Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
14. Shipley LA, Eacho PI, Sweeny DJ and Weiner M, Inhibition of glucuronidation and sulfation by dibutyryl cyclic AMP in isolated rat hepatocytes. *Drug Metab Dispos* **14**: 526–531, 1986.
15. Orrenius S, Andersson B, Jernstrom B and Moldeus P, Isolated hepatocytes as an experimental tool in the study of drug conjugation reactions. In: *Conjugation Reactions in Drug Biotransformation* (Ed. Aitio A), pp. 273–282. Elsevier/North Holland, Amsterdam, 1978.
16. Hers HG, The control of glycogen metabolism in the liver. *Annu Rev Biochem* **45**: 167–189, 1976.
17. Agius L, Peak L and Alberti KGMM, Regulation of glycogen synthesis from glucose and gluconeogenic precursors by insulin in periportal and perivenous rat hepatocytes. *Biochem J* **266**: 91–102, 1990.
18. De Wulf H and Hers HG, The stimulation of glycogen synthesis and of glycogen synthase in the liver by the administration of glucose. *Eur J Biochem* **2**: 50–56, 1967.
19. Kitagawa S, Ito Y, Oda Y and Kametani F, Inhibitory effects of phenol derivatives on bovine platelet aggregation and their effects on Ca^{2+} mobilization. *Biochim Biophys Acta* **1011**: 52–57, 1989.
20. Price VF and Jollow DJ, Mechanism of decreased acetaminophen glucuronidation in the fasted rat. *Biochem Pharmacol* **37**: 1067–1075, 1988.
21. Mulder GJ, Temmink TJM and Koster HJ, The effect of fasting on sulfation and glucuronidation in the rat *in vivo*. *Biochem Pharmacol* **31**: 1941–1944, 1982.
22. Conway JG, Kauffman FC and Thurman RG, Effect of glucose on 7-hydroxycoumarin glucuronide production in periportal and pericentral regions of the liver lobule. *Biochem J* **226**: 749–756, 1985.
23. Angus PW, Mihaly GW, Morgan DJ and Smallwood RA, Synergistic effects of hypoxia and fasting on harmol elimination in the isolated perfused rat liver. *Biochem Pharmacol* **37**: 1207–1212, 1988.

Biochemical Pharmacology, Vol. 42, No. 6, pp. 1302–1306, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

Effects of morphine tolerance-dependence and abstinence on κ-opiate receptors of rat brain and spinal cord

(*Received* 2 *November* 1990; *accepted* 25 *April* 1991)

Several attempts have been made to understand the possible role of opiate receptors in morphine tolerance-dependence and abstinence processes; however, the picture is far from clear. As indicated in a previous publication [1], factors which could give rise to different results include the degree of tolerance-dependence induced, the animal species used, the selectivity of various ligands used to characterize the receptors, whether or not the animals were undergoing abstinence and whether whole brain or various brain regions were used for the study of a specific receptor. In more recent studies using D-Ala^2,MePhe^4,Gly-ol^5-enkephalin (DAMGO), a highly selective ligand for the μ-opiate receptor [2], it has been shown that μ-receptors are down-regulated in the spinal cord and specific areas of the brain of morphine tolerant-dependent rats and guinea pigs [1, 3]; however, they are unaffected in the morphine abstinence process.

Little is known about the role of κ-opiate receptors in morphine tolerance-dependence and abstinence processes. Several lines of evidence suggest that endogenous or exogenous κ-opiate receptor agonists antagonize the actions of morphine in non-tolerant animals but potentiate them in morphine tolerant-dependent animals [4–7]. The intensity of dynorphin-(1-13)-induced catalepsy was found to be greater in sufentanil (a μ-opiate agonist) -tolerant rats than in non-tolerant rats [8]. Therefore, it can be expected that there may be changes in κ-opiate receptors induced by chronic treatment with morphine.

The present studies were undertaken to determine the binding of [^{3}H]ethylketocyclazocine ([^{3}H]EKC) and [^{3}H]-U-69,593 to membranes of discrete brain regions and spinal cord of morphine tolerant-dependent and abstinent rats. In addition, the effect of U-50,488H, a κ-opiate receptor agonist, on the thermoregulatory behavior in non-tolerant and morphine-tolerant rats also was determined.

