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Pharmacology

Long-term sensitization to the activation of cerebral δ-opioid receptors by the deltorphin Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2 in rats exposed to morphine

(locomotor activity/stereotypy behavior)

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ABSTRACT **In experiments to evaluate responses to the activation of cerebral δ-opioid receptors, repeated daily injection of the selective δ-opioid agonist Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2 ([D-Ala²]deltorphin II) into rat brain resulted in the development of tolerance, whereas repeated daily injection or continuous infusion of morphine resulted in sensitization to the behavioral activating effects of the δ-opioid agonist. Although the rats did not modify their spontaneous locomotor activity after morphine withdrawal, they became markedly hyperresponsive to the locomotor and stereotypy-producing effects of a challenge dose of the δ-opioid agonist. Sensitization to activation of δ-opioid receptors persisted for at least 60 days after discontinuing morphine treatment. These results show that the development of tolerance and long-term sensitization to opioids involves δ-opioid as well as μ-opioid receptors.**

In rats, systemic morphine administration produces both "depressant" and "stimulant" effects. Repeated injections of morphine result in the development of tolerance to the depressant effects (analgesia, sedation, catalepsy, and respiratory depression) but lead to a progressive hypersensitivity (sensitization) to stimulant effects such as locomotor activity and stereotyped behavior (1–5). This sensitization to the motor effects of peripherally administered morphine develops alongside dependence to the drug and persists for 8 mo after morphine withdrawal (6–8). More recent experiments have demonstrated behavioral stimulation after a single injection of opioids into the rat brain (9–13). Furthermore, as reported with repeated systemic administration of morphine, repeated microinjections of opioids into the ventral tegmental area (VTA) produce sensitization to the motor response (14–16). In contrast, although repeated injections of opioids into the nucleus accumbens (NA) initially increased locomotion, these injections did not subsequently enhance the stimulant effects (17, 18). Which of the three main classes of opioid receptors (μ, κ, and δ) so far identified in the central nervous system is involved in the motor response and sensitization to opioids is still unclear. When [D-Ala²,Phe(Me)⁴,Gly-ol⁵]enkephalin (DAGO), a selective μ-opioid agonist, is injected into rat VTA, it can produce both behavioral activation and sensitization, but when it is injected into rat NA, it produces behavioral activation alone without sensitization (18, 19). In rats, injections of δ-opioid-selective agonists into the brain lateral ventricles, VTA, or NA caused a dose-related increase in locomotor activity and stereotyped behavior, without depressant effects (20–23). Thus, at least in rats, selective activation of cerebral δ-opioid receptors predominantly results in stimulant effects. However, because the time course of the behavioral response to repeated injections of δ-opioid agonists was not evaluated, sensitization to stimulant effects could not be revealed. We recently isolated from amphibian skin a family of opioid peptides with high affinity and selectivity for δ-opioid receptors and named them deltorphins (24, 25). Deltorphins appear to possess many of the attributes of an ideal δ-opioid probe. They lack biological activity on both μ and κ receptors and possess a very high affinity for δ receptors (K_d = 0.1–0.8 nM). In addition, the presence in their sequence of a D-amino acid residue in position 2 and an amide group at the C terminus offers good resistance to hydrolysis by cerebral aminopeptidase and carboxy-peptidase. Injections of deltorphin Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 ([D-Ala²]deltorphin I) and Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2 ([D-Ala²]deltorphin II) into the lateral ventricles or into the NA of rat brain stimulate locomotor activity as well as stereotyped and social behaviors (26–28). In the present study we chose [D-Ala²]deltorphin II as a challenge peptide to selectively activate δ-opioid receptors in rat brain. Our purpose was to evaluate the response to the activation of cerebral δ-opioid receptors in rats exposed to a δ-opioid agonist or morphine. The first approach was to determine whether repeated daily injection of a selective δ-opioid agonist in rats produces tolerance or sensitization to the behavioral activating effects. In a second experimental design, we measured the time course of the behavioral response to δ-opioid receptor activation in rats preexposed to a single systemic injection of morphine. A third approach was to examine whether tolerance or sensitization to δ-opioid receptor activation develops in rats when they are chronically exposed to morphine or are abstinent from morphine. For this purpose, we measured the behavioral effects produced by injecting [D-Ala²]deltorphin II into rats, at defined intervals during morphine exposure and after discontinuation of treatment.

