 suppressed the body weight loss that normally occurs in rats withdrawn from morphine. The rats showed catalepsy after morphine was replaced with

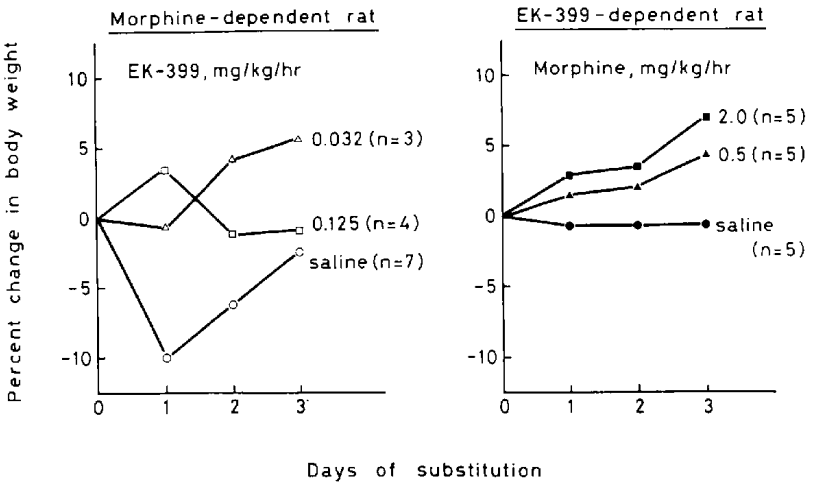

Fig. 4. Body weight changes in morphine-dependent and EK-399-dependent rats when morphine and EK-399 were substituted for EK-399 and morphine, respectively.

0.125 mg/kg/hr of EK-399.

The EK-399-dependent rats were given 0.5 or 2.0 mg/kg/hr of morphine in place of EK-399. Morphine suppressed body weight loss in rats withdrawn from EK-399. No abnormalities in general condition were seen in these animals.

Discussion

Physical dependence on EK-399 could be produced in rats by intravenously infusing the drug for 3–7 days using an administration schedule with a short interval between each dose. In precipitated withdrawal tests after 3 days of treatment with EK-399, naloxone precipitated withdrawal signs such as a decrease in spontaneous activity, ptosis, and weight loss. The weight loss in the EK-399-treated rats was less than that in the morphine-treated rats and was comparable to that in the pethidine- and the pentazocine-treated rats. In abrupt withdrawal tests after 7 days of treatment, slight weight loss was noted in the EK-399-treated rats, but it was less than that in the morphine-, pethidine-, or pentazocine-treated rats. Weight loss is considered a good index of withdrawal because of its objectivity, sensitivity, and dose responsiveness (13, 14). The degree of dependence in EK-399-treated rats appears to be much lower than that in morphine-treated rats and slightly lower than that in pethidine-treated and pentazocine-treated rats.

In substitution tests, cross physical dependence was seen between morphine and EK-399. EK-399 suppressed the weight loss due to the withdrawal of morphine, and vice versa. This suggests that EK-399 has a morphine-like property in its dependence-producing effect. EK-399 had high affinity for both μ and δ opioid receptors in a rat brain receptor binding assay (1). Morphine is a well-known μ opioid agonist, and the μ opioid receptor is thought to mediate physical dependence (10). It is not though clear whether or not δ opioid receptors are involved in the dependence produced by enkephalins and enkephalin analogs. The μ opioid receptors, not the δ opioid receptors, may mediate the physical dependence produced by EK-399.

In conclusion, these results suggest that EK-399 has a morphine-like physical dependence potential in rats that is weaker than that of morphine, pethidine, or pentazocine.

Acknowledgments: I am grateful to Drs. S. Chiba, H. Mayahara and S. Sato for giving me helpful advice. I also thank Mr. Y. Yasuhara for his technical assistance.

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