Materials and Methods

Animals. Male Sprague–Dawley rats weighing 225–250 g (Sasco-King Animal Co., Oregon, WI) were acclimatized to a room with controlled ambient temperature (23 ± 10%), and a 12-hr dark–light cycle (light 6:00 a.m. to 6:00 p.m.). The animals were housed under these conditions for at least 4 days prior to being used and were given food and water continuously.

Chemicals. Morphine and placebo pellets, U-50,488H, U-69,593 and [^{3}H]EKC (sp. act. 35.0 Ci/mmol) were supplied by the National Institute on Drug Abuse, Rockville, MD, through the courtesy of Mr. Robert Walsh. [^{3}H]U-69,593 (sp. act. 58 Ci/mmol) was purchased from the New England Nuclear–Du Pont Corp., Boston, MA. Unlabeled levorphanol was a gift from the Hoffmann-LaRoche, Nutley, NJ.

Induction of tolerance to and physical dependence on morphine. Rats were rendered tolerant to, and physically dependent on, morphine by s.c. implantation of six morphine pellets during a 7-day period as described previously [9–11]. On the evening of day 7, the pellets were removed from one group of rats and they were killed 18 hr later (abstinent rats). The other set of rats in which pellets were left intact were killed on day 8 (tolerant-dependent but non-abstinent rats). The spinal cord (cervical to lumbar region) and brain regions (amygdala, hippocampus, hypothalamus, corpus striatum, midbrain, pons and medulla, and cortex) were isolated.

Determination of the binding of [^{3}H]*EKC and* [^{3}H]*U-69,593 to opiate receptors in discrete brain regions and*

spinal cord of morphine-tolerant-dependent and -abstinent rats. The binding of [^{3}H]EKC was performed according to a procedure described previously [1, 12] using a 2.5 nM concentration and 250–300 μg protein in each sample. The binding of [^{3}H]EKC was carried out in a total volume of 0.25 mL which contained 0.05 M Tris–HCl buffer and 0.1 mL of the homogenate. The binding of [^{3}H]EKC to μ and δ receptors was suppressed by using 100 nM DAMGO and 100 nM cyclic [D-penicillamine^2,D-penicillamine^5] enkephalin (DPDPE), respectively. All binding assays were carried out in triplicate at 37° for 30 min. Specific binding was defined as the difference in binding observed in the absence and presence of 10 μM levorphanol. The concentration of protein in the samples was determined by the method of Lowry *et al.* [13]. The amount of specific binding of the ligand was expressed as femtomoles per milligram of protein (mean ± SEM). The binding of [^{3}H]-U-69,593 was carried out in a manner analogous to that for [^{3}H]EKC except that the concentration range used for [^{3}H]-U-69,593 was 0.10 to 12.0 nM and nonspecific binding was defined by using 1 μM unlabeled U-69,593. The B_{max} and K_d values were calculated using the LIGAND program [14]. Five rats were used for each treatment group. The data were analyzed by analysis of variance followed by Scheffe's S-test. A value of $P < 0.05$ were considered to be significant.

Effect of U-50,488H on the body temperature of rats implanted with placebo and morphine pellets. To seek a

