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Naltrexone In Vivo Protects μ Receptors From Inactivation by β-Funaltrexamine, But Not κ Receptors From Inactivation by Nor-Binaltorphimine

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PARONIS, C. A., A. B. WADDELL AND S. G. HOLTZMAN. *Naltrexone in vivo protects μ receptors from inactivation by β-funaltrexamine, but not κ receptors from inactivation by nor-binaltorphimine.* PHARMACOL BIOCHEM BEHAV **46**(4) 813–817, 1993.—The ability of the competitive opioid antagonist, naltrexone, to protect opioid receptors from inactivation by the nonequilibrium antagonists, β-funaltrexamine (β-FNA) and nor-binaltorphimine (nor-BNI), was examined in vivo. Male rats were injected SC with 10 mg/kg naltrexone or saline, 30 min before being injected intracisternally (IC) with water, 10 μg β-FNA, or 1.0 or 10 μg nor-BNI. The rats were tested for analgesic responses to either U69,593 (nor-BNI groups) or morphine (β-FNA groups), on a 50°C hot plate, 24 h later. Morphine produced dose-related increases in the latency to paw lick in rats that received water (IC) (mean ED_{50} = 3.2 mg/kg). Little or no analgesia occurred after 1.0–30 mg/kg of morphine in animals that had received saline (SC) and 10 μg β-FNA (IC) 24 h earlier. Pretreatment with 10 mg/kg naltrexone attenuated the antagonist effects of β-FNA (morphine ED_{50} = 10.8 mg/kg). U69,593 also produced analgesia in animals that received water (IC) (ED_{50} = 0.97 mg/kg). This analgesia was dose-dependently blocked by nor-BNI for up to 7 days. Naltrexone did not inhibit the actions of nor-BNI. Thus, naltrexone prevented inactivation of μ receptors by β-FNA but not inactivation of κ receptors by nor-BNI, suggesting that antagonist interactions with μ receptors are different from those with κ receptors.

β-Funaltrexamine Nor-binaltorphimine In vivo protection

NOR-BINALTORPHIMINE (nor-BNI) is a κ-selective antagonist both in vivo and in vitro (13,15). Nor-BNI does not have an alkyl moiety that would suggest it is capable of covalently binding to a receptor. Nevertheless, the κ-opioid antagonist effects of nor-BNI persist at least 3 weeks in vivo following a single central injection of the drug (7,9). Similarly, although the effects of nor-BNI in smooth muscle preparations are reversible, 3 h of repeated washings are necessary for the return of the preparations to their basal activity (2). The mechanisms responsible for the long duration of action of nor-BNI are not yet known, though several explanations have been suggested. Included among these possibilities are the sequestration of nor-BNI, slow diffusion of the molecule out of the central nervous system, or a conformational change of the κ-opioid receptor.

The opioid antagonist, β-funaltrexamine (β-FNA), will bind to μ, δ, and κ sites, although it has a relatively higher affinity for μ-opioid receptors (4). In contrast to the uncertainty regarding the longevity of nor-BNI activity, the noncompetitive nature of β-FNA is believed to be caused by alkylation of μ-opioid receptors. The number of μ binding sites is decreased following β-FNA treatment either in vitro or ex vivo (16,18), and a single injection of β-FNA will antagonize the analgesic effects of morphine for up to 5 days (17).

Inactivation of receptors by irreversible antagonists can be prevented by pretreatment with competitive ligands. This technique is often used in vitro to protect specific receptor populations in a tissue. For example, in the presence of dynorphin A, the alkylating agent β-chlornaltrexamine will only inactivate δ- and μ-opioid receptors, resulting in a tissue that contains κ-opioid receptors almost exclusively (8). Similarly, the irreversible binding of the μ-opioid antagonist β-FNA to guinea pig brain membranes can be prevented by pretreatment with naloxone or morphine (16). Few studies have examined drug-induced protection of receptors in vivo, although some investigators have had success with this method (3,14). In the present experiments, we sought to determine in vivo whether pretreatment with naltrexone, an opioid antagonist that binds with

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high affinity to all opioid receptors, would protect μ- or κ-opioid receptors from inactivation by β-FNA or nor-BNI, respectively. Protection was assessed by the ability of morphine or U69,593, μ- and κ-opioid agonists, respectively, to elicit an analgesic response in rats that had been pretreated with the various opioid antagonists.

