Morphine Withdrawal Syndrome and its Prevention With Baclofen: Autoradiographic Study of μ-Opioid Receptors in Prepubertal Male and Female Mice

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ABSTRACT Although the expression of the morphine (MOR) withdrawal syndrome is more marked in male mice than in females, we have demonstrated that the $GABA_B$ agonist baclofen (BAC) is able to attenuate MOR withdrawal signs in either sex. In order to extend these previous observations, the aim of the present study was to evaluate the μ-opioid receptor labeling in various brain areas in mice of either sex, during MOR withdrawal and its prevention with BAC. Prepubertal Swiss-Webster mice were rendered dependent by intraperitonial (i.p.) injection of MOR (2 mg/kg) twice daily for 9 days. On the 10th day, dependent animals received naloxone (NAL; 6 mg/kg, i.p.) 60 min after MOR, and another pool of dependent mice received BAC (2 mg/kg, i.p.) previous to NAL. Thirty minutes after NAL, mice were sacrificed and autoradiography with [^{3}H]-[D-Ala^2, *N*-Me-Phe^4, -glycol^5] enkephalin (DAMGO) was carried out on mice brains at five different anatomical levels. Autoradiographic mapping showed a significant increase of μ-opioid receptor labeling during MOR withdrawal in nucleus accumbens core (NAcC), caudate putamen (CPu), mediodorsal thalamic nucleus (MDTh), basolateral and basomedial amygdala, and ventral tegmental area vs. respective control groups in male mice. In contrast, opiate receptor labeling was not significantly modified in any of the brain areas studied in withdrawn females. BAC reestablished μ-opioid receptor binding sites during MOR withdrawal only in NAcC of males, and a similar tendency was observed in CPu and MDTh, even when it was not statistically significant. The sexual dimorphism observed in the present study confirms previous reports indicating a greater sensitivity of males in response to MOR pharmacological properties. The present results suggest that the effect of BAC in preventing the expression of MOR withdrawal signs could be related with the ability of BAC to reestablish the μ-opioid receptor labeling in certain brain areas. **Synapse 60:132–140, 2006.**

INTRODUCTION

Chronic administration of morphine (MOR) induces a dependent state characterized by neuroadaptive intracellular changes to an altered pharmacological condition. Spontaneous or pharmacologically precipitated abstinence from chronic opiate administration results in a variety of physiological and psychological withdrawal signs based on these adaptations of the neuronal system (Kirschke et al., 2002). A vast body of evidence points to an important role of endogenous opioids and their receptors in the mechanism of drug

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addiction (Turchan et al., 1999). In particular, the opioid antagonist naloxone (NAL) failed to induce any withdrawal classical sign in MOR-dependent mice lacking the μ-opioid receptor gene (Matthes et al., 1996).

Mounting evidence suggests that males are more sensitive than females to the MOR withdrawal syndrome (Craft et al., 1999; Diaz et al., 2001). Although the underlying mechanisms for these sex differences are still unknown, it has been suggested that changes in μ-opioid binding sites may account for this sexual dimorphism (Cicero et al., 1997; Craft, 2003).

Considering that MOR dependence as well as MOR withdrawal syndrome are due to both processes of homologous and heterologous regulation of neurotransmitter systems (Koob and Bloom, 1988), several brain areas have been related with these states. In a previous binding study (Diaz et al., 2004), we have observed that μ-opioid receptor density increased in the cortex (Cx) of NAL-precipitated withdrawn male mice as well as in the striatum of withdrawn male and female mice; therefore, both brain areas appear to be modified by the MOR withdrawal syndrome. The caudate-putamen (CPu), a part of dorsal striatum (Heimer et al., 1995), participates in planning and programming of voluntary movements, while the Cx, closely connected to the CPu, is involved in processing sensory information, delivery motor commands, behavioral planning, and cognitive capabilities of the brain (Kandel et al., 2000).

Adaptive changes have been observed after repeated opiates use in the ventral tegmental area (VTA), part of the mesolimbic dopaminergic system, a neuronal network critical for drug reward (Bonci and Williams, 1996, 1997; Self et al., 1995). Further, it is well known that drugs of abuse, like opiates, induce a positive reward by releasing dopamine (DA) from the VTA into the nucleus accumbens (NAc) (Koob, 1992).

The amygdala, a part of the limbic system, has been also implicated in the expression of MOR withdrawal signs (Stinus et al., 1990). The basolateral amygdala (BLA) could specifically mediate the negative motivational component of opiate withdrawal (Frenois et al., 2002). In addition, the bed nucleus of stria terminalis (BNST), a part of the extended amygdala, has also been involved in the mechanisms of opiate withdrawal (Maldonado, 1997). Another brain area suggested to play an important role in the precipitation of the physical signs of opiate withdrawal is the periaqueductal gray matter (PAG), mainly related with the expression of its motor component (Maldonado et al., 1992, 1995).

Hammer (1985) has reported a sexual dimorphism related to the density of μ-opioid binding sites in the medial preoptic area (MPOA), with a more dense concentration of labeling located in the MPOA of normal female and neonatally castrated male rats at 5 days old. To our knowledge, no data about a relation between MPOA and MOR withdrawal has been reported.

On the other hand, we have previously observed that the selective $GABA_B$ agonist baclofen (BAC) decreased the expression of the MOR withdrawal syndrome in male as well as female mice (Diaz et al., 2001), which is in agreement with previous results (Zarrindast and Mousa-Ahmadi, 1999). Additionally, we have also reported that BAC was able to reestablish the decreased μ-opioid receptor density induced by the NAL-precipitated withdrawal syndrome in the Cx of male mice and in the striatum of mice of either sex (Diaz et al., 2004).

To examine the μ-opioid receptor population in other brain areas and to enlighten our previous binding results, the aim of the present study was to analyze, using quantitative autoradiography, μ-opioid binding sites in several brain regions of mice during MOR dependence, NAL-precipitated withdrawal, and BAC pretreatment. Considering that many brain areas are known to be affected by the MOR-dependent and withdrawal states, it would be interesting to analyze if previous behavioral effect demonstrated for BAC is mediated by acting on μ-opioid receptor levels. Another purpose of this study was to evaluate if μ-opioid receptor labeling is sex-specific in our experimental conditions, given that sexually dimorphic results would provide a basis for the comprehension of sex differences in response to MOR withdrawal syndrome.