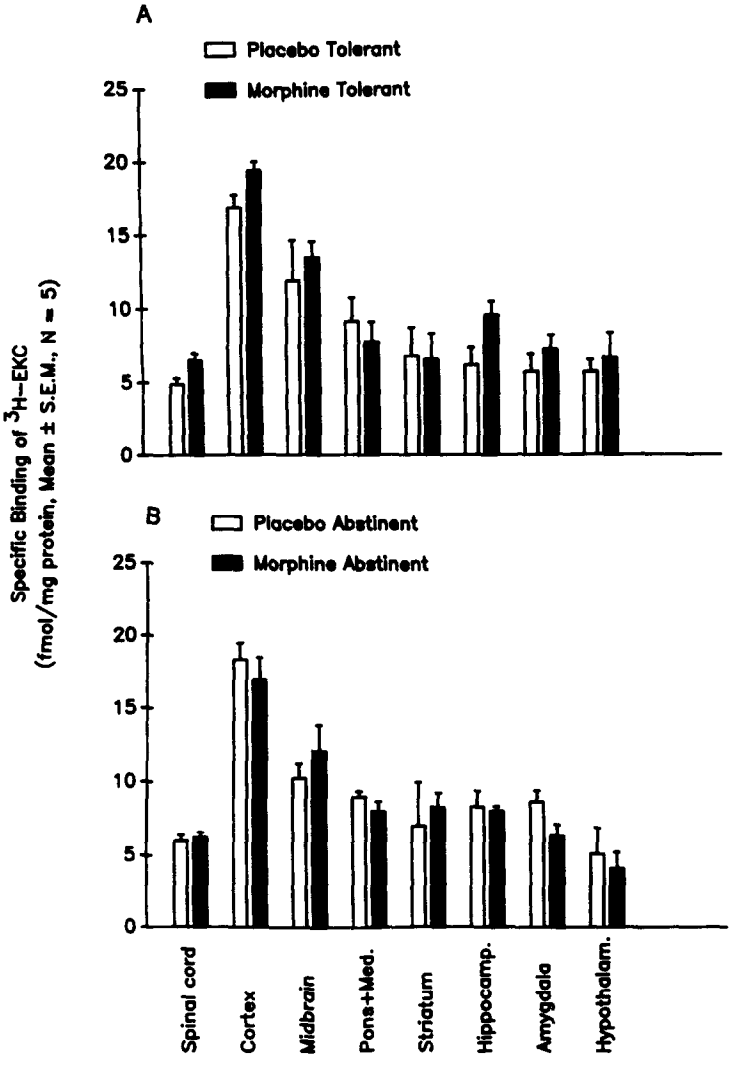

Fig. 1. Effects of morphine tolerance-dependence (A) and abstinence (B) on the binding of [^{3}H]EKC to κ-opiate receptors in spinal cord and discrete brain regions of the rat. Male Sprague–Dawley rats were implanted with six morphine or six placebo pellets as described in the text. The binding of [^{3}H]-EKC to tissue membranes was carried out at a 2.5 nM concentration. The specific binding of [^{3}H]EKC was defined as the difference in binding in the absence and presence of a 10 μM concentration of unlabeled levorphanol.

correlation between the receptor binding data and the behavioral data, the effect of a highly selective k-opiate receptor agonist, U-50,488H (10, 20 and 40 mg/kg, i.p.), on the colonic temperature of rats implanted with placebo and morphine pellets was determined. The colonic temperature of each rat was determined using a telethermometer [15]. The colonic temperature was measured prior to and at 30-min intervals after the injection of U-50,488H for a period of 300 min in rats from which placebo and morphine pellets had been removed 18 hr earlier. The change in temperature following U-50,488H administration was recorded at each time point and the area under the time–response curve ($AUC_{0\text{-}300\,min}$) was calculated. The data are expressed as mean $AUC_{0\text{-}300\,min} \pm$ SEM. Eight rats were used for each dose of U-50,488H. The difference in AUC for placebo and morphine-treated groups was determined by Student's *t*-test. A value of $P < 0.05$ was considered to be significant.

Results

Effects of morphine tolerance-dependence and abstinence on the binding of [^{3}H]*EKC and* [^{3}H]U-69,593 *to κ-opiate receptors on membranes of rat spinal cord and discrete brain regions.* The amount of [^{3}H]EKC specifically bound to brain regions and spinal cord of placebo and morphine pellet implanted rats did not differ (Fig. 1A) nor did the abstinence from morphine alter the binding of [^{3}H]EKC to any brain region or the spinal cord (Fig. 1B). The B_{max} and K_d values for the binding of [^{3}H]U-69,593 to brain regions and spinal cord of morphine tolerant-dependent (Fig. 2A) and morphine abstinent (Fig. 2B) rats did not differ from their respective control groups.

Effect of U-50,488H on the body temperature of rats implanted with placebo or morphine pellets. Intraperitoneal injection of U-50,488H into rats produced a hypothermic effect. The intensity and duration of the effect of different doses of U-50,488H administered to placebo pellet implanted rats are shown in Fig. 3A. The drug produced a dose-dependent decrease in the colonic temperature. The hypothermic response transformed into $AUC_{0\text{-}300\,min}$ is shown in Fig. 3B. The hypothermic effect of U-50,488H at any dose did not differ in placebo and morphine pellet implanted rats.