METHOD

Subjects

Male rats of Sprague–Dawley descent (Charles River, Inc., Raleigh, NC), weighing 250–400 g at the start of the experiment, were used. Rats were group housed in standard laboratory cages and kept in a temperature-controlled colony room with a 12L : 12D cycle (lights on at 7:00 a.m.). Food (Purina Rodent Chow; Purina Mills, St. Louis, MO) and water were available ad lib. All testing was done between 9:30 a.m. and 12:30 p.m.

Experimental Procedure

Animals were divided into nine groups of eight rats each. On day 0 of the experiment, the animals were weighed and injected SC with either saline or 10 mg/kg naltrexone. Thirty minutes later they were injected intracisternally (IC) with 1.0 or 10 μg nor-BNI (1.51 or 15.1 nmol, respectively), 10 μg β-FNA (20.4 nmol), or distilled water. Twenty-four hours after the IC injections, the animals were tested for an analgesic response to either U69,593 (nor-BNI groups) or morphine (β-FNA groups), using a cumulative dosing procedure. The rats that received nor-BNI were retested with U69,593 7 days later.

Analgesia Testing

Rats were placed on a 50°C hot plate surrounded by clear Plexiglas walls (26.5 × 29 × 28.5 cm). A lick of a rear paw, or a jump (all four paws off the surface of the hot plate) signified a response and ended the trial. A foot-activated timer was used to measure the latency to paw lick or jump. Rats were given two habituation trials, separated by at least 15 min. The third trial was used as the baseline measure, and was immediately followed by an injection of the lowest dose of either U69,593 or morphine. Testing continued at 25-min intervals, each trial followed by the next cumulative dose of drug, until the animals failed to respond within 35 s or until the maximum dose of drug was given.

Intracisternal Injections

Animals were lightly anesthetized with halothane (Halocarbon Laboratories, North Augusta, SC). The back of the rat's neck was shaved and cleaned with alcohol, and the rat was placed in the earbars of a stereotaxic device. The head of the animal was held perpendicular to the body axis as a 25-ga needle, attached to a 50-μl Hamilton syringe, was inserted in the cisterna magna to a depth of 5 mm. A 5–10-μl injection was delivered over 20–30 s and the needle was held in place for an additional 20–30 s before being withdrawn.

Data Analysis

Latencies to paw lick are expressed as percent maximum possible effect (%MPE) (5):

$$\%\text{MPE} = \frac{\text{test latency} - \text{baseline latency}}{\text{cut-off time (35 s)} - \text{baseline latency}} \times 100\%$$

The transformed data were used to calculate ED_{50} values for individual animals. Where an ED_{50} could not be calculated reliably in individual animals (e.g., for rats that failed to show a greater than 50% MPE), an ED_{50} was calculated from pooled data of the entire group. Baseline paw lick latencies of all groups on day 1 were subjected to a one-factor ANOVA. Baseline paw lick latencies of animals tested with U69,593 were subjected to a two-factor ANOVA (pretreatment × day of test), with repeated measures on one factor (day of test). The ANOVA was followed by a "protected" t-test (i.e., Student's t-test corrected for multiple pairwise comparisons) to identify significant differences across the pretreatment and the test days.