MATERIALS AND METHODS

Subjects

Experiments were performed on naïve prepubertal (indicated by vaginal smears) male and female Swiss–Webster albino mice weighing 20 g at the beginning of the treatment. On the day of the experiment (10th day), mice weighed 23–27 g. Animals were housed in groups of five under conditions of constant temperature (22 ± 2°C) and humidity (55 ± 15%) and in a 12-h light–dark cycle (light on 08:00 a.m.), according to local regulations (SENASA). The animals had free access to food and water up to the beginning of the experiments.

Drugs

Morphine hydrochloride (Chemotecnia Sintyal, Buenos Aires, Argentina), naloxone (Endo Laboratories, USA) and (±) baclofen (Ciba-Geigy, Basel, Switzerland) were used to develop this study. All of them were dissolved in isotonic (NaCl, 0.9%) saline solution. The dose of morphine refers to the salt form. The drugs were injected intraperitonially (i.p.) in a volume of 0.1 ml/10 g body weight of the animals.

Drug treatment

Mice of either sex were rendered dependent on MOR by i.p. injection of MOR (2 mg/kg), twice daily (8.00 a.m. and 8.00 p.m.), for 9 consecutives days.

On the day of the experiment (10th day), dependent male and female mice received the last dose of MOR only at 8.00 a.m. and then were randomly divided into three groups as follows:

- Dependent group: 30 and 60 min after the last dose of MOR, animals received a saline i.p. injection.
- MOR withdrawal group: 30 and 60 min after the last dose of MOR, mice received saline and NAL (6 mg/kg, i.p.) respectively, in order to precipitate the MOR withdrawal.
- Prevention group: 30 and 60 min after the last dose of MOR, animals received BAC (2 mg/kg, i.p.) and NAL (6 mg/kg, i.p.), respectively.

The control groups (male and female mice) received saline i.p. twice daily for 9 consecutives days. On the 10th day, they received the last injection of saline at 8.00 a.m. and were divided into three control groups:

- Saline control: 30 and 60 min after the last dose of saline, animals received a saline i.p. injection.
- NAL control: 30 and 60 min after the last dose of saline, mice received saline and NAL (6 mg/kg, i.p.), respectively.
- BAC control: 30 and 60 min after the last dose of saline, mice received BAC (2 mg/kg) and saline, respectively.

Preparation of brain sections

Thirty minutes after the last injection corresponding to each experimental group, mice ($n = 5$) were sacrificed and intact whole brains were removed immediately following cervical dislocation. Brains were rapidly frozen by immersion in Freon (-40°C) and stored at -80°C. Frozen coronal sections (12 μm) were cut at five different anatomical levels in a cryostat at -20°C, thawed, mounted onto gelatin-coated microscopic slides, and stored at -80°C until use (Antonelli et al., 1989).

Quantitative autoradiographic assays

Sections were processed for opioid autoradiography based on the technique previously described by Kitchen et al. (1997). Briefly, on the day of the experiment, slides were thawed at room temperature. Slides-mounted tissue sections were first preincubated for 30 min at room temperature in 50 mM Tris-HCl, pH 7.4. Sections were incubated for 60 min at room temperature in Tris HCl containing 4 nM [^{3}H] [D-Ala^2-*N*-methyl-Phe^4-Gly^{5}ol] enkephalin (DAMGO, specific activity = 69 Ci/mmol; Amersham, UK) to label the μ-opioid receptors. Nonspecific binding was determined with 4 μM DAMGO (RBI, Natick, MA). After incubation, slides were washed (3×5 min) in ice-cold buffer, dipped in ice-cold water, and air dried.

Film exposure and image analysis

Radiolabeled slides and calibrated tritium standards (Amersham Radiolabeled Chemicals, St. Louis, MO) were exposed to tritium-sensitive films Biomax MR1 at 4°C for 16 weeks. Films were developed in Kodak D-19 developer and fixative. Optical densities (OD) in brain regions of interest were measured using a computer-based densitometer, image analyzer (MCID-M4, Imaging Research Inc., St. Catherines, Ontario, Canada). Brain regions of interest were outlined and their ODs measured. Left and right side of four contiguous sections (eight measurements per subject-brain) represented total binding and four other sections represented nonspecific binding; the eight determinations were averaged for each subject, with $n = 4$–5 subjects for each treatment condition. Receptor binding levels were taken for the following regions: nucleus accumbens core (NAcC) and shell (NAcSh); CPu; motor cortex, deep layer (Cx); MPOA; lateral preoptic area (LPOA); BNST; BLA; basomedial amygdala (BMA); dorsomedial hypothalamus; mediodorsal thalamic nucleus (MDTh); VTA; substantia nigra pars reticular and pars compacta; periaqueductal gray. A single measurement was taken from the central structure interpeduncular nucleus. Structures were identified with reference to the mouse atlas of Franklin and Paxinos (1997). OD was converted to nCi/mg of tissue with calibrated methacrylate tritium standards, and after subtracting nonspecific from total binding, specific binding was expressed as fmol/mg tissue. Male and female mice of the six different experimental groups were processed together to ensure a paired protocol for binding, film apposition, and image analysis. The operator measuring the optical density was unaware of the experimental condition of each section.

Statistical analysis

To determine differences between the experimental groups for each brain area, two-way analysis of variance (ANOVA) with sex and treatment as the main factors was used, followed by corresponding one-way ANOVAs. Previously, normality of distribution and homoscedasticity were verified by Shapiro-Wilks test and Levene's test, respectively. Duncan test was used for post hoc comparisons. Differences were considered significant if the probability of error was less than 1%.

RESULTS

Statistical analysis is reported in Table I. Binding levels of MOR-withdrawn male mice significantly

TABLE I. Statistical analysis of [^{3}H] DAMGO autoradiographic study of μ-opioid receptor binding in different brain regions

