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UPREGULATION OF MU OPIOID RECEPTORS BY VOLUNTARY MORPHINE ADMINISTRATION IN DRINKING WATER*

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Morphine was provided to rats in drinking water for 21 days. Profound analgesic tolerance was detected both in hot-plate and tail-flick tests. The density of [^{3}H]DAMGO binding sites increased by 76% in spinal cord membranes due to morphine exposure compared to those in opioid naive animals. Slightly augmented [^{3}H]DAMGO binding was measured in the synaptic plasma membranes, with a concomitant decrease in the microsomal membranes, of morphine tolerant/dependent brains. These observations suggest that the regulation of spinal mu opioid receptors might be different from those in the brain. It is emphasized that the molecular changes underlying tolerance/dependence are influenced by several factors, such as the tissue or subcellular fractions used, besides the obvious importance of the route of drug administration. Results obtained after voluntary morphine intake further support the growing number of experimental data that chronic morphine does not internalize/downregulate the mu opioid receptors in the central nervous system.

Keywords: Opioid – upregulation – tolerance – morphine – spinal cord

INTRODUCTION

Opioid addiction is a central issue in neurobiology whose cellular and biochemical bases have not been fully elucidated. Tolerance implies that repeated drug administration decreases the sensitivity to opioids, with the consequence that higher doses are required to elicit the same effect. Dependence is defined as a need for continuous administration of a drug to avoid withdrawal symptoms. The abstinence syndromes are generally qualitatively opposite to the effect of the drug that induced the dependence. Thus, because the acute opioid effects are usually depressant, morphine dependence has been referred to as a state of "latent hyperexcitability" [19].

Both the supersensitivity and the subsensitivity of most G protein coupled receptors (GPCRs) are mutual expressions of cellular homeostatic mechanism that compensate for chronic changes in the net stimulus the cells receive. Maria Wollemann became interested in receptor sensitization and desensitization in the 1970s, and

* Dedicated to Professor Maria Wollemann on the occasion of her 80th birthday.

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revealed that hypersensitivity to isoproterenol in rabbit heart decreases guanine nucleotide effect on adenylate cyclase [24]. In analogy to other GPCRs, the prevailing hypothesis of opioid tolerance assumed receptor desensitization (G protein uncoupling) and downregulation as underlying mechanisms of the hyposensitivity that follows chronic opioid exposure [6, 26]. However, numerous studies have demonstrated no substantial downregulation in the number of mu opioid receptors, even in profoundly tolerant animals [6, 25, 26]. We have shown that the density of surface mu opioid receptors was not changed after 5-day *subcutaneous* morphine treatment, but significantly increased with a higher dose upon 10-day drug injection [11]. Moreover, the number of mu binding sites in a light vesicle or microsomal fraction (MI) was elevated by 68% and 30% after 5 and 10 days of morphine exposure, respectively [11].

Multiple forms of tolerance to and dependence on opioids have been characterized in terms of time of development and specificity characteristics (for a review see [23]). When comparing different data one must pay careful attention to the strain and gender used, length of the treatment and the total dose of the drug [7]. The route of administration (oral, *intraperitone*al, *subcutaneous* or continuous with osmotic minipump or pellet implantation) is an obvious variable of potential importance (see above and [9]). Preclinical data show that tolerance develops differently following intermittent or continuous administration [10].

The goal of the present work was to mimic the voluntary drug seeking behavior of human addicts in rats freely exposed to morphine in drinking water. Membrane homogenate fractions of the brains and spinal cords of morphine tolerant/dependent animals were prepared, and changes in mu opioid receptor levels were determined by ligand binding experiments. Surface mu opioid receptors were augmented both in the spinal cord and rat brain membranes, albeit to a lesser extent in the latter.