Drugs

Morphine sulfate (Penick Corp., Newark, NJ) and naltrexone hydrochloride (Sigma Chemical Co., St. Louis, MO) were dissolved in normal (0.9%) saline. U69,593 [National Institute on Drug Abuse (NIDA), Rockville, MD] was dissolved in a 3 : 2 solution of 8.5% lactic acid and 1.0 N NaOH. All of the above drugs were injected SC in a volume of 1.0 ml/kg. β-FNA (NIDA) and nor-BNI (Research Biochemicals Inc., Natick, MA) were dissolved in distilled water. Drug doses are expressed as the free base.

RESULTS

Administration of saline or naltrexone, 24.5 h before the start of analgesic testing, and IC injections of water, β-FNA, or nor-BNI, 24 h before testing, did not significantly alter baseline latencies to paw lick, $F(8, 63) = 1.26$, $p = 0.276$ (Table 1). A two-way ANOVA of the baseline latencies of animals tested on days 1 and 7 with U69,593 revealed a significant interaction between pretreatments and day of testing, $F(4, 35) = 3.25$, $p = 0.022$; however, there was no distinct pattern of differences among baseline measures, and there were no differences for the main effects of treatment, $F(4, 35) = 0.851$, $p = 0.505$, or day of test, $F(1, 35) = 0.900$, $p = 0.649$.

Morphine produced dose-dependent analgesia in animals that had received IC injections of distilled water 24 h earlier (Fig. 1). The mean analgesic ED_{50} of morphine (with 95% C.L.) was 3.2 mg/kg (2.6, 3.7) in animals pretreated with

TABLE 1

BASELINE LATENCIES TO PAW LICK

Pretreatment	Day 1*	Day 7†
Sal/H_20	10.4 ± 0.7	9.6 ± 0.8
Ntx/H_20	9.4 ± 0.7	9.0 ± 0.8
Nor-BNI (1 μg)	11.4 ± 1.0	8.4 ± 0.8
Sal/nor-BNI	10.2 ± 1.2	9.8 ± 0.8
Ntx/nor-BNI	8.0 ± 0.6	10.2 ± 0.8
Sal/β-FNA	9.1 ± 0.6	
Ntx/β-FNA	9.8 ± 1.0	

Values are mean ± SEM in seconds (n = 7–16).
*Twenty-four hours after IC injection.
†Seven days after IC injection. Data are only for rats tested with U69,593.

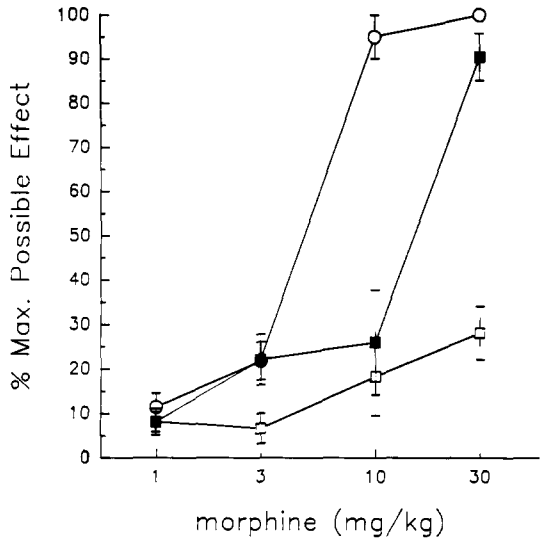

FIG. 1. Dose-effect curve of morphine in animals that received injections of: (○) distilled water (IC), (□) 10 μg β-FNA (IC) + saline (SC), or (■) 10 μg β-FNA + 10 mg/kg naltrexone (SC) 24 h before analgesic testing. Abscissa: cumulative morphine dose in mg per kg. Ordinate: Percentage of M.P.E. Each point represents the mean of 8–15 animals and vertical lines are 1 SEM.