	Two-way ANOVA						One-way ANOVA			
	Treatment		Sex		Interaction		Males		Females	
Brain region	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Nucleus accumbens core	$F_{(5, 41)} = 5.54$	<0.001	$F_{(1, 41)} = 0.14$	NS	$F_{(5, 41)} = 1.50$	NS	$F_{(5, 21)} = 5.80$	<0.001	$F_{(5, 20)} = 2.10$	NS
Nucleus accumbens shell	$F_{(5, 39)} = 2.67$	<0.05	$F_{(1, 39)} = 0.29$	NS	$F_{(5, 39)} = 0.79$	NS	$F_{(5, 20)} = 2.72$	0.05	$F_{(5, 19)} = 0.9$	NS
Caudate putamen	$F_{(5, 42)} = 2.21$	0.07	$F_{(1, 42)} = 0.87$	NS	$F_{(5, 42)} = 1.35$	NS	$F_{(5, 21)} = 3.92$	<0.05	$F_{(5, 21)} = 0.49$	NS
Motor cortex (deep layers)	$F_{(5, 42)} = 0.68$	NS	$F_{(1, 42)} = 3.03$	NS	$F_{(5, 42)} = 0.57$	NS	–	–	–	–
Medial preoptic area	$F_{(5, 42)} = 9.45$	<0.001	$F_{(1, 42)} = 4.50$	<0.05	$F_{(5, 42)} = 1.06$	NS	$F_{(5, 21)} = 6.75$	<0.001	$F_{(5, 21)} = 3.45$	<0.05
Lateral preoptic area	$F_{(5, 45)} = 8.71$	<0.001	$F_{(1, 45)} = 5.1$	<0.05	$F_{(5, 45)} = 1.07$	NS	$F_{(5, 21)} = 4.80$	<0.01	$F_{(5, 24)} = 4.83$	<0.01
Bed nucleus stria terminalis	$F_{(5, 46)} = 5.87$	<0.001	$F_{(1, 46)} = 1.02$	NS	$F_{(5, 46)} = 0.45$	NS	$F_{(5, 22)} = 2.47$	NS	$F_{(5, 24)} = 4.64$	<0.01
Basolateral amygdala	$F_{(5, 43)} = 2.13$	0.08	$F_{(1, 43)} = 0.23$	NS	$F_{(5, 43)} = 0.64$	NS	$F_{(5, 20)} = 3.62$	<0.05	$F_{(5, 23)} = 0.21$	NS
Basomedial amygdala	$F_{(5, 44)} = 2.18$	0.07	$F_{(1, 44)} = 0.77$	NS	$F_{(5, 44)} = 1.14$	NS	$F_{(5, 21)} = 2.77$	<0.05	$F_{(5, 23)} = 0.65$	NS
Dorsomedial hypothalamus	$F_{(5, 45)} = 4.10$	<0.01	$F_{(1, 45)} = 0.15$	NS	$F_{(5, 45)} = 0.26$	NS	$F_{(5, 22)} = 2.83$	0.05	$F_{(5, 23)} = 1.85$	NS
Mediodorsal thalamic nucleus	$F_{(5, 43)} = 2.3$	0.06	$F_{(1, 43)} = 1.40$	NS	$F_{(5, 43)} = 1.20$	NS	$F_{(5, 20)} = 2.79$	<0.05	$F_{(5, 23)} = 0.96$	NS
Periaqueductal gray	$F_{(5, 40)} = 2.21$	NS	$F_{(1, 40)} = 0.15$	NS	$F_{(5, 40)} = 0.44$	NS	–	–	–	–
Ventral tegmental area	$F_{(5, 37)} = 3.69$	<0.01	$F_{(1, 37)} = 3.29$	NS	$F_{(5, 37)} = 0.36$	NS	$F_{(5, 20)} = 2.57$	<0.05	$F_{(5, 17)} = 1.63$	NS
Substantia nigra pars reticular	$F_{(5, 40)} = 1.88$	NS	$F_{(1, 40)} = 2.18$	NS	$F_{(5, 40)} = 0.65$	NS	–	–	–	–
Substantia nigra pars compacta	$F_{(5, 40)} = 2.73$	NS	$F_{(1, 40)} = 0.38$	NS	$F_{(5, 40)} = 0.26$	NS	–	–	–	–
Interpeduncular nucleus	$F_{(5, 32)} = 0.52$	NS	$F_{(1, 32)} = 0.66$	NS	$F_{(5, 32)} = 1.00$	NS	–	–	–	–

Two-way ANOVA with treatment and sex as main factors and subsequent one-way ANOVA to find differences between treatments for each sex. See Materials and Methods for details.

increased in NAcC ($P < 0.01$) compared with that of respective SAL control group. Additionally, BAC pretreatment (prevention group) induced a significant decrease in binding levels of NAcC compared with that of withdrawal group ($P < 0.01$). In contrast, no differences were found between female experimental groups for NAcC (Figs. 1, 2, and Table II).

Binding levels in MOR-withdrawn male mice significantly increased in CPu, MDTh, BLA, BMA, and VTA ($P < 0.01$) compared with that of respective SAL control groups. BAC pretreatment (prevention group) showed a tendency to decrease binding levels compared with withdrawal group in CPu and MDTh ($P < 0.05$), although it was not significant. No differences were found between female experimental groups neither for CPu, MDTh, VTA, BLA, nor for BMA (Figs. 1, 2, and Table II).

Binding levels in MPOA of NAL control, MOR withdrawal and prevention groups significantly increased ($P < 0.01$) in males compared with that in the SAL control group, and LPOA binding levels in prevention group significantly increased ($P < 0.01$) with respect to the SAL control group. In females, LPOA binding levels in NAL control group significantly increased ($P < 0.01$) compared with the SAL control group, and BNST binding levels in NAL control and prevention groups significantly increased ($P < 0.01$) with respect to the SAL control group (Table II).

No significant changes were observed for binding levels in any of the other brain areas studied (Tables I and II).

DISCUSSION

The present experimental results suggest that μ-opioid binding sites are dramatically affected in specific brain regions of male mice, but not of females, during NAL-precipitated withdrawal syndrome. In addition, the $GABA_B$ agonist, BAC is able to reestablish μ-opioid levels modified by MOR withdrawal only in NAcC, and showed a similar tendency in CPu and MDTh, suggesting that these brain areas might play a role in the prevention of the expression of MOR withdrawal signs previously reported for BAC (Diaz et al., 2001).

In the present study, mice received low doses of MOR (2 mg/kg), and although this experimental protocol positively induced MOR dependence (Diaz et al., 2001), we observed herein that μ-opioid binding sites were modified during the dependent state in neither brain area nor sex. These results are in agreement with previous studies, where it was observed that chronic treatment with low intrinsic efficacy agonists like MOR may cause blunting of receptor signaling without changes in surface receptor number (Abdelhamid and Takemori, 1991; Diaz et al., 2004; Patel et al., 2002). Similar results in dependent animals

