Baclofen Did Not Modify Sexually Dimorphic c-Fos Expression During Morphine Withdrawal Syndrome

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ABSTRACT In previous studies, we have reported sex-related differences during morphine withdrawal. We have also shown that the $GABA_B$ agonist baclofen (BAC) was able to prevent the morphine withdrawal syndrome in male as well as in female mice. Considering that early gene expression is induced by drugs of abuse, we evaluated the expression of c-Fos in several brain areas, in mice of either sex during naloxone (NAL)-precipitated withdrawal, and after pretreatment with BAC. Swiss-Webster prepubertal mice were rendered dependent by i.p. injection of morphine (2 mg/kg), twice daily for 9 days. On the 10th day, dependent mice were divided into two groups: the withdrawal group received NAL (6 mg/kg, i.p.) after the last dose of morphine, while the prevention group received BAC (2 mg/kg, i.p.) before NAL. Thirty minutes after NAL, animals were sacrificed by transcardial perfusion. Brains were removed and slices were obtained to perform immunohistochemical studies. Our results show a significant decrease in c-Fos expression in hippocampal dentate gyrus, CA3, and CA1 areas of morphine withdrawn males, vs. their control group. Conversely, in females, the number of c-Fos positive nuclei was not modified in any of the areas studied. BAC pretreatment had no effect on the decreased c-Fos expression in morphine withdrawn males. The sexual dimorphism observed here confirms the greater sensitivity of males over females in their response to morphine. The preventive action of BAC on the expression of morphine withdrawal would not be related to an effect on c-Fos expression. **Synapse 67:118–126, 2013.**

INTRODUCTION

Previous studies in experimental animal models have demonstrated marked sex differences related to many pharmacological properties of the opioid agonist morphine (MOR), i.e., antinociception (Candido et al., 1992; Cicero et al., 1996, 1997), tolerance to analgesia (Craft et al., 1999), conditioned place preference (CPP) (Cicero et al., 2000), self-administration (Cicero et al., 2003), etc. Most of these studies refer to a greater sensitivity in males than in females. Studies from our laboratory have shown that female prepubertal mice were less prone to develop signs of MOR withdrawal syndrome than males (Diaz et al., 2001), a finding that is in agreement with previous results (Craft et al., 1999; Yoshimura and Yamamoto, 1979). We have also demonstrated that during MOR withdrawal syndrome, striatal and cortical endogenous levels of dopamine (DA) and its metabolite, dihydroxyphenyl acetic acid (DOPAC), decreased significantly in male mice (Diaz et al., 2003), but not in females (Diaz et al., 2005). We have also observed an increase in μ-opioid receptor density during MOR withdrawal syndrome in male mice, but not in females (Diaz et al., 2004, 2006). Finally, we observed that these sex differences in naloxone (NAL) precipitated withdrawal could not be attributed to pharmacokinetic

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factors (Diaz et al., 2007). Our group has also extensively worked on the effect of baclofen (BAC), a $GABA_B$ agonist, on the expression of MOR withdrawal syndrome. We have demonstrated that BAC prevented MOR withdrawal in males as well as female mice (Diaz et al., 2001) and was able to reestablish dopaminergic activity (Diaz et al., 2003) and μ-opioid receptor levels (Diaz et al., 2004, 2006), modified during NAL-precipitated MOR withdrawal.

At a genomic level, all drugs of abuse have been found to induce the expression of immediate early genes (IEGs) in the brain (Harlan and Garcia, 1998). IEGs are those genes that are expressed at an early stage in response to a stimulus, and function as transcription factors (Sheng and Greenberg, 1990). Studies in rats have shown that acute administration of morphine induces multiple alterations in the expression patterns of several IEGs products (Chang et al., 1988; Garcia et al., 1995; Liu et al., 1994). Particularly regarding c-Fos, a marker of neural activation (Kovács, 1998), a greater induction was evidenced in the dorsomedial caudate putamen (CPu) in male compared to female rats, after a single MOR administration (D'Souza et al., 1999), and this response was independent of circulating gonadal hormones (D'Souza et al., 2002). In addition, MOR withdrawal also increases c-Fos expression in areas reported to correlate with the negative affective component of MOR withdrawal, i.e. the extended amygdala (Frenois et al., 2002; Gracy et al., 2001; Le Guen et al., 2001; Stornetta et al., 1993; Veinante et al., 2003).