Discussion

The present studies clearly indicate that κ-opiate receptors of discrete brain regions and spinal cord of morphine tolerant-dependent or abstinent rats were unaffected. κ-Opiate receptors were labeled by using two ligands, [^{3}H]EKC and [^{3}H]U-69,593. Several studies have shown that EKC labels all three opiate receptors, namely, μ, δ and κ; however, under μ and δ receptor suppressed

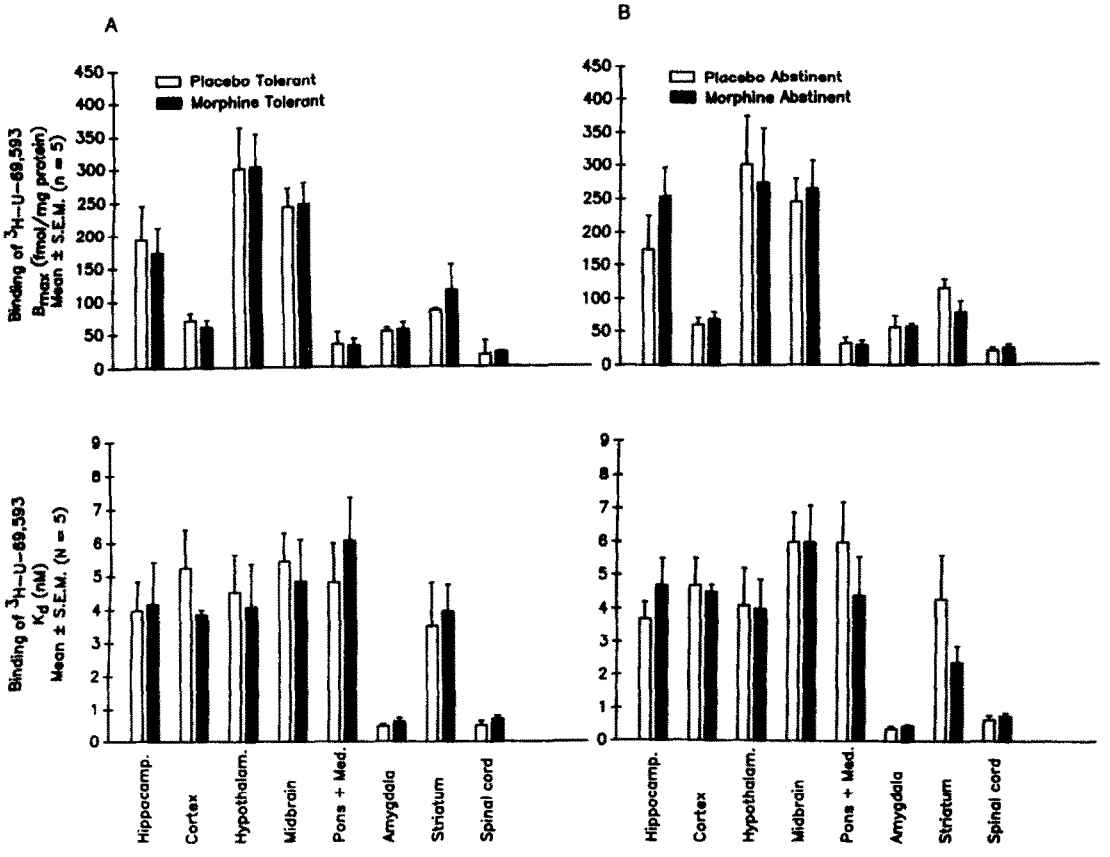

Fig. 2. Effects of morphine tolerance-dependence (A) and abstinence (B) on the binding of [^{3}H]U-69,593 to κ-opiate receptors in spinal cord and discrete brain regions. Male Sprague–Dawley rats were implanted with placebo or morphine pellets as described in the text.