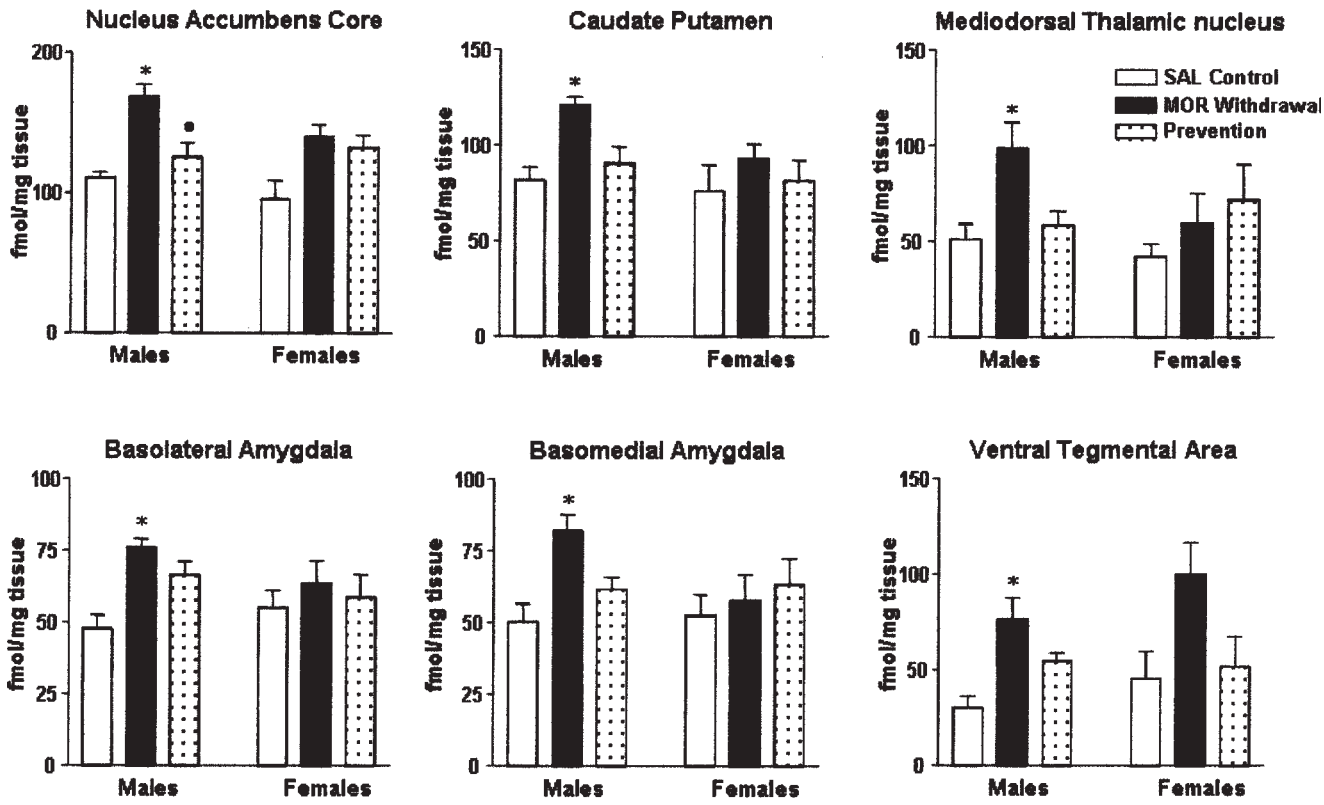

Fig. 1. Autoradiography of μ-opioid receptor binding (fmol/mg tissue) in male and female mice following MOR withdrawal and its prevention with BAC in the nucleus accumbens core, caudate putamen, mediodorsal thalamic nucleus, basolateral and basomedial amygdala, and ventral tegmental area. Data are expressed as mean ± SEM (n = 4–5 for each group). *$P < 0.01$ compared with saline (SAL) control group; •$P < 0.01$ compared with MOR withdrawal group (two-way ANOVA; Duncan test).

have lead to the concept that changes in receptor density cannot completely explain the mechanism of dependence development (van Bockstaele and Commons, 2001).

Chronic MOR administration has been shown to up-regulate the cAMP system in the NAc (Terwilliger et al., 1991). These changes could be viewed as a homeostatic compensatory adaptation to the chronic opioid receptor-induced inhibition of cAMP activity in the NAc, which leads to overstimulation of this system during withdrawal (Self and Nestler, 1995), and thereby underlies the negative motivational state during withdrawal (Koob et al., 1989; Stinus et al., 1990). In this context, the effect of the NAL-precipitated withdrawal on the μ-opioid population of the NAcC of male mice could also be considered to contribute to the aversive state of withdrawal. Considering that the psychological symptoms characteristic of the opiate withdrawal syndrome have been mapped partly to the mesolimbic DA system, which arises from dopaminergic neurons in the VTA and projects to the NAc and other forebrain limbic structures (Koob et al., 1998), the increase in μ-opioid receptor labeling in VTA and NAcC could be regarded as a compensatory mechanism tending to alleviate the abrupt absence of the reinforcing stimulus. Since the time between NAL injection and brain removal (30 min) argues against the possibility of μ-opioid receptor synthesis as the cause of the increase in binding levels, we rule out an enhancement of μ-opioid receptor expression during NAL-precipitated withdrawal.

The striatal distribution of μ-opioid receptors observed herein in mice of either sex showed a clearly patchy appearance irrespective of the drug treatment, which is in good agreement with earlier reports in rodents (Caboche et al., 1991; Loughlin et al., 1992; Smith et al., 1993). We have recently observed that μ-opioid receptor density increased during MOR withdrawal in male and female striatum as well as in male Cx (Diaz et al., 2004). In concordance with that, the present study shows a μ-opioid labeling increase in male CPu during NAL-precipitated withdrawal. Conversely, we neither observed increased [^{3}H]DAMGO binding in the CPu of withdrawn females nor in the Cx of males. By means of saturation binding studies, only extracellular receptors can be detected, whereas in autoradiographic studies, extracellular as well as intracellular receptors are labeled (Kuhar and Unner-