MATERIALS AND METHODS

Materials

Morphine-HCl was obtained from Alkaloida Ltd. (Tiszavasvári, Hungary). Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO) and [^{3}H]DAMGO were obtained from Multiple Peptide System (San Diego, CA, USA) via the Drug Supply Program of NIDA (Rockville, MD, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

In vivo *morphine treatments*

Female Wistar rats were locally bred. The animals were housed three per cage on a 12-h light/dark cycle, with free access to food and water. All animal procedures met the guidelines of the European Community directives regulating animal research and

were approved by the Animal Care Committee of Szeged University and the Biological Research Center, Szeged, Hungary. The animals were randomly assigned to treatment groups and the observer was blind to the treatment administered. Control animals were maintained on normal tap water. Chronically morphine-treated rats were provided with increasing concentration of morphine-HCl in drinking water for 21 days *ad libitum* as presented in Table 1 according to a published protocol [2]. The total amount of morphine consumed was 1104 ± 224mg/kg/rat. Body weight was recorded for all rats, whereas fluid intake was estimated for each rat from average consumption by the three rats housed in each cage.

Table 1
The paradigm of chronic morphine treatment

Days	1	3	5	7	9	11	13	15	17	19–21
Morphine (mg/ml)	0.1	0.15	0.2	0.3	0.4	0.5	0.6	0.8	1.0	1.2

Hot-plate and tail-flick tests

Nociceptive sensitivity for control and morphine-treated animals was assessed on day 21 of the treatment using hot-plate and tail-flick techniques. The latency of licking one of the hind paws or jumping was measured on the hot-plate (52.5 °C, cut-off time: 60 s). The reaction time in the tail-flick test was determined by immersing the lower 5 cm portion of the tail in the hot water until the typical tail-withdrawal response was observed (51.5 °C water, cut-off time: 20 s). Baseline latencies were obtained immediately before and 30, 60 and 120 min after the drug injection (saline or 10 mg/kg morphine *s.c.*). Analgesic latencies were converted to percentage maximum possible effect (%MPE) by using the formula: %MPE = [(observed latency – baseline latency)/(cut-off time – baseline latency)] × 100.

Rat spinal cord membranes

Whole spinal cords of rats (usually 4) were homogenized in 10 volumes (v/w) of 50 mM Tris-HCl buffer (pH 7.4) at 700 rpm with 5 up-and-down strokes in a Braun Teflon-glass homogenizer on ice and centrifuged at 1,000 × g for 10 min. The resulting supernatants were stored on ice while this centrifugation step was repeated with the pellet resuspended in the original volume of buffer. The combined supernatants were spun at 20,000 × g for 25 min. Pellets were suspended in buffer, incubated at 37 °C for 30 min and centrifuged as above. The final pellets (P_2) were homogenized in 5 volumes (v/w) of 50 mM Tris-HCl (pH 7.4) buffer containing 0.32 M sucrose and stored at –80 °C for several months. Membranes were thawed prior to use, diluted in Tris buffer and spun at 20,000 × g for 25 min to remove sucrose. Final pellets

were suspended in appropriate volume of Tris buffer to yield 0.3–0.6 mg protein/ml and used for binding studies. Protein concentration was determined with the method of Bradford using bovine serum albumin as standard [4].

Subcellular fractionation of rat brain

Subcellular fractions of rat brains were prepared as described [11, 21]. Briefly, fresh forebrains were gently homogenized, and the homogenate centrifuged at 1,000 × g for 10 min. This centrifugation step was repeated with the resulting pellets. The combined supernatants were spun at 12,000 × g for 20 min. The pellets were suspended in 10% sucrose and subjected to consecutive centrifugations at 20,000 × g for 25 min and 14,000 × g for 20 min twice resulting in crude synaptic plasmamembranes (SPM). Crude microsomes (MI) were obtained from the 12,000 × g supernatant by consecutive 20,000 × g for 25 min and 165,000 × g for 1 h centrifugations. Purified SPM fractions were finally resolved on a 10%, 28.5% and 34% sucrose density step gradient centrifuged at 100,000 × g for 2 h, whereas purified MIs were obtained from a 10% and 28.5% gradient centrifuged at the same speed. SPM and MI fractions were diluted 3-fold with Tris buffer, pelleted at 100,000 × g for 1 h, suspended in 50 mM Tris-HCl (pH 7.4) buffer and used for binding experiments.