Regarding the relationship between IEGs expression and the effect of BAC, intra-ventral tegmental area (VTA) injections of this $GABA_B$ agonist inhibited MOR-induced locomotor hyperactivity and MOR-induced increase in c-Fos immunoreactivity in the nucleus accumbens (Leite-Morris et al., 2002). Furthermore, MOR-induced CPP was associated with an increased c-Fos expression in the anterior cingulate cortex (ACg), prelimbic cortex, nucleus accumbens shell (AcbSh), and nucleus accumbens core (AcbC) regions, and this effect was inhibited by the pretreatment with BAC (Kaplan et al., 2003).

Considering our previous results, and in order to better characterize sexual dimorphism during morphine withdrawal, the aim of the present study was to evaluate c-Fos expression in various brain areas, in mice of either sex during NAL-precipitated MOR withdrawal and, if changes were found, whether they could be reverted with BAC.

MATERIALS AND METHODS

Subjects

Experiments were performed on naïve prepubertal male and female (as evidenced by vaginal smears) Swiss Webster albino mice (Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina), weighing 20 g at the beginning of the treatment. On the day of the experiment (Day 10) mice weighed 23–27 g. Animals were housed in groups of five per cage under conditions of controlled temperature (22 ± 2°C) and humidity (55 ± 15%) and a 12-h light/dark cycle (lights on 08:00 h) according to local regulations (SENASA). The animals had free access to food and water up to the beginning of the experiments. All experiments and animal care were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH, publication no. 85-23, revised 1985). Animals were habituated to manipulation by four daily handling session in the experimental room, for four days prior to MOR treatment onset, in order to minimize the possible contribution of stress to withdrawal behavior and c-Fos expression in specific brain areas (de Medeiros et al., 2005; Wan et al., 1994).

Drugs

Morphine hydrochloride (Chemotecnia Sintyal, Buenos Aires, Argentina), NAL (Sigma Aldrich, Saint Louise, Missouri), and (±) BAC (Novartis, Basel, Switzerland) were used in this study. All drugs were dissolved in isotonic (NaCl 0.9%) saline solution and administered intraperitoneally (i.p.), in a volume of 0.1 ml/10 g body weight of the animals. The dose of morphine refers to the salt form.

Drug treatment

Mice of either sex were rendered dependent by i.p. injection of MOR (2 mg/kg), twice daily at 8:00 a.m. and 8:00 p.m., for nine consecutive days.

On the day of the experiment (Day 10), dependent male and female mice received the last dose of MOR at 8:00 a.m. and then were randomly divided into three groups (n = 5–6) as follows:

- **Dependence group**: 30 and 60 min after the last dose of MOR, animals received a saline i.p. injection.
- **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.

Control groups (male and female mice) received saline i.p. twice daily for nine consecutive days. On the 10th day, they received the last injection of saline at 8:00 a.m. and were randomly divided into three control groups:

- **Saline (SAL) Control**: 30 and 60 min after the last dose of saline, mice 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, i.p.) and saline, respectively.

c-Fos experiments

Tissue preparation

Mice were deeply anesthetized 30 min after the last injection using a mixture of ketamine (70 mg/kg, Holliday-Scott S.A., Argentina) and xylazine (10 mg/kg, König, Argentina). They were then perfused transcardially with heparinized PB (0.1 M phosphate buffer, pH 7.4), followed by a cold solution of 4% paraformaldehyde delivered with a peristaltic pump. Brains were removed, postfixed for 2 h in the same fixative, and cryoprotected overnight in a 30% sucrose solution. Coronal frozen sections were made at 40 μm on a freezing microtome. They were collected in three serial groups of free-floating sections and stored at 4°C.

c-Fos immunohistochemistry

The procedure was adapted from previously described protocols (Bester et al., 2001). All reactions were performed on floating sections agitated on a shaker. Sections from different experimental groups were processed in parallel to minimize variations in immunohistochemical labeling. Free-floating sections were rinsed in 0.1 M phosphate buffered saline with 0.15% Triton X-100 (PBS-T; pH 7.4) and then incubated with 3% hydrogen peroxide in PBS-T for a period of 30 min to remove endogenous peroxidase activity. After rinsing again in PBS-T, sections were incubated for 30 min in 2% normal goat serum in PBS-T. Then, sections were incubated