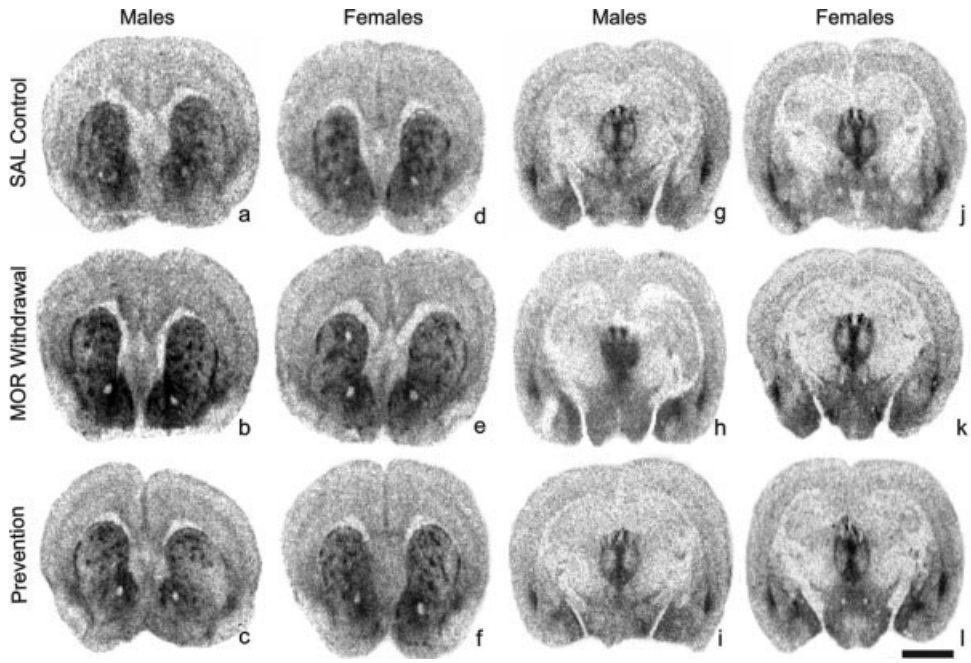

Fig. 2. [^{3}H]DAMGO autoradiograms of μ-opioid receptor binding in mice of SAL control, MOR withdrawal and prevention groups. The first and second columns show sections cut at the level of the nucleus accumbens core and caudate putamen (bregma 1.18 mm) in males (**a**, **b**, and **c**) and females (**d**, **e**, and **f**). The third and fourth columns show sections cut at the level of the mediodorsal thalamic nucleus (bregma −1.70 mm) in males (**g**, **h**, and **i**) and females (**j**, **k**, and **l**). Scale, 2 mm.

stall, 1990). Additionally, these authors stated that autoradiography can be three to five orders of magnitude more sensitive than biochemical method. Therefore, results provided by both methodologies are complementary and can be useful in this case to better characterize the changes observed. Based on the differences between homogenate preparations and tissue slices, we hypothesize that the increased density observed in female striatum and male Cx in our previous homogenate binding study might have reflected in part a process of increased expression at the plasma membrane of μ-opioid receptor contained in cytoplasmic vesicles, which cannot be evidenced by tissue slices binding. Another source of variability between saturation binding assays and autoradiography refers to the regions covered by each technique, i.e., while Cx homogenates represent this entire brain area, only the deep layer of a specific anatomical level was evaluated in the present study.

A decrease in the number of *c-fos* expressing neurons has been described in the BLA of rats during the NAL-precipitated withdrawal (Frenois et al., 2002), but there is a paucity of data about the participation of the BLA, BMA, and DMTh in the motivational component or the somatic aspect of withdrawal syndrome. In the light of the present results, these brain areas appear to be affected by the NAL-precipitated withdrawal; therefore, it would be interesting to further investigate the role of these brain regions in the development of the MOR withdrawal syndrome.

The dose of NAL (6 mg/kg) administered was selected after trying increasing doses of NAL in order to precipitate MOR withdrawal in mice chronically treated with MOR (2 mg/kg) twice daily. The NAL control group did not show any significant difference with respect to the SAL control group, indicating that the dose of NAL was not able to precipitate an abstinence syndrome in nondependent animals (Diaz et al., 2001). Therefore, the changes observed in MOR-abstinent mice depend only on the combination of the MOR-dependent state plus the administration of NAL. The results obtained in the male MPOA and LPOA, and in the female LPOA and BNST indicate that NAL could have a per se effect, given the fact that groups receiving NAL (i.e., NAL control, MOR withdrawal and prevention) suffered an increase in binding levels in these particular brain areas. Even when these NAL effects are difficult to explain, pharmacological properties may vary significantly from one brain region to another (Sadée et al., 2005). Furthermore, to our knowledge, no relations have been established between these brain areas and the MOR withdrawal state. On the other hand, it has been demonstrated that in basal conditions, the density of μ-opioid binding sites is sexually dimorphic in the MPOA of rats (Hammer et al., 1985), but our results failed to

TABLE II. [^{3}H]DAMGO autoradiography of μ-opioid receptor binding (fmol/mg tissue) in different brain regions of male and female mice