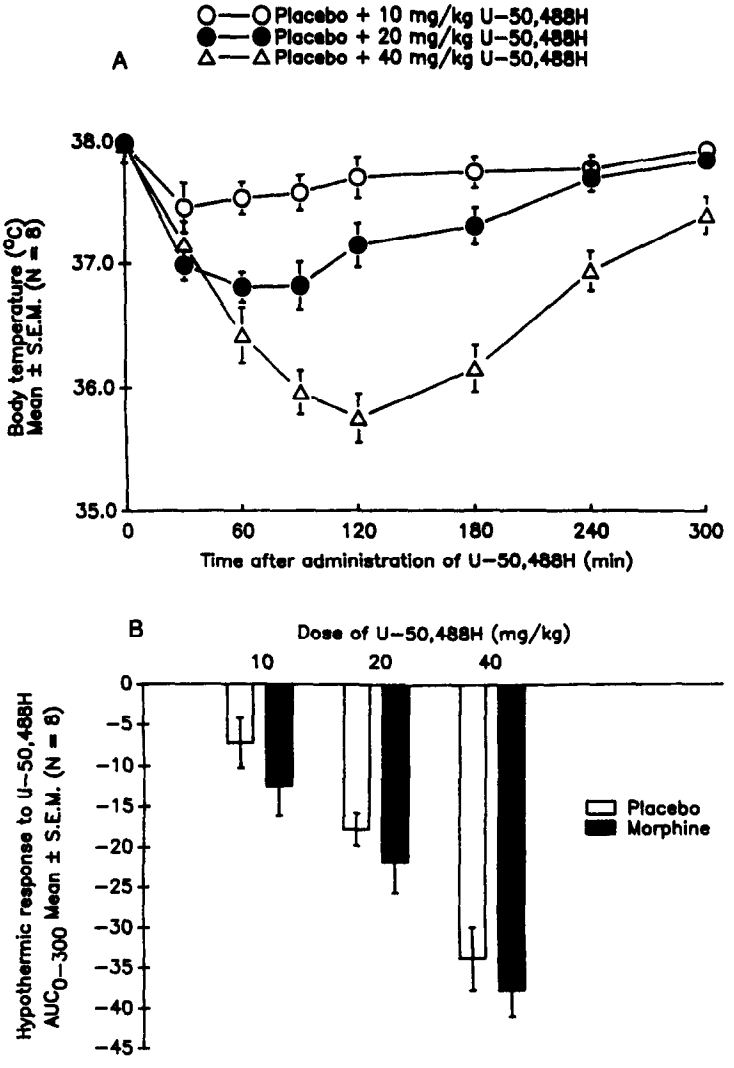

Fig. 3. Effect of different doses of U-50,488H on the time course of changes in colonic temperature of placebo pellet implanted rats (A) and an $AUC_{0-300min}$ for changes in temperature in placebo and morphine-abstinent rats (B).

conditions, it labels a high-affinity κ-opiate receptor. Since the binding of [^{3}H]EKC was unchanged, studies were carried out with a more selective ligand, [^{3}H]U-69,593, to get direct proof for the involvement of κ-opiate receptors in morphine actions. However, neither the receptor density nor the affinity for the binding of [^{3}H]U-69,593 to κ-opiate receptors was altered in the spinal cord or brain regions. Heterogeneity of κ-opiate receptors has been suggested [16]. The high-affinity sites labeled by [^{3}H]EKC and [^{3}H]-U-69,593 have been shown to be identical [17] and have been labeled as κ_1 sites [18, 19]. Based on this observation, it would appear that central, κ_1-opiate receptors are unaltered in morphine tolerance-dependence and abstinence processes. Werling *et al.* [3] showed indirectly by competition experiments for the binding of [^{3}H]-diprenorphine by U-50,488H in morphine tolerant guinea pig cortex that κ-receptors are not affected.

Behavioral evidence was also provided to substantiate the results of the binding experiments. U-50,488H, a highly selective κ-opiate agonist, produced a dose-dependent hypothermic response in non-tolerant rats. This response was unaltered in morphine tolerant-dependent rats.

Studies from this laboratory indicate that activation of κ-opiate receptors by U-50,488H does not alter the development of tolerance to the pharmacological effects of morphine [20]. Similarly, U-50,488H treatment fails to affect the development of dependence on morphine in the rat [21], giving further credence to the results obtained in the present studies.

In summary, the present studies demonstrate, using biochemical and behavioral experiments, that κ-opiate receptors in discrete brain regions and spinal cord are unaffected in morphine-induced tolerance-dependence and abstinence processes.

Acknowledgements—These studies were supported by United States Public Health Grant DA-02598 from the National Institute on Drug Abuse. The authors thank George A. Matwyshyn for technical assistance and Josephine M. Diaz for secretarial assistance.

Department of Pharmacodynamics (M/C 865)
The University of Illinois at Chicago
Health Sciences Center
Chicago
IL 60612, U.S.A.