METHODS

Animals. Male Wistar rats weighing 220–230 g when received from the supplier (Charles River Breeding Laboratories) and 250–270 g at surgery were housed singly in 25 cm × 35 cm cages placed in a thermostatically controlled cabinet at an environmental temperature of 21°C. The cabinet was ventilated at 10-min intervals and illuminated between 0830 and 1730. Rats were accustomed to the 9-hr light/15-hr dark cycle for at least 15 days before testing. Water was available ad libitum, but daily food intake was restricted to one-tenth body weight.

Surgery. Under light ethyl ether anesthesia, each rat was implanted surgically with a plastic guide cannula (Linca, Tel

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Abbreviations: [D-Ala²]deltorphin I, Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2; [D-Ala²]deltorphin II, Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2; i.c.v., intracerebroventricular; VTA, ventral tegmental area; NA, nucleus accumbens.

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Aviv) screwed into a skull hole drilled over the left lateral cerebral ventricle (anterioposterior, −0.5 mm and lateral, +1.8 mm relative to bregma; ventral, −1.0 mm relative to skull surface). The cannula was secured to bone with dental cement, and the rat was returned to its cage to recover from surgery for at least a week.

Locomotor Activity. Locomotor activity of rats in open field was monitored by a television camera connected to a videotape recorder. Rats were placed singly in an arena consisting of a wooden box (100 cm × 100 cm × 50 cm), the floor and side walls of which were painted black. The boxes were arranged into recording units; a recording unit consisted of a set of four boxes illuminated by a 16 W fluorescent bulb and monitored by a television camera located 3 m above the boxes. The recording unit was kept in a ventilated, sound-attenuating chamber. Four recording units were used in each experimental session, so that the motor activity of 16 rats could be recorded concomitantly. A four-channel videotape recorder and a picture-analyzing apparatus were situated in an adjacent room. A digital converter was connected to the videotape recorder, and digitized pictures were analyzed by a computer program running on an Intel 386 computer. The program calculated the distance traveled by each rat, its velocity, and the time spent in locomotor activity. Locomotor activity was classified according to velocity of the locomotion, as walking or running (walking <0.5 m·sec^{-1} and running >0.5 m·sec^{-1}). Frequency of stereotyped behavior (rearing) was calculated by an observer with the program THE OBSERVER (Noldus Information Technology, Wageningen, The Netherlands). Each recording session lasted 2 hr, 1 hr before and 1 hr after injection of [D-Ala^2]deltorphin II challenge dose. Rats were always tested during the 9-hr light period. The response of each animal to activation of δ-opioid receptors was calculated by subtracting the values of distance traveled and rearing frequency recorded before [D-Ala^2]deltorphin II injection from those obtained after peptide administration in the same test session. Data obtained from opioid-exposed rats on each test day are represented in the figures by two columns—one white and the other hatched. The white column represents rat motor activity recorded during the hour preceding activation of δ-opioid receptors; the hatched column represents the behavioral response to activation of δ-opioid receptors, calculated as described above. When measured in control rats, the time course of the response to activation of δ-opioid receptors is represented in the figures by a solid line connecting the data points (closed circles). All data shown in the figures are the mean of eight rats, and the error bars represent the SEM.