Brain Region	SAL control	Dependence	NAL control	Withdrawal	BAC control	Prevention
Nucleus accumbens core						
Male	110.7 ± 4.1	115.6 ± 12.3	140.1 ± 9.58	168.4 ± 8.8*	107.7 ± 13.2	125.5 ± 9.9**
Female	95.3 ± 13.5	112.0 ± 13.6	138.2 ± 10.7	139.4 ± 9.0	136.4 ± 13.3	132.1 ± 8.7
Nucleus accumbens shell						
Male	97.8 ± 5.5	100.4 ± 10.2	118.2 ± 10.9	140.0 ± 13.0	87.7 ± 10.5	115.2 ± 11.8
Female	83.3 ± 15.6	96.7 ± 14.5	116.6 ± 13.5	114.7 ± 9.8	111.6 ± 14.9	113.3 ± 4.2
Caudate putamen						
Male	81.8 ± 6.9	84.2 ± 10.0	95.6 ± 6.6	120.8 ± 4.3*	74.9 ± 14.5	90.4 ± 8.6
Female	76.0 ± 13.7	86.0 ± 10.6	86.6 ± 8.4	92.9 ± 7.7	95.0 ± 7.2	81.5 ± 10.6
Motor cortex (deep layers)						
Male	20.2 ± 3.2	21.5 ± 6.5	27.9 ± 5.0	32.4 ± 5.8	24.0 ± 9.0	25.5 ± 6.7
Female	15.4 ± 2.0	22.8 ± 4.9	19.0 ± 2.1	18.1 ± 3.7	26.9 ± 5.6	18.7 ± 3.6
Medial preoptic area						
Male	14.7 ± 1.8	16.6 ± 2.7	46.6 ± 7.1*	50.4 ± 6.6*	20.4 ± 2.5	43.6 ± 12.2*
Female	19.2 ± 2.0	12.3 ± 3.2	35.1 ± 4.5	34.4 ± 8.1	20.7 ± 1.6	27.8 ± 6.6
Lateral preoptic area						
Male	34.7 ± 8.4	39.6 ± 7.9	61.9 ± 6.1	65.9 ± 7.1	35.0 ± 7.0	85.3 ± 16.9*
Female	37.2 ± 4.6	28.7 ± 3.9	74.0 ± 6.7*	66.3 ± 11.6	50.3 ± 7.4	66.7 ± 11.8
Bed nucleus stria terminalis						
Male	44.5 ± 12.7	47.0 ± 7.7	78.2 ± 14.0	70.7 ± 7.3	48.3 ± 12.0	93.2 ± 17.8
Female	37.9 ± 3.6	35.6 ± 6.9	74.3 ± 8.0*	68.4 ± 12.6	57.1 ± 5.0	71.8 ± 8.5*
Basolateral amygdale						
Male	47.7 ± 4.8	52.0 ± 8.3	60.4 ± 5.5	76.1 ± 2.9*	61.7 ± 4.0	66.4 ± 4.8
Female	55.1 ± 5.9	55.3 ± 12.4	60.4 ± 3.6	63.6 ± 7.8	60.5 ± 4.3	58.8 ± 7.7
Basomedial amygdale						
Male	50.3 ± 6.3	52.8 ± 8.7	64.8 ± 6.9	82.0 ± 5.7*	56.7 ± 8.0	61.5 ± 4.3
Female	52.5 ± 7.3	48.7 ± 9.9	61.7 ± 2.6	57.9 ± 8.8	62.5 ± 5.4	63.4 ± 9.0
Dorsomedial hypothalamus						
Male	52.4 ± 4.6	56.5 ± 9.2	84.5 ± 5.7	86.5 ± 8.8	63.3 ± 9.7	74.5 ± 12.2
Female	56.6 ± 7.7	62.6 ± 15.2	76.0 ± 6.8	88.9 ± 15.1	58.0 ± 6.6	86.3 ± 10.7
Mediodorsal thalamic nucleus						
Male	51.1 ± 8.0	53.1 ± 17.7	53.0 ± 7.1	98.7 ± 13.5*	55.9 ± 8.6	58.5 ± 7.4
Female	41.9 ± 6.6	41.0 ± 9.1	47.8 ± 5.3	59.3 ± 16.0	60.5 ± 11.4	71.8 ± 18.5
Periaqueductal gray						
Male	55.7 ± 6.7	59.8 ± 12.0	73.5 ± 8.7	85.2 ± 11.8	59.2 ± 19.8	88.8 ± 12.2
Female	56.5 ± 13.0	75.2 ± 23.5	58.7 ± 17.4	98.9 ± 17.2	70.4 ± 10.9	80.5 ± 6.3
Ventral tegmental area						
Male	30.1 ± 6.0	45.2 ± 12.4	48.3 ± 11.7	76.5 ± 11.2*	47.3 ± 10.6	54.5 ± 4.4
Female	45.4 ± 14.2	73.7 ± 26.7	59.2 ± 14.2	99.9 ± 16.7	54.1 ± 10.7	51.8 ± 15.8
Substantia nigra pars reticular						
Male	55.7 ± 10.7	70.4 ± 12.0	69.6 ± 12.4	74.5 ± 7.2	50.9 ± 7.6	75.9 ± 7.4
Female	56.2 ± 11.9	76.0 ± 24.9	76.1 ± 18.7	107.9 ± 18.9	74.4 ± 11.2	72.2 ± 11.7
Substantia nigra pars compact						
Male	50.3 ± 7.2	54.3 ± 13.6	69.6 ± 10.7	87.8 ± 13.6	57.3 ± 16.8	77.1 ± 15.9
Female	49.5 ± 10.0	70.4 ± 21.1	67.1 ± 14.0	99.2 ± 13.9	68.3 ± 9.7	69.5 ± 5.7
Interpeduncular nucleus						
Male	95.3 ± 28.2	124.6 ± 31.1	140.9 ± 21.3	140.5 ± 14.2	96.8 ± 29.8	100.7 ± 20.7
Female	126.2 ± 24.6	104.5 ± 19.3	124.1 ± 21.1	126.4 ± 29.5	120.0 ± 20.4	166.0 ± 9.3

Values are expressed as mean ± SEM of 4 to 5 animals per experimental group.*$P < 0.01$ vs. SAL control group; **$P < 0.01$ vs. MOR withdrawal group (Two-way ANOVA; Duncan test).

extend this sexual dimorphism to Swiss-Webster mice, considering that no significant difference in μ-opioid labeling was detected between either sex in SAL control groups.

The data obtained in the present study is difficult to analyze, given that, as each brain area was analyzed with a separate two-way ANOVA, the possibility of making a type-I error increases. Classical procedures that control experimental-wise errors in the strong sense in single-comparison problems tend to have substantially less power than the per comparison procedure of the same levels (Benjamini and Hochberg, 1995). This is the reason why we combined a post hoc test with a good power (Duncan test) and set the type-I error in 0.01. However, in that case, there are less chances of finding statistically significant differences. The $GABA_B$ agonist BAC was able to reestablish the basal levels of μ-opioid labeling decreased by the NAL-precipitated withdrawal syndrome only in the NAcC of male mice, and a similar tendency was observed in CPu and MDTh. On the contrary, BAC was not able to reestablish the changes induced by the MOR withdrawal syndrome in ABL, ABM, or VTA. These effects could anticipate which brain areas would be more relevant for the attenuation of MOR withdrawal signs. The dose of BAC (2 mg/kg) was selected based on our previous reports (Diaz et al., 2001, 2003, 2004), and this dose did not have intrinsic effect in nondependent animals. In agreement, a similar dose of BAC was reported by other authors (Fadda et al., 2003; Suzuki et al., 2005; Zarrindast and Mousa-Ahmadi, 1999). In the present study, BAC (2 mg/kg, i.p.) did not modify per se the basal population of μ-opioid receptors in any of the brain areas studied, neither in males nor in females.

It is remarkable that NAc μ-opioid receptors have been proposed to be responsible of aversive effects characteristic of opiates withdrawal (Koob et al., 1989). Given that ultrastructural studies have shown that in the NAc, μ-opioid receptors are localized prominently on GABA-containing neurons (Svingos et al., 1997), the possibility exists that the effect of BAC is achieved by interacting with these particular neurons.