HEMENDRA N. BHARGAVA*
ANIL GULATI
NAFASAT H. RAHMANI

REFERENCES

1. Bhargava HN and Gulati A, Down-regulation of brain and spinal cord μ-opiate receptors in morphine-tolerant-dependent rats. *Eur J Pharmacol* **190**: 305–311, 1990.
2. Goldstein A and Naidu A, Multiple opioid receptors: Ligand selectivity profiles and binding site signatures. *Mol Pharmacol* **36**: 265–272, 1989.
3. Werling LL, McMahon PN and Cox BM, Selective changes in μ-opiate receptor properties induced by chronic morphine exposure. *Proc Natl Acad Sci USA* **86**: 6393–6397, 1989.
4. Tulunay FC, Jen MF, Chang JK, Loh HH and Lee NM, Possible regulatory role of dynorphin on morphine and β-endorphin-induced analgesia. *J Pharmacol Exp Ther* **219**: 296–298, 1981.
5. Rezvani A and Way EL, Dynorphin (1-13) restores the potency of morphine on tolerant guinea pig ileum. *Eur J Pharmacol* **102**: 475–479, 1984.
6. Ramarao P, Jablonski HI Jr, Rehder KR and Bhargava HN, Effect of κ-opioid receptor agonists on morphine analgesia in morphine-naive and morphine-tolerant rats. *Eur J Pharmacol* **156**: 239–246, 1988.
7. Bhargava HN, Kremer EK, Gibbons MO, Philips BJ, Driver JW and Chou M, Stereospecific effects of a κ-opiate antagonist on the actions of morphine in morphine-tolerant rats. *Eur J Pharmacol* **173**: 159–164, 1989.
8. Herman BH and Goldstein A, Cataleptic effects of dynorphin-(1-13) in rats made tolerant to a mu opiate receptor agonist. *Neuropeptides* **2**: 13–22, 1981.
9. Bhargava HN, Rapid induction and quantitation of morphine dependence in the rat by pellet implantation. *Psychopharmacology* **52**: 55–62, 1977.
10. Bhargava HN, Quantitation of morphine-tolerance induced by pellet implantation in the rat. *J Pharm Pharmacol* **30**: 133–135, 1978.
11. Bhargava HN and Gulati A, Modification of brain and spinal cord dopamine D_1 receptors labeled with [^{3}H]-SCH 23390 after morphine withdrawal from tolerant and physically dependent rats. *J Pharmacol Exp Ther* **252**: 901–907, 1990.
12. Bhargava HN and Gulati A, *Kappa* opioid receptor activity in spontaneously hypertensive rats. *J Pharmacol Exp Ther* **245**: 460–465, 1988.
13. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
14. Munson PJ and Rodbard D, LIGAND: A versatile computerized approach for the characterization of ligand binding systems. *Anal Biochem* **107**: 220–239, 1980.
15. Bhargava HN, Gulati A and Ramarao P, Effect of chronic administration of U-50,488H on tolerance to its pharmacological actions and on multiple opioid receptors in rat brain regions and spinal cord. *J Pharmacol Exp Ther* **251**: 21–26, 1989.
16. Su T-P, Further demonstration of *kappa* opioid binding sites in the brain: Evidence for heterogeneity. *J Pharmacol Exp Ther* **232**: 144–148, 1985.
17. Tiberi M, Payette P, Mongeau R and Magnan J, [^{3}H]-U69,593 binding in guinea-pig brain: Comparison with [^{3}H]ethylketazocine binding at the κ-opioid sites. *Can J Physiol Pharmacol* **66**: 1368–1372, 1988.
18. Zukin RS, Eghbali M, Olive D, Unterwald EM and Tempel A, Characterization and visualization of rat and guinea pig brain κ opioid receptors: Evidence for κ_1 and κ_2 opioid receptors. *Proc Natl Acad Sci USA* **85**: 4061–4065, 1988.
19. Tiberi M and Magnan J, Quantitative analysis of multiple κ-opioid receptors by selective and nonselective ligand binding in guinea pig spinal cord: Resolution of high and low affinity states of the κ_2 receptors by a computerized model-fitting technique. *Mol Pharmacol* **37**: 694–703, 1990.
20. Bhargava HN, Matwyshyn G and Ramarao P, The effect of U-50,488H, a κ-opioid receptor agonist on tolerance to the analgesic and hyperthermic effects of morphine in the rat. *Gen Pharmacol* **22**: 429–434, 1991.
21. Fukagawa Y, Katz JL and Suzuki T, Effects of a selective κ-opioid agonist, U-50,488H, on morphine dependence in rats. *Eur J Pharmacol* **170**: 47–51, 1989.

* Correspondence: Dr. Hemendra N. Bhargava (M/C 865), Department of Pharmacodynamics, The University of Illinois at Chicago, 833 South Wood St., Chicago, IL 60612.