Radioligand binding assays

Homologous displacement assays were performed with 1 nM [^{3}H]DAMGO (Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol), 11 concentrations ($10^{-10} – 10^{-5}$ M) of unlabelled DAMGO and the membrane suspensions (200–300 μg protein) in 50 mM Tris-HCl pH 7.4 buffer in a final volume of 1 ml. The tubes were incubated at 25 °C for 1 h. The reaction was stopped by vacuum filtration through GF/C glass fibre filters (Whatman, NJ, USA) using a Brandel M24-R Cell Harvester (Gaithersburg, MD, USA). Filters were rapidly washed twice with 10 ml ice-cold 50 mM Tris-HCl (pH 7.4) buffer, air-dried and counted in a toluene-based scintillation cocktail in a Beckman LS 5000TD counter.

Data analysis

Untransformed binding data from homologous displacement curves were analyzed by means of the non-linear least-squares regression computer program, LIGAND to obtain K_D (dissociation constant) and B_{max} (densities of binding sites) values [15]. The data reported are means ±S.E.M.; the significances were calculated using Student's *t*-test. Analysis of variance (ANOVA) of data for repeated measures was used for overall behavioral effects, with the Newman–Keuls test for post-hoc comparison for differences between means.

RESULTS

The average daily fluid intake per rat (in ml, means ± S.E.M. of 9 rats in two independent experiments) is shown in Fig. 1. Fluid consumption slightly decreased from day 1 to day 21 for both control and morphine-treated animals. Consumption of the morphine containing water was consistently and significantly ($p < 0.0001$) lower

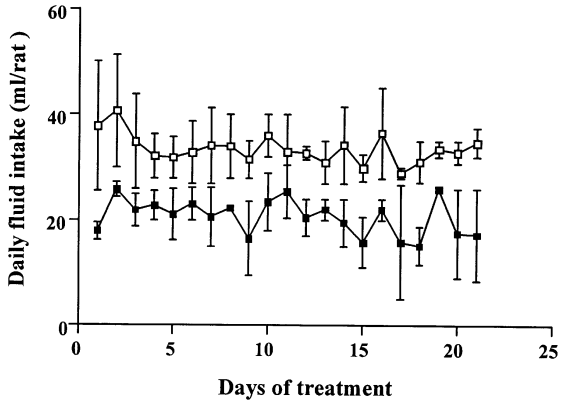

Fig. 1. Fluid intake by control and morphine-treated rats. Control rats (open squares) received drinking water, whereas morphine-treated animals (filled squares) received increasing concentrations of the drug for 21 days starting from 0.1 mg/ml on day 1 that was gradually elevated to 1.2 mg/ml by day 19, which was then kept constant until day 21 as described in Methods. Values are means ± S.E.M. of 9 rats in two independent experiments

Table 2
Binding parameters of [^{3}H]DAMGO in opioid-naive and morphine tolerant/dependent rat CNS

	K_D (nM)	B_{max} (fmol×mg protein^{-1})
Brain		
Control SPM	2.2±0.5	239±47
Control MI	2.4±0.9	310±32
Tolerant SPM	1.0±0.2	305±51
Tolerant MI	2.0±0.9	226±35
Spinal cord		
Control P_2	4.1±0.8	47±12
Tolerant P_2	4.0±1.5	83±19*

Rats were rendered tolerant by increasing doses of morphine-HCl in their drinking water for 21 days. Membrane preparation and subcellular fractionation was performed as in Methods. K_D and B_{max} values of [^{3}H]DAMGO homologous displacement experiments were calculated by computer program LIGAND. Values are means ± S.E.M., $n \geq 2$; *: $p < 0.1$.