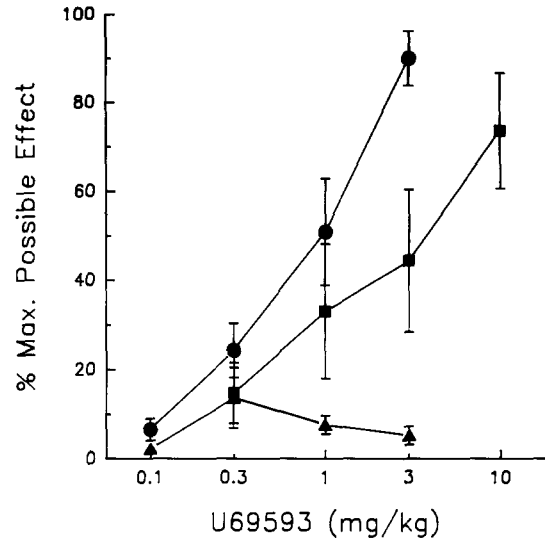

FIG. 2. Dose-effect curve of U69,593 in animals that received injections of: (●) distilled water (IC), (■) 1.0 μg nor-BNI (IC), or (▲) 10 μg nor-BNI (IC) 24 h before analgesic testing. Abscissa: cumulative U69,593 dose in mg per kg. Ordinate: Percentage of M.P.E. Each point represents the mean of 7–8 animals and vertical lines are 1 SEM.

saline (SC), and 4.8 mg/kg (2.6, 7.0) in animals that received 10 mg/kg naltrexone (SC); these values were not statistically different, $t(14) = 1.313$. Because naltrexone did not have an effect on the morphine dose-response curve, the data from the two groups were combined.

An IC injection of 10 μg β-FNA blocked the analgesic activity of up to 30 mg/kg morphine (Fig. 1). Pretreatment with 10 mg/kg naltrexone, 30 min before the β-FNA injection, attenuated the actions of β-FNA. Thus, morphine produced a full analgesic response in animals that had received both naltrexone and β-FNA, albeit the morphine dose-response curve was shifted more than twofold to the right of the curve established in control animals. The ED_{50} of morphine in animals that received naltrexone and β-FNA was 10.8 mg/kg (6.4, 15.2).

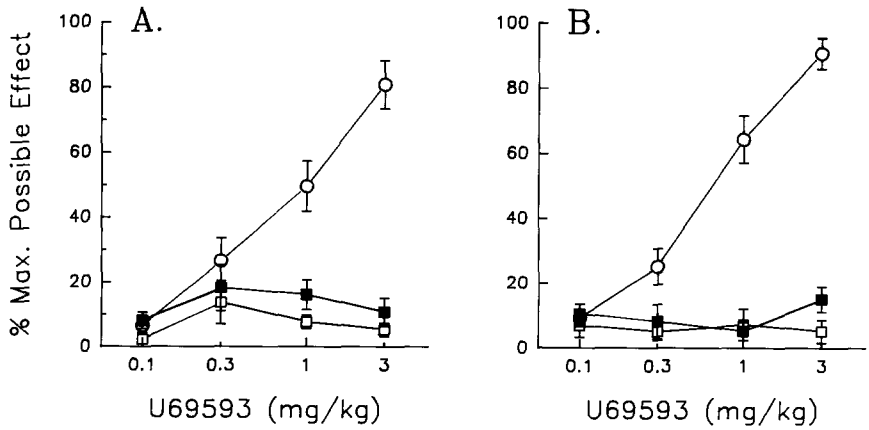

FIG. 3. Dose-effect curve of U69,593 in animals that received injections of: (○) distilled water (IC), (□) 10 μg nor-BNI (IC) + saline (SC), or (■) 10 μg nor-BNI (IC) + 10 mg/kg naltrexone (SC). (A) Depicts the analgesic activity of U69,593, 24 h after the IC injections of water or nor-BNI, (B) depicts the analgesic activity of nor-BNI 7 days after the IC injections. Abscissae: cumulative U69,593 dose in mg per kg. Ordinates: Percentage of M.P.E. Each point represents the mean of 7–15 animals and vertical lines are 1 SEM. One animal that received naltrexone before the IC injection of water did not have an analgesic response to U69,593 24 h later; another animal in that group did not respond to U69,593 on day 7. Because these data differed by more than 3 SEM from the group means, they were excluded from further study.