Activation of δ-Opioid Receptors. Selective activation of cerebral δ-opioid receptors was evoked by a single intracerebroventricular (i.c.v.) injection of [D-Ala^2]deltorphin II. The peptide was dissolved in saline (0.9% NaCl in sterile water), and 2 μl of solution was injected into the brain lateral ventricle. In all test sessions, the challenge dose of [D-Ala^2]deltorphin II was 0.5 nmol per rat. On each test day, two animal groups, one comprising saline- and the other opioid-exposed rats, were injected at the same time with the challenge dose of [D-Ala^2]deltorphin II. To avoid changes in response from repeated administration of the challenge dose in the same animal, each animal group was tested with [D-Ala^2]deltorphin II once only. Thus, on different test days a different pair of animal groups was used. The test started at 0830, when two saline- and two opioid-group-allotted animals were transferred to each of the four recording units and left undisturbed to become accustomed to the arena until 0930. Basal locomotor activity and stereotyped behavior of rats were recorded from 0930 to 1030. The animals were then i.c.v. injected with the [D-Ala^2]deltorphin II challenge dose, and the motor response was recorded for 1 hr. At the end of recording, animals were returned to the home cages and, if due, the daily opioid dose was given.

Animal Groups. As the study occurred over several months, it was split into experimental groupings; control animals were in each grouping. Because activation of δ-opioid receptors by [D-Ala^2]deltorphin II was tested once only in the same rat group, in each experimental design the number of animal groups equaled the number of test sessions. Chronic treatment with [D-Ala^2]deltorphin II lasted 5 days. Rats were divided into seven groups of eight rats, and each rat received one daily i.c.v. injection of [D-Ala^2]deltorphin II (1 nmol per rat) at 1300 for 5 consecutive days. According to the same time schedule, eight groups of eight rats each were i.c.v. injected with saline. Activation of δ-opioid receptors was evoked in one of the eight groups of control rats before beginning the opioid treatment. Stimulation of δ-opioid receptors was then tested on day 1, 2, 4, and 5 of treatment and 3, 7, and 15 days after treatment discontinuation. Three different dosage schedules were used for morphine treatment. Five groups of eight rats each were injected with a single s.c. dose of morphine hydrochloride at 10 mg/kg. An additional six groups of eight rats each acted as controls and received a s.c. injection of saline. Rats were tested with [D-Ala^2]deltorphin II before and 6, 12, 24, 120, or 360 hr after the single morphine exposure. Rats that received one daily injection of morphine (10 mg/kg, s.c.) for 10 consecutive days were divided into six groups of eight rats each. Seven more groups of eight rats each received a s.c. injection of saline instead of morphine. Activation of δ-opioid receptors was evoked in one of the seven groups of control rats before beginning morphine treatment. In the remaining animal groups, the δ-opioid response was evoked on the third, fifth, and tenth day of morphine or saline exposure and 2, 7, and 15 days after discontinuation of the treatment. Finally, morphine was delivered continuously to rats by an osmotic pump (Alza) implanted s.c. in the dorsal region. Model 2ML1 Alzet osmotic pumps with a mean fill volume of 2.3 ± 0.05 ml and mean pumping rate of 10.3 ± 0.3 μl/hr were used. Morphine hydrochloride was dissolved in distilled water at concentrations of 50 mg/ml. Because a single osmotic pump delivered ≈240 μl of morphine solution per day, each rat received a daily dose of 12 mg of morphine. The osmotic pumps delivered morphine solution at this infusion rate for 9 days. Development of tolerance was monitored by determining the analgesic response to a s.c. challenge dose of morphine at 10 mg/kg. Response to the challenge dose of morphine was tested with the tail-flick test at 1600 (29). A rat was considered tolerant when its reaction time was smaller than its control reaction time plus three SDs of the mean control reaction time of all animals in the group. Control rats were implanted with saline-filled osmotic pumps. After the 9-day infusion, osmotic pumps were removed, and the rats were considered to be abstinent thereafter. To study δ-opioid receptor activation, chronically infused rats were divided into 17 groups of eight rats each—eight groups were implanted with morphine, and nine groups were implanted with saline-filled osmotic pumps. Activation of δ-opioid receptors was evoked in one of the nine groups of control rats 2 hr after pump implantation. The other rats were tested with [D-Ala^2]deltorphin II on the second, fifth, and ninth day of morphine or saline infusion and on the second, seventh, twentieth, and sixtieth day after pump removal.

Drugs. Morphine hydrochloride was bought from Salars (Varese, Italy), and [D-Ala^2]deltorphin II was synthesized as described (25).