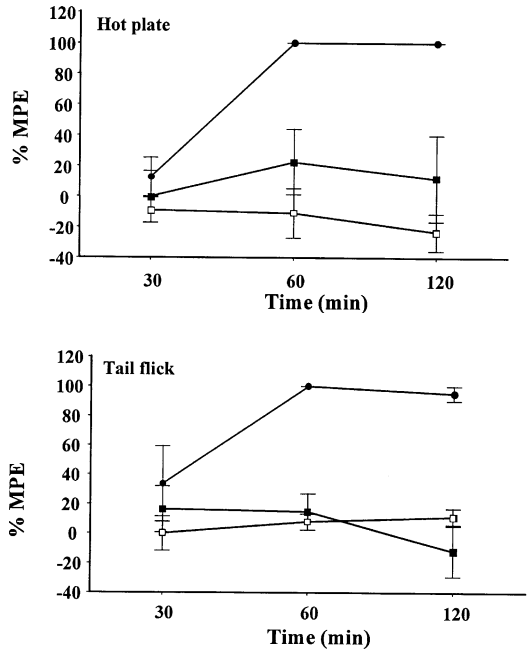

Fig. 2. Hot-plate and tail-flick analgesia tests of control and chronic morphine-treated rats. The % maximal possible effects (%MPE) of a challenge dose (10 mg/kg) of morphine at different times after the injection are shown. Water drinking for 21 days + saline *s.c.* on day 21 (open squares), water drinking +10 mg/kg morphine *s.c.* on day 21 (filled circle), morphine drinking for 21 days + 10 mg/kg morphine *s.c.* on day 21 (filled squares)

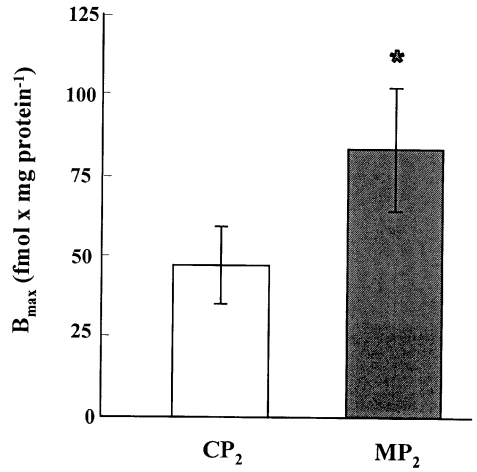

Fig. 3. Chronic morphine induced changes in the B_{max} of [^{3}H]DAMGO binding in rat spinal cord membranes. Animals were maintained on drinking water (C) or morphine solution (M) for 21 days as described in Materials and Methods. Results are means ± S.E.M. Significance was determined by Student's *t*-test, *: $p < 0.1$

than that of control rats during the 3-week period apparent already on day 1 where only 0.1 mg/ml morphine was added to the water. The body weight of control rats increased from 205 ± 13 to 229 ± 11 ($n = 9$, $p < 0.05$) in 21 days, whereas the body weight of morphine-treated animals was 197 ± 11 and 197 ± 8 ($n = 9$) on days 1 and 21, respectively.

As shown in Fig. 2, a single injection of 10 mg/kg morphine caused significant increases in the latency of the antinociceptive response in both the tail-flick and hot-plate tests in morphine-naive rats. Chronic administration of morphine for 21 days in drinking water significantly decreased the antinociceptive effect of morphine, thus resulting in morphine tolerant/dependent animals.

Control (C) and morphine-treated (M) tissue homogenates were simultaneously assessed in every experiment. The binding parameters (K_D, B_{max}) of [^{3}H]DAMGO binding were determined with homologous displacement experiments in spinal cord membranes. The receptor affinity (K_D) did not change due to chronic morphine treatment in these fractions (Table 2). There was an about 76% increase in the density of mu opioid receptors in spinal cord membranes due to chronic morphine exposure (Fig. 3).