The κ-opioid agonist, U69,593, also produced analgesia as measured in the hot plate assay. Naltrexone, administered 30 min before the IC injection of water, had no effect on the dose-response curve of U69,593 and so the data were combined with those of the animals that received saline pretreatments. The mean analgesic ED_{50} of U69,593, 24 h after IC injections of water, was 0.97 mg/kg (0.59, 1.35).

Nor-BNI blocked the analgesic activity of U69,593 in a dose-dependent fashion (Fig. 2). A 1.0 μg injection of nor-BNI shifted the dose-response curve of U69,593 twofold to the right; the group ED_{50} of U69,593 was 1.92 mg/kg. Nor-BNI (10 μg) administered 24 h before testing eliminated the analgesic activity of up to 3.0 mg/kg U69,593. The antagonism of U69,593 was not diminished in the 7 days following the nor-BNI injections (Fig. 3). The ED_{50} of U69,593 in the 1 μg nor-BNI group was 1.52 mg/kg on day 7, and the higher dose of nor-BNI continued to block the effects of up to 3.0 mg/kg U69,593. The 10 μg dose of nor-BNI antagonized U69,593 to a greater degree than was seen with 1 μg nor-BNI. Hence, the higher dose of nor-BNI was used to assess the ability of naltrexone to protect κ receptors from a nor-BNI-induced inactivation. Naltrexone pretreatment did not affect the antagonism of U69,593 by 10 μg nor-BNI on either of days 1 or 7 (Fig. 3).

DISCUSSION

The dose of β-FNA used in the present experiments antagonized the analgesic effects of morphine 24 h later. These results agree with previous reports that central administration of 10–20 nmol β-FNA will lower the slope of the morphine dose-response curve (1,12). A loss of morphine-induced analgesia has also been reported following much lower doses of β-FNA (0.2–0.6 nmol) (14). Moreover, Sánchez-Blázquez and Garzón demonstrated that the μ-opioid agonists, morphine and [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin (DAMGO), in mice could protect μ receptor-mediated analgesia from antagonism by 0.2 nmol β-FNA. Our results support and extend these findings. That is, a 30-min pretreatment of naltrexone was capable of protecting the analgesic effects of morphine from antagonism by a dose of β-FNA two orders of magnitude greater than that used by Sánchez-Blázquez and Garzón (14). These results are in accordance with results from other investigators (3), demonstrating that the effects of irreversible antagonists can be inhibited in vivo by pretreatment with competitive antagonists. Comer et al. (3) further demonstrated that the degree of the parallel rightward shift of the morphine dose-response curve, following pretreatment with naloxone and clocinnamox, was dependent on the dose of naloxone. However, even doses as high as 100 mg/kg naloxone failed to eliminate completely the effects of clocinnamox. That is, even in the presence of naloxone, the alkylating agent was able to effect a slight shift to the right of the morphine dose-response curve.

Nor-BNI antagonized the analgesic activity of the κ-opioid agonist, U69,593, in a dose-dependent fashion. The degree of antagonism by nor-BNI did not change over 7 days. Other investigators have already described the long duration of action of nor-BNI. Indeed, nor-BNI has been shown to antagonize the analgesic effects of U69,593 in mice for up to 4 weeks, while it was able to decrease the binding of [^{3}H]U69,593 to mouse brain homogenates for up to 56 days (7). The long-lasting antagonism by nor-BNI of another κ agonist, spiradoline, in rats also has been reported (9). Our results extend these observations, demonstrating that nor-BNI will block, for at least 7 days, the analgesia elicited by U69,593 in rats.