Statistics. Data were evaluated by analysis of variance, and Dunnett's *t* test was used for post hoc comparison between animal groups. Control-treated animals were included in each test group and, therefore, statistical comparisons were only made within individual groupings.

RESULTS

Chronic Treatment with [D-Ala²]deltorphin II. Repeated daily injection of [D-Ala²]deltorphin II into rat brain (1 nmol per rat) led to a decrease in motor response to the peptide challenge dose after 4 days of treatment (Fig. 1). Response to the challenge dose evoked 7 and 15 days after discontinuation of treatment did not differ significantly from that in control animals. Spontaneous motor activity of rats recorded on each test day before daily injection of [D-Ala²]deltorphin II (Fig. 1, open columns) was not modified by peptide administration.

Single Morphine Injection. Fig. 2 illustrates the activation of δ-opioid receptors by [D-Ala²]deltorphin II at different time points after a single s.c. injection of morphine, at 10 mg/kg, or saline. From 6 hr onward after morphine injection, the locomotor response and stereotypy frequency produced by δ-opioid receptor activation progressively increased. The response reached a maximum 24 hr after morphine injection and persisted undiminished for at least 5 days. Fifteen days after morphine injection the hypersensitivity to the δ-opioid agonist was no longer detectable.

Chronic Morphine Treatment. In rats that received one daily injection of morphine at 10 mg/kg for 10 consecutive days (Fig. 3), sensitization to the locomotor response and stereotyped behavior evoked by δ-opioid receptor activation was already present on day 5, increased on day 10 of morphine exposure, and persisted for at least 15 days after morphine withdrawal. Rats implanted with morphine-delivering osmotic pumps (Fig. 4) progressively increased their spontaneous motor activity during the first 5 days of continuous morphine infusion and maintained it at higher levels than controls, until the pumps stopped. Continuous morphine delivery produced tolerance to the antinociceptive effect of a challenge dose of morphine at 10 mg/kg from treatment day 3 onward (data not shown). In these animals, significant enhancement of the motor response to δ-opioid receptor activation was recorded only on day 9 of morphine infusion, at which tolerance to the analgesic effect of morphine was already established. When morphine infusion was stopped, sensitization to the stimulant effects of the δ-opioid agonist increased abruptly from the seventh day of abstinence onward. In response to the [D-Ala²]deltorphin II challenge dose, morphine-abstinent rats showed complex multiphasic changes in motor activity. They engaged in frequent and intense running, followed by sudden freezing from which they slowly recovered by entering a stereotypy phase with recurrent rearing (data not shown). This pronounced enhancement in locomotor activity and stereotypy frequency was not evident in the spontaneous motor behavior of the abstinent rats but was revealed only by the challenge injection of [D-Ala²]deltorphin II. The hypersensitivity of the δ-opioid system remained practically unchanged 60 days after morphine withdrawal. On day 9 of morphine infusion, i.c.v. injection of [D-Ala²]deltorphin II significantly reduced reaction time in the tail-flick test (Table 1). The hyperalgesic response to the δ-opioid agonist was still present 2 and 7 days after discontinuing the morphine regimen.