Brain homogenates of control and morphine-treated rats were subjected to subcellular fractionation to result in highly enriched synaptic plasma membranes (SPM) and a 'light vesicle' also called microsomal (MI) fraction, the latter representing intracellular sites. The densities of mu binding sites measured with [^{3}H]DAMGO were 239 ± 47 and 309 ± 32 fmol × mg protein^{-1} in opioid naive SPM and MI fractions, respectively (Fig. 4). Although the B_{max} values were slightly elevated in the SPMs, with a concomitant decrease in the MIs, of morphine tolerant rat brains compared to those in control brains, these changes did not reach a statistically significant extent (Fig. 4, Table 2). Receptor affinities (K_D) did not change due to chronic morphine exposure in either SPM or MI fractions (Table 2).

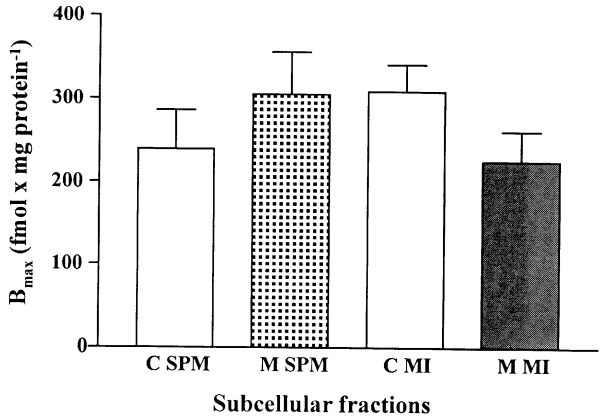

Fig. 4. Chronic morphine induced changes in the B_{max} of [^{3}H]DAMGO binding in rat brain membranes. Animals were maintained on drinking water (C) or morphine solution (M) for 21 days as described in Materials and Methods. Subcellular fractionations of brain homogenates were performed to prepare synaptic plasma membrane (SPM) and microsomal (MI) membranes. Results are means ± S.E.M.

DISCUSSION

Here we show that voluntary morphine intake in the drinking water results in a 76% upregulation of [^{3}H]DAMGO binding in spinal cord membranes. There was a trend toward increased [^{3}H]DAMGO binding in highly purified brain SPMs of the same morphine tolerant/dependent rats that, however, was not statistically significant. These data contribute to the growing number of experimental results revealing that the development of analgesic tolerance to morphine is not accompanied by internalization/downregulation of surface mu opioid receptors, changes hypothesized in accordance with the prevailing model of tolerance/dependence. Most studies on receptor regulation and trafficking have involved brain areas and periferal tissues, thus the present work extend the validity of the above statement to the spinal cord, an equally important site of opioid action.

The results for the surface opioid sites shown here agree with our previous data [11]. There, injection of morphine *subcutaneously* for 10 days resulted in 70% and 30% upregulation of the mu binding sites in the SPM and MI of brain homogenates, respectively. It should be noted that shorter treatment with lower drug dose (5 day, *s.c.*) did not affect the density of surface mu binding sites, but elevated those in the tolerant/dependent MI by 68% [11]. Such increase of the intracellular sites was not noticed in the present work where morphine was voluntarily taken by the animals for 21 days in the drinking water.

Conceivably, several variables can influence the results of such studies. Factors such as drug dose, length of the treatment and interdrug interval have been explored on tolerance development [8, 22]. Route of drug administration is an obvious variable of potential importance. It is interesting that while the majority of experiments involve intermittent daily drug injection (*subcutaneous* or *intraperitoneal*) in the literature dealing with the pharmacological characterization of morphine tolerance, continuous drug administration (with the pellet implantation technique) is more frequently used in studies examining the biochemical consequences of chronic morphine exposure. However, these procedures are stressful and may therefore release catecholamines, glucocorticoids or endogenous opioids, which then act at appropriate receptors thereby influencing the biochemical changes being measured [1]. The second disadvantage of the above drug administrations is that the experiments are performed many hours after stopping the drug administration thus the animals might already be in a state of withdrawal. Oral administration in drinking water may be a better alternative also because it mimics the voluntary nature of drug administration of human addicts. The aversion of the animals to consume morphine solution, presumably because of its bitter taste, was overcame by the initial provision of a very weak solution of the drug (0.1 mg/ml) followed by the continuous increase in its dose up to 1.2 mg/ml for 21 days according to Badawy et al. [2].