To date, the mechanisms responsible for the long-lasting effects of nor-BNI are unknown. We speculated that if the antagonism induced by nor-BNI is due to the irreversible binding of nor-BNI to κ-opioid receptors, then pretreatment with naltrexone should prevent the effects of nor-BNI. Although naltrexone was able to protect μ-opioid receptors from inactivation by β-FNA, the actions of nor-BNI were not inhibited by the prior administration of naltrexone. It might be argued that naltrexone failed to protect κ-opioid receptors adequately because it does not have a high selectivity for κ-opioid receptors; it is a nonselective opioid antagonist, with an approximately tenfold higher affinity for μ- than for κ-opioid receptors (10). Nonetheless, naltrexone will antagonize κ-opioid-mediated effects in vivo. For example, doses of less than 0.3 mg/kg naltrexone will inhibit the analgesic activity of the κ-opioid agonists, U50,488H and U69,593, in rats (11), and as little as 0.1 mg/kg produces a fourfold shift to the right of the spiradoline curve in a drug discrimination procedure (6). Therefore, it can be assumed that in the present experiments we used a dose of naltrexone (10 mg/kg) capable of occupying κ-opioid receptors in vivo.

Because naltrexone was unable to protect κ-opioid receptors from inactivation by nor-BNI, our results do not support the hypothesis that nor-BNI binds irreversibly to the κ-receptor. It is possible, however, that nor-BNI binds to a site near the receptor, but not on it, thus causing a steric hindrance of agonist binding. Such a situation is also suggested by the binding studies of Horan and colleagues, in which nor-BNI decreased the affinity of [^{3}H]U69,593 binding, but had no effect on the number of available binding sites.

Our results, furthermore, do not exclude the possibility that the longevity of antagonism by nor-BNI is due to a long elimination half-life of the drug. To date, there are no pharmacokinetic data available for nor-BNI; however, it has been proposed that nor-BNI diffuses slowly across the blood–brain barrier (15). The simple explanation of slow diffusion across the blood–brain barrier, however, is probably not the sole mechanism for the long duration of action of nor-BNI. Systemic administration of nor-BNI will antagonize the analgesic effects of κ-opioid agonists within 90–120 min after injection (2,15). Whereas this 1–2-h lag time does support a slow diffusion rate, it also demonstrates that 2 h is time enough to allow diffusion of nor-BNI into the central nervous system. Thus, it is unlikely that 3 weeks are needed for the drug to diffuse out of the central nervous system, if its movement is dictated solely by the law of mass action.

It is possible that the elimination of nor-BNI is prolonged by partitioning into cell membranes, resistance to metabolism, or even formation of an active metabolite that is less able to diffuse across the blood–brain barrier. Finally, the long duration of action of nor-BNI could be a consequence of multiple factors acting in concert, none of which alone provides a satisfactory explanation for the 3–8-week effects of nor-BNI.

In conclusion, we have shown that the opioid antagonist, naltrexone, is capable of protecting μ-opioid receptors from inactivation by the irreversible antagonist, β-FNA, in behaving rats. However, naltrexone pretreatment was unable to attenuate the actions of the κ-opioid antagonist, nor-BNI. Thus, our results indicate that the interactions of naltrexone and β-FNA at the receptor level appear to be different from the interactions of naltrexone and nor-BNI.