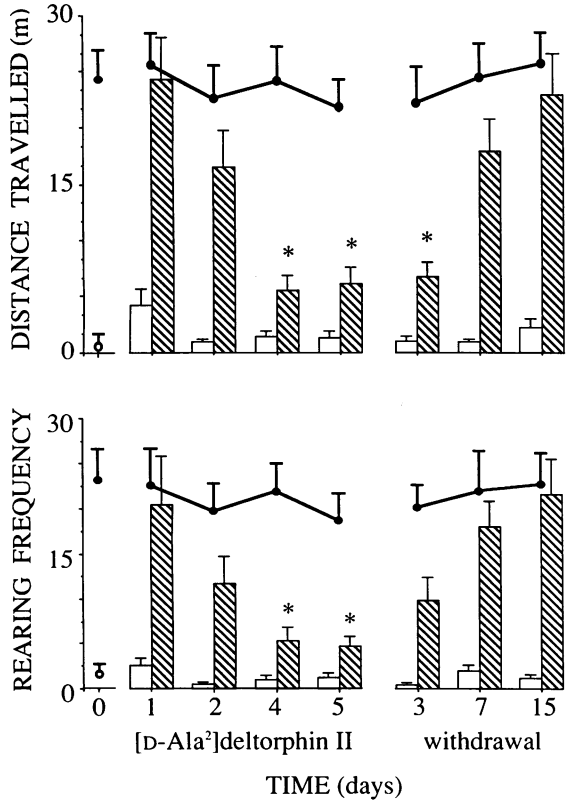

FIG. 1. Time course of behavioral response to repeated daily injection of [D-Ala²]deltorphin II. Each column represents locomotor activity of rats injected with [D-Ala²]deltorphin II once daily. Open columns refer to spontaneous motor activity before daily injection of [D-Ala²]deltorphin II; hatched columns represent locomotor response to a challenge dose (0.5 nmol per rat i.c.v.) of [D-Ala²]-deltorphin II. ○, Spontaneous motor activity of control rats; ●, locomotor response of control rats to the challenge dose of [D-Ala²]deltorphin II. *, $P < 0.05$ vs. controls (Dunnett's test).

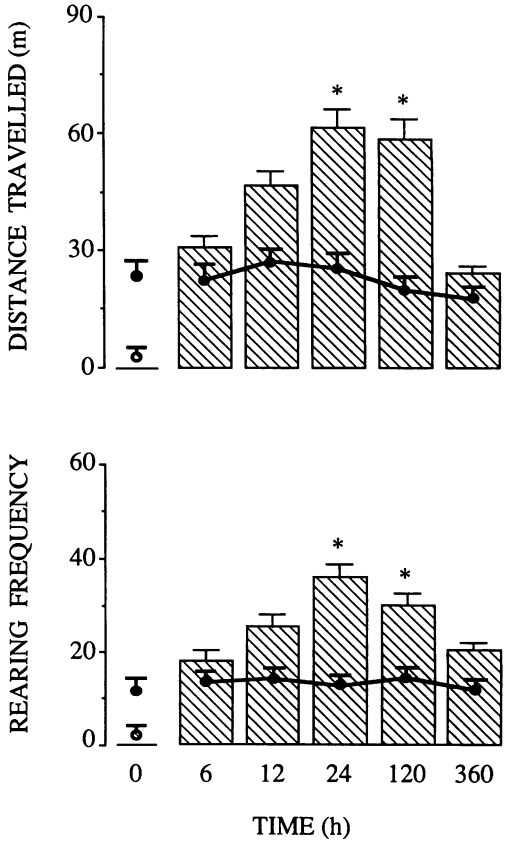

FIG. 2. Time course of behavioral response to a challenge dose (0.5 nmol/rat i.c.v.) of [D-Ala²]deltorphin II in rats injected with a single s.c. dose of morphine (10 mg/kg). ○, Spontaneous motor activity of control rats; ●, locomotor response of control rats to [D-Ala²]deltorphin II. Hatched columns, locomotor response of morphine-exposed rats to [D-Ala²]deltorphin II. *, $P < 0.05$ vs. controls (Dunnett's test).

DISCUSSION

The role of μ-opioid receptors in development of opiate tolerance and physical dependence is well documented (30–