We show here that this paradigm results in a high degree of morphine tolerance both spinally and supraspinally (Fig. 2). Yet, the resulting changes (increase) in receptor numbers were qualitatively similar but quantitatively weaker in brain membranes. One can hypothesize that coupling of the mu opioid receptors with receptor-

regulating proteins (β-arrestin, dynamin, G protein-coupled receptor kinases) is somehow less effective in the brain than in the spinal cord. Alternatively, coupling of these receptor-associated signaling proteins may be loosen during the lengthy centrifugation process involved in the subcellular fractionation protocol applied to prepare brain SPM and MI, whilst the crude membrane preparation of the spinal cord involved less centrifugation steps. Previously, we detected translocation of dynamin immunoreactivity from intracellular pools to the SPM upon 5-day *s.c.* morphine injection [17]. The importance of β-arrestin in tolerance development was determined by showing that desensitization of the mu opioid receptor did not occur after chronic morphine treatment in mice lacking β-arrestin-2. In addition, these experiments have provided *in vivo* evidence that the mu opioid receptor is differentially regulated spinally and supraspinally [3]. Others hypothesize the presence of distinct mu receptor subtypes in the brain and spinal cord [18].

The present study is in accordance with a growing number of experimental data showing the inability of morphine to induce receptor internalization and downregulation. He et al. [13] proposed that endocytosis of mu opioid receptors can reduce the development of tolerance. Thus, the development of morphine tolerance must involve adaptational changes within receptor-associated signal transduction pathways [16]. Several groups have detected reduced mu opioid receptor signaling in the CNS following chronic morphine treatment [5, 20]. Others reported increased efficacy of opioid agonists at the mu opioid receptors in periaqueductal gray area, increased G protein coupling in intracellular membranes, or altered signaling as a consequence of sustained morphine treatment [11, 12, 14]. An upregulated number of receptors in the morphine tolerant/dependent state might play a role in opening up new signal transduction pathways that do not manifest in opioid naive tissues. The fact that multiple signaling pathways can be activated via a single agonist using a single receptor subtype through the G-protein transducers provides mechanisms to simultaneously involve many components in the process of cellular adaptation.

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REFERENCES

1. Adams, J. U., Andrews, J. S., Hiller, J. M., Simon, E. J., Holtzman, S. G. (1987) Effects of stress and beta-funaltrexamine pretreatment on morphine analgesia and opioid binding in rats. *Life Sci. 41*, 2835–2844.
2. Badawy, A. A.-B., Evans, C. M., Evans, M. (1982) Production of tolerance and physical dependence in the rat by simple administration of morphine in drinking water. *Br. J. Pharmac. 75,* 485–491.
3. Bohn, L. M., Lefkowitz, R. J., Caron, M. G. (2002) Differential mechanisms of morphine antinociceptive tolerance revealed in β arrestin-2 knock-out mice. *J. Neurosci. 22,* 10494–10500.
4. Bradford, M. M. (1986) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye-binding. *Anal. Biochem. 72*, 248–254.