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REFERENCES

1. Adams, J. U.; Paronis, C. A.; Holtzman, S. G. Assessment of relative intrinsic activity of *mu*-opioid analgesics *in vivo* by using beta-funaltrexamine. J. Pharmacol. Exp. Ther. 255:1027–1032; 1990.
2. Birch, P. J.; Hayes, A. G.; Sheehan, M. J.; Tyers, M. B. Norbinaltorphimine: Antagonist profile at κ opioid receptors. Eur. J. Pharmacol. 144:405–408; 1987.
3. Comer, S. D.; Burke, T. F.; Lewis, J. W.; Woods, J. H. Clocinnamox: A novel, systemically active, irreversible opioid antagonist. J. Pharmacol. Exp. Ther. 262:1051–1056; 1992.
4. Corbett, A. D.; Kosterlitz, H. W.; McKnight, A. T.; Paterson, S. J.; Robson, L. E. Pre-incubation of guinea-pig myenteric plexus with *beta*-funaltrexamine: Discrepancy between binding assay and bioassays. Br. J. Pharmacol. 85:665–673; 1985.
5. Dewey, W. L.; Harris, L. S. The tail flick test. In: Ehrenpreis, S.; Neidle, A., eds. Methods in narcotic research. New York: Marcel Dekker; 1975:101–109.
6. Holtzman, S. G.; Cook, L.; Steinfels, G. F. Discriminative stimulus effects of spiradoline, a *kappa*-opioid agonist. Psychopharmacology (Berlin) 105:447–452; 1991.
7. Horan, P.; Taylor,J.; Yamamura, H. I.; Porreca, F. Extremely long-lasting antagonistic actions of nor-binaltorphimine (nor-BNI) in the mouse tail-flick test. J. Pharmacol. Exp. Ther. 260: 1237–1243; 1992.
8. James, I. F.; Goldstein, A. Site-directed alkylation of multiple opioid receptors. Mol. Pharmacol. 25:337–342; 1984.
9. Jones, D. N. C.; Holtzman, S. G. Long-term k-opioid receptor blockade following nor-binaltorphimine. Eur. J. Pharmacol. 215: 345–348; 1992.
10. Magnan, J.; Patterson, S. J.; Tavani, A.; Kosterlitz, H. W. The binding spectrum of narcotic analgesic drugs with different agonist and antagonist properties. Naunyn Schmiedebergs Arch. Pharmacol. 319:197–205; 1982.
11. Millan, M. J. *Kappa*-opioid receptor-mediated antinociception in the rat. I. Comparative actions of *mu*- and *kappa*-opioids against noxious thermal, pressure and electrical stimuli. J. Pharmacol. Exp. Ther. 251:334–341; 1989.
12. Mjanger, E.; Yaksh, T. L. Characteristics of dose-dependent antagonism by β-funaltrexamine of the antinociceptive effects of intrathecal *mu* agonists. J. Pharmacol. Exp. Ther. 258:544–550; 1991.
13. Portoghese, P. S.; Lipowski, A. W.; Takemori, A. E. Binaltorphimine and nor-binaltorphimine, potent and selective κ-opioid receptor antagonists. Life Sci. 40:1287–1292; 1987.
14. Sánchez-Blázquez, P.; Garzón, J. Evaluation of δ receptor mediation of supraspinal opioid analgesia by in vivo protection against the β-funaltrexamine antagonist effect. Eur. J. Pharmacol. 159: 9–23; 1989.
15. Takemori, A. E.; Ho, B. Y.; Naeseth, J. S.; Portoghese, P. S. Nor-binaltorphimine, a highly selective *kappa*-opioid antagonist in analgesic and receptor binding assays. J. Pharmacol. Exp. Ther. 246:255–258; 1988.
16. Tam, S. W.; Liu-Chen, L-Y. Reversible and irreversible binding of β-funaltrexamine to *mu*, *delta*, and *kappa* opioid receptors in guinea pig brain membranes. J. Pharmacol. Exp. Ther. 239:351–357; 1986.
17. Ward, S. J.; Portoghese, P. S.; Takemori, A. E. Pharmacological characterization *in vivo* of the novel opiate, β-funaltrexamine. J. Pharmacol. Exp. Ther. 220:494–498; 1982.
18. Ward, S. J.; Fries, D. S.; Larson, D. L.; Portoghese, P. S.; Takemori, A. E. Opioid receptor binding characteristics of the non-equilibrium μ antagonist, β-funaltrexamine (β-FNA). Eur. J. Pharmacol. 107:323–330; 1985.