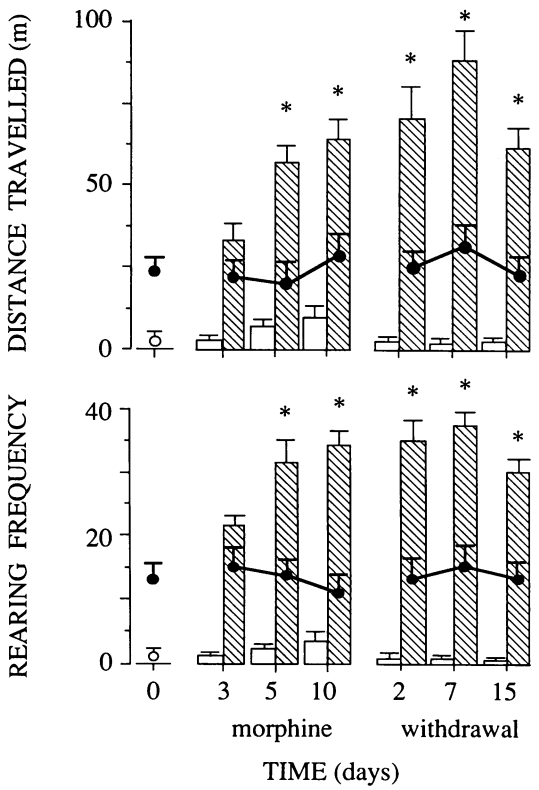

FIG. 3. Time course of behavioral response to a challenge dose (0.5 nmol/rat i.c.v.) of [D-Ala²]deltorphin II in rats injected daily with morphine. Open columns refer to spontaneous motor activity before the daily injection of morphine; hatched columns represent locomotor response of morphine-exposed rats to the [D-Ala²]deltorphin II challenge dose. ○, Spontaneous motor activity of control rats; ●, locomotor response of control rats to the [D-Ala²]deltorphin II challenge dose. *, $P < 0.05$ vs. controls (Dunnett's test).

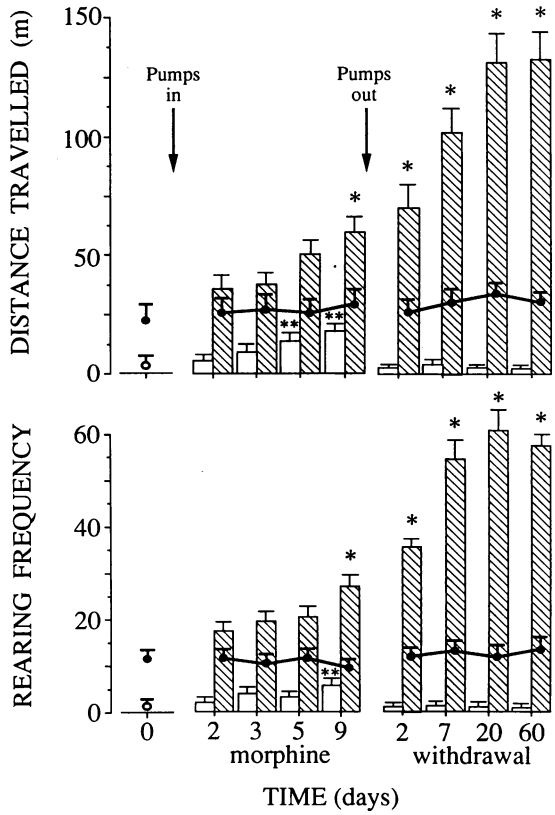

FIG. 4. Time course of behavioral response to a challenge dose (0.5 nmol/rat i.c.v.) of [D-Ala²]deltorphin II in rats infused continuously with morphine. Open columns refer to spontaneous motor activity of morphine-infused rats; hatched columns represent locomotor response of morphine-infused rats to the [D-Ala²]deltorphin II challenge dose. ○, Spontaneous motor activity of control rats; ●, locomotor response of control rats to the [D-Ala²]deltorphin II challenge dose. * and **, $P < 0.05$ vs. controls (Dunnett's test).