5. Christie, M. J., Williams, J. T., North, R. A. (1987) Mechanisms of tolerance to opiates in locus coeruleus neurons. *NIDA Res Monogr. 78,* 158–168.
6. Cox, B. M. (1993) Opioid receptor-G protein interactions: acute and chronic effects of opioids. In: Born et al. (eds) *Handbook of Exp. Pharmacol. Opioids I.* Springer-Verlag, London, 145–188.
7. Craft, R. M., Stratmann, J. A., Bartok, R. E., Walpole, T. I., King, S. J. (1999) Sex differences in the development of morphine tolerance and dependence in rat. *Psychopharmacology 143,* 1–7.
8. Dafters, R. I., Odber, J. (1989) Effects of dose, interdose interval, and drug-signal parameters on morphine analgesic tolerance: Implications for current theories of tolerance. *Behav. Neurosci. 103,* 1082–1090.
9. Du, X., Skopp, G., Aderjan, R. (1999) The influence of the route of administration: a comparative study at steady state of oral sustained release morphine and morphine sulfate suppositories. *Ther. Drug Monit. 21*, 208–214.
10. Duttaroy, A., Yoburn, B. C. (1995) The effect of intrinsic efficacy on opioid tolerance. *Anesthesiology 82*, 1226–1236.
11. Fábián, G., Bozó, B., Szikszay, M., Horváth, G., Coscia, C. J., Szűcs, M. (2002) Changes in the subcellular distribution of mu opioid receptors and G-proteins in morphine-tolerant rat brains. *J. Pharmacol. Exp. Therap. 302*, 774–780.
12. Gintzler, A. R., Chakrabarti, S. (2000) Opioid tolerance and the emergence of new opioid receptor-coupled signaling. *Mol. Neurobiol. 21,* 21–33.
13. He, L., Fong, J., von Zastrow, M., Whistler, J. L. (2002) Regulation of opioid receptor trafficking and morphine tolerance by receptor oligomerization. *Cell 108,* 271–282.
14. Ingram, S. L., Vaughan, C. W., Bagley, E. E., Connor, M., Christie, M. J. (1998) Enhanced opioid efficacy in opioid dependence is caused by an altered signal transduction pathway. *J. Neurosci. 18,* 10269–10276.
15. Munson, P. J., Rodbard, D. (1980) Ligand, a versatile computerized approach for characterization of ligand binding systems. *Anal. Biochem. 107*, 220–239.
16. Nestler, E. J., Aghajanian, G. K. (1997) Molecular and cellular basis of addiction. *Science 278*, 58–63.
17. Noble, F., Szűcs, M., Kieffer, B., Roques, B. P. (2000) Overexpression of dynamin is induced by chronic stimulation of mu but not delta opioid receptors: relationship with mu-related morphine dependence. *Mol. Pharmacol. 58,* 159–166.
18. Paul, D., Bodnar, R. J., Gistrak, M. A., Pasternak, G. W. (1989) Different mu receptor subtypes mediate spinal and supraspinal analgesia in mice. *Eur. J. Pharmacol. 168*, 307–314.
19. Seevers, M. H., Deneau, G. A. (1963) Physiological aspects of tolerance and physical dependence. In: Root, W. S., Hoffmann, F. G. (eds) *Physiological Pharmacology.* Academic Press, New York, 565–640.
20. Selley, D. E., Nestler, E. J., Breivogel, C. S., Childers, S. R. (1997) Opioid receptor coupled G-proteins in rat locus coeruleus membranes: decrease in activity after chronic morphine treatment. *Brain Res. 746*, 10–18.
21. Szűcs, M., Coscia, C. J. (1992) Differential coupling of opioid binding sites to guanosine triphosphate binding regulatory proteins in subcellular fractions of rat brain. *J. Neurosci. Res. 31*, 565–572.
22. Tao, P. L., Lee, C. R., Law, P. Y., Loh, H. H. (1993) The interaction of the mu-opioid receptor and G protein is altered after chronic morphine treatment in rats. *Naunyn Schmiedebergs Arch. Pharmacol. 348,* 504–508.
23. Taylor, D. A., Fleming, W. W. (2000) Unifying perspectives of the mechanisms underlying the development of tolerance and physical dependence to opioids. *J. Pharmacol. Exp. Therap. 297*, 11–18.
24. Tkachuk, V. A., Wollemann, M. (1979) Hypersensitivity to isoproterenol in rabbit heart decreases guanine nucleotide effect on adenylate cyclase. *Biochem Pharmacol. 28*, 2097–2100.
25. Williams, J. T., Christie, M. J., Manzoni, O. (2001) Cellular and synaptic adaptations mediating opioid dependence. *Physiol. Rev. 81*, 299–343.
26. Zadina, J. E., Kastin, A. J., Harrison, L. M., Chang, S. L. (1995) Opiate receptor changes after chronic exposure to agonists and antagonists. *Annals N.Y. Acad. Sci. 757*, 353–360.