Previous reports demonstrated that BAC is able to prevent the expression of certain NAL-precipitated withdrawal signs like jumping (Zarrindast and Mousa-Ahmadi, 1999), hopping activity (Belozertseva and Andreev, 2000), stereotyped head movements, ptosis, weight loss, chewing (Bexis et al., 2001), diarrhea, sniffing, and wet-dog shakes (Diaz et al., 2001). On the other hand, Capasso (1999) reported that BAC reduced opiates abstinence in vitro, which is similar to the in vivo observed effects. Therefore, if the same effects of BAC are obtained in vivo as well as in vitro conditions, we could, at first, lay aside that the effect of BAC depends on its influence over NAL pharmacokinetic parameters. The effect of BAC on μ-opioid receptor population observed herein in certain brain areas of MOR-withdrawn male mice could partially explain BAC ability to attenuate the expression of MOR-withdrawal signs, by decreasing μ-opioid receptor levels available to bind NAL. To our knowledge, no mechanism has been proposed to date to explain the way by which this $GABA_B$ agonist reestablishes the changes induced by the MOR withdrawal state. Decreases in receptor labeling could be a result of three different mechanisms: internalization, proteolysis (down-regulation), or conformational changes (desensitization) (Tsao and von Zastrow, 2001). BAC has been proved to enhance the expression of the proenkephalin gene at least in neocortical neurons (Morl et al., 2003), and in addition, it is well known that endogenous opioid peptides, but not MOR, induce μ-opioid receptors internalization (Eckersell et al., 1998; Minnis et al., 2003; Sternini et al., 2000). Taken together, one possible explanation for the present result in NAcC, and to a lesser extent in CPu and MDTh of males, is that BAC may enhance the release of endogenous opioids, which, in turn, induce the internalization of μ-opioid receptors. It has been reported that the ubiquitin/proteasome pathway, but not the lysosomal pathway, plays a role in agonist-induced down-regulation of μ-opioid receptors (Chaturvedi et al., 2001). The decrease of μ-opioid labeling induced by BAC in the areas described above could be a consequence of a proteosomic degradation of these receptors after internalization as well as conformational changes of μ-opioid receptors.

The influence of $GABA_A$ receptors on NAL-precipitated withdrawal has been also studied and results have been controversial: while the $GABA_A$ agonist muscimol was able to decrease the NAL behavioral response in MOR-dependent mice (El-Kadi and Sharif, 1995; Zarrindast and Mousa-Ahmadi, 1999), other authors found that the same $GABA_A$ agonist dose dependently increased μ-opiate withdrawal in vitro (Capasso and Sorrentino, 1997). On the contrary, there are no previous reports studying the effect of $GABA_A$ agonists on μ-binding parameters during MOR withdrawal.

It has been suggested that females may be less sensitive to MOR properties because they have either lesser μ-opioid receptor density or affinity or mediated signal transduction than males (Cicero et al., 1997; Craft, 2003). Based on the present results no basal differences in μ-receptor labeling between either sex has been found in any of the brain areas studied. However, significant increases in binding levels was observed only in specific brain areas of MOR-withdrawn male mice, suggesting that μ-opioid receptors' changes in response to NAL-precipitated withdrawal might play a role in the differential sensitivity described between sex.

Understanding how biological factors such as sex influence opioid effects may facilitate more rapid development toward novel mechanism-based pharmacotherapy of opiate-induced clinical syndrome. Additionally, further studies about the neural basis of BAC effect over addictive behaviors may aid in the development of treatments targeting opioid addiction.

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REFERENCES

Abdelhamid EE, Takemori AE. 1991. Characteristics of μ and δ opiod sites in striatal slices of morphine-tolerant and -dependent mice. Eur J Pharmacol 198:157–163.

Antonelli MC, Bassin DG, Garland M, Stahl WL. 1989. Localization and characterization of binding sites with high affinity for [^{3}H]Ouabain in cerebral cortex of rabbit brain using quantitative autoradiography. J Neurochem 52:193–200.

Belozertseva IV, Andreev BV. 2000. The effect of GABA-positive agents on the formation of morphine dependence and on the manifestations of a withdrawal syndrome. Eksp Klin Farmakol 63:19–23.

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Royal Stat Soc Series B Stat Meth 57:289–300.

Bexis S, Ong J, White J. 2001. Attenuation of morphine withdrawal signs by the GABA(B) receptor agonist baclofen. Life Sci 70:395–401.

Bonci A, Williams JT. 1996. A common mechanism mediates long-term changes in synaptic transmission after chronic cocaine and morphine. Neuron 16:631–639.

Bonci A, Williams JT. 1997. Increased probability of GABA release during withdrawal from morphine. J Neurosci 17:796–803.

Caboche J, Rogard M, Besson MJ. 1991. Comparative development of D1-dopamine and μ opiate receptors in normal and in 6-hydroxydopamine lesioned neonatal rat striatum: dopaminergic