32), although the mechanism of tolerance is still unknown. Tolerance to the antinociceptive and cataleptic effects of morphine has been found to develop alongside sensitization to the behavioral activating effects of the drug, at least in rats. Behavioral sensitization could be reproduced by selective μ-opioid agonists (17). In our experiments we showed that exposure of rats to morphine also induces a long-lasting sensitization to the behavioral stimulant effects of the selective δ-opioid agonist [D-Ala²]deltorphin II. Thus cerebral δ- as well as μ-opioid receptors appear to participate in the sensitization process. Previous experiments suggest that repeated activation of opioid receptors in the VTA of the rat brain is responsible for the development of sensitization, whereas activation of opioid receptors in the NA elicits increased locomotion without sensitization (18). Injection of the δ-opioid selective agonist [D-Ala²]deltorphin II into the rat NA produced locomotor effects and stereotyped behavior (27), and, accordingly, a high density of δ-opioid binding sites have been found in the NA (33, 34). In the present study, repeated i.c.v. injections of [D-Ala²]deltorphin II induced tolerance instead of sensitization to the stimulant effects, confirming that sensitization needs activation of μ-opioid receptors in the VTA. Thus, an explanation of our findings may be that morphine sensitizes δ-opioid receptors in the NA by activating μ-opioid receptors of VTA neurons that project to the NA via the mesolimbic pathway. The present results show that hypersensitivity of the cerebral δ-opioid system occurs a discrete time interval after opioid exposure (≈8–12 hr) and persists for at least 2 mo after opioid withdrawal. This finding implies the existence of cellular mechanisms that couple the opioid-elicited stimulus to a long-term alteration in the neuronal phenotype. The existence of a measurable time lapse between opioid exposure and the appearance of the hypersensitivity to δ-opioid receptor activation seems to indicate that the underlying cellular mechanisms depend on protein synthesis. A growing body of evidence indicates that extracellular stimuli can elicit the transcriptional activation of a number of cellular immediate-early genes (c-*fos*, c-*jun*), which produce crucial intermediates in a complex cascade that links stimulation of membrane receptors to long-term alterations in cellular phenotype. Morphine has been found to activate c-*fos* transcription in the rat striatum (35). Fos is known to act in heterodimeric association with other mem-

Table 1. Tail-flick response of rats continuously infused with morphine (12 mg/day)

Days of infusion	Tail-flick response, sec	
	Challenge dose of morphine hydrochloride (10 mg/kg)	Challenge dose of [D-Ala²]deltorphin II (0.5 nmol)
0	>10	3.2 ± 0.2
Morphine		
2	6.4 ± 0.5*	5.3 ± 0.3
5	4.1 ± 0.2*	
9	3.9 ± 0.2*	2.2 ± 0.1*
Withdrawal		
2		1.6 ± 0.1*
7		1.8 ± 0.2*
20		3.4 ± 0.2

*$P < 0.05$ vs. day 0 (Dunnett's test).

bers of the Jun/AP-1 family of nuclear proteins to control transcriptional activity of several other genes, including those of the opioid system (36, 37). A molecular sequel to this c-*fos* induction may involve up-regulation of the δ-opioid system. An increase in δ-opioid receptor density has been recently reported in the whole brain and striatal slices during chronic morphine treatment in rats and mice (38, 39).

During the present study, Abdelhamid *et al.* (40) published a paper showing that the blockage of δ-opioid receptors by naltrindole, a selective δ-opioid antagonist, prevents development of morphine tolerance and dependence in mice. In our study, we demonstrated that activation of cerebral δ-opioid receptors resulted in a reaction time to painful stimuli that was shorter in morphine-tolerant mice than in naive rats. These results show that δ-opioid as well as μ-opioid receptors are involved in the development of tolerance, sensitization to opioids, and dependence on opioids. Hypersensitivity of the δ-opioid system during repeated morphine exposure could enhance responses, such as increased motor activity and hyperalgesia, compensatory to sedation, catalepsy, and antinociception and eventually contribute to tolerance. In a one-trial inhibitory avoidance task, we have previously shown that selective activation of δ-opioid receptors in mice improves memory consolidation (41). Stewart and Vezina (42) and Vezina *et al.* (43) have shown that the degree of sensitization induced by repeated morphine administration depends on the environment in which morphine is administered. Memory reinforcement of the pleasant effects of morphine may contribute to the development of long-term dependence, vulnerability, and craving. In both animals and humans, withdrawal phenomena, short-term and long-term opiate dependence, and relief of withdrawal symptoms can be conditioned to environmental stimuli. Thus, the high level of δ-opioid receptor hypersensitivity persisting for months after morphine withdrawal may be partially responsible for the appearance of the abstinence syndrome and for relapse to drug use.

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