fibers regulate μ but not D1 receptor distribution. Brain Res Dev Brain Res 58:111–122.
Capasso A. 1999. GABAB receptors are involved in the control of acute opiate withdrawal in isolated tissue. Prog Neuropsychopharmacol Biol Psychiatry 23:289–299.
Capasso A, Sorrentino L. 1997. $GABA_A$ receptor antagonists reduce acute opiate withdrawal in isolated tissue. Prog Neuropsychopharmacol Biol Psychiatry 21:315–330.
Chaturvedi K, Bandari P, Chinen N, Howells RD. 2001. Proteasome involvement in agonist-induced down-regulation of μ and δ opioid receptors. J Biol Chem 276:12345–12355.
Cicero TJ, Nock B, Meyer ER. 1997. Sex-related differences in the morphine's antinociceptive activity: relationship to serum and brain morphine concentrations. J Pharmacol Exp Ther 282:939–944.
Craft RM. 2003. Sex differences in opioid analgesia: "from mouse to man". Clin J Pain 19:175–186.
Craft RM, Stratmann JA, Bartok RE, Walpole TI, King SJ. 1999. Sex differences in development of morphine tolerance and dependence in rat. Psychopharmacology 143:1–7.
Diaz SL, Kemmling AK, Rubio MC, Balerio GN. 2001. Lack of sex-differences in the prevention by baclofen of the morphine withdrawal syndrome in mice. Behav Pharmacol 12:75–79.
Diaz SL, Kemmling AK, Balerio GN. 2003. Baclofen reestablishes striatal and cortical dopamine concentrations during naloxone-precipitated withdrawal. Neurochem Int 42:293–298.
Diaz SL, Kemmling AK, Bonavita CD, Rubio MC, Balerio GN. 2004. Baclofen reestablishes the μ-opioid receptor levels modified by the morphine withdrawal syndrome in either sexes. Synapse 54:24–29.
Eckersell CB, Popper P, Micevych PE. 1998. Estrogen-induced alteration of μ-opioid receptor immunoreactivity in the medial preoptic nucleus and medial amygdala. J Neurosci 18:3967–3976.
El-Kadi AO, Sharif SI. 1995. Modification by muscimol of naloxone-precipitated withdrawal symptoms in morphine-dependent mice. Gen Pharmacol 26:1579–1581.
Fadda P, Scherma M, Fresu A, Collu M, Fratta W. 2003. Baclofen antagonizes nicotine-, cocaine-, and morphine-induced dopamine release in the nucleus accumbens of rat. Synapse 50:1–6.
Franklin KBJ, Paxinos G. 1997. The Mouse Brain in Stereotaxic Coordinates. San Diego: Academic Press.
Frenois F, Cador M, Caille S, Stinus L, Le Moine C. 2002. Neural correlates of the motivational and somatic components of naloxone-precipitated morphine withdrawal. Eur J Neurosci 16:1377–1389.
Hammer RP, Jr. 1985. The sex hormone-dependent development of opiate receptors in the rat medial preoptic area. Brain Res 360: 65–74.
Heimer L, Zahm DS, Alheid GF. 1995.Basal ganglia. In:Paxinos G, editor. The Rat Nervous System. San Diego: Academic Press. Chapter 23.
Kandel ER, Schwartz JH, Jessell TM. 2000. Principles of Neural Science, 4th Ed. New York: McGraw-Hill.
Kirschke C, Schadrack J, Zieglgänsberger W, Spanagel R. 2002. Effects of morphine withdrawal on μ-opioid receptor-stimulated guanylyl 5′-[γ-[35S]thio]-triphosphate autoradiography in rat brain. Eur J Pharmacol 446:43–51.
Kitchen I, Slowe S, Matthes H, Kieffer B. 1997. Quantitative autoradiographic mapping of μ-, δ- and κ-opioid receptors in knockouts mice lacking the μ-opioid receptor gene. Brain Res 778:73–88.
Koob GF. 1992. Drugs of abuse: anatomy, pharmacology and function of reward pathways. Trends Pharmacol 13:177–184.
Koob GF, Bloom FE. 1988. Cellular and molecular mechanisms of drug dependence. Science 242:715–723.
Koob GF, Wall TL, Bloom FE. 1989. Nucleus accumbens as a substrate for the aversive stimulus effects of opiate withdrawal. Psychopharmacology 98:530–534.
Koob GF, Sanna PP, Bloom FE. 1998. Neuroscience of addiction. Neuron 21:467–476.
Kuhar MJ, Unnerstall JR. 1990.Receptor autoradiography. In:Yamamura HI, Enna SJ,Kuhar MJ, editors. Methods in Neurotransmitter Receptor Analysis. New York: Raven Press. p 177–178.
Loughlin SE, An A, Leslie FM. 1992. Opioid receptor changes in weaver mouse striatum. Brain Res 585:149–155.
Maldonado R. 1997. Participation of noradrenergic pathways in the expression of opiate withdrawal: biochemical and pharmacological evidence. Neurosci Biobehav Rev 21:91–104.
Maldonado R, Stinus L, Gold LH, Koob GF. 1992. Role of different brain structures in the expression of the physical morphine withdrawal syndrome. J Pharmacol Exp Ther 261:669–677.
Maldonado R, Stinus L, Gold LH, Koob GF, Maldonado R, Valverde O, Garbay C, Roques BP. 1995. Protein kinases in the locus coeruleus and periaqueductal gray matter are involved in the expression of opiate withdrawal. Naunyn Schmiedebergs Arch Pharmacol 352:565–575.
Matthes HW, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I, Befort K, Dierich A, Le Meur M, Dolle P, Tzavara E, Hanoune J, Roques BP, Kieffer BL. 1996. Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the μ-opioid-receptor gene. Nature 383:819–823.
Minnis JG, Patierno S, Kohlmeier SE, Brecha NC, Tonini M, Sternini C. 2003. Ligand-induced μ opioid receptor endocytosis and recycling in enteric neurons. Neuroscience 119:33–42.
Morl F, Leemhuis J, Lindemeyer K, Grass N, Norenberg W, Meyer DK. 2003. Stimulation of $GABA_B$ receptors increases the expression of the proenkephalin gene slice cultures of rat neocortex. Naunyn Schmiedebergs Arch Pharmacol 367:640–647.
Patel MB, Patel CN, Rajashekara V, Yoburn BC. 2002. Opioid agonists differentially regulate μ-opioid receptors and trafficking proteins in vivo. Mol Pharmacol 62:1464–1470.
Sadée W, Wang D, Bilsky EJ. 2005. Basal opioid receptor activity, neutral antagonists, and therapeutic opportunities. Life Sci 76: 1427–1437.
Self DW, Nestler EJ. 1995. Molecular mechanisms of drug reinforcement and addiction. Ann Rev Neurosci 18:463–495.
Self DW, McClenahan AW, Beitner-Johnson D, Terwilliger RZ, Nestler EJ. 1995. Biochemical adaptations in the mesolimbic dopamine system in response to heroin self-administration. Synapse 21:312–318.
Smith JA, Leslie FM, Broide RS, Loughlin SE. 1993. Long-term changes in striatal opioid systems after 6-hydroxydopamine lesion of rat substantia nigra. Neuroscience 55:935–951.
Sternini C, Brecha NC, Minnis J, D'Agostino G, Balestra B, Fiori E, Tonini M. 2000. Role of agonist-dependent receptor internalization in the regulation of μ-opioid receptors. Neuroscience 98:233–241.
Stinus L, Le Moal M, Koob GF. 1990. Nucleus accumbens and amygdala are possible substrates for the aversive stimulus effects of opiate withdrawal. Neuroscience 37:767–773.
Suzuki T, Nurrochmad A, Ozaki M, Khotib J, Nakamura A, Imai S, Shibasaki M, Yajima Y, Narita M. 2005. Effect of a selective GABA(B) receptor agonist baclofen on the μ-opioid receptor agonist-induced antinociceptive, emetic and rewarding effects. Neuropharmacology 49:1121–1131.
Svingos AL, Moriwaki A, Wang JB, Uhl GR, Pickel VM. 1997. μ-Opioid receptors are localized to extrasynaptic plasma membranes of GABAergic neurons and their targets in the rat nucleus accumbens. J Neurosci 17:2585–2594.
Terwilliger RZ, Beitner-Johnson D, Sevarino Ka, Crain SM, Nestler EJ. 1991. A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. Brain Res 548:100–110.
Tsao PI, von Zastrow M. 2001. Diversity and specificity in the regulated endocytic membrane trafficking of G-protein-coupled receptors. Pharmacol Ther 89:139–147.
Turchan J, Przewlocka B, Toth G, Lason W, Borsodi A, Przewlocki R. 1999. The effect of repeated administration of morphine, cocaine and ethanol on μ and δ opioid receptor density in the nucleus accumbens and striatum of the rat. Neuroscience 91:971–977.
van Bockstaele EJ, Commons KG. 2001. Internalization of μ-opioid receptors produced by etorphine in the rat locus coeruleus. Neuroscience 108:467–477.
Zarrindast MR, Mousa-Ahmadi E. 1999. Effects of GABAergic system on naloxone-induced jumping in morphine-dependent mice. Eur J Pharmacol 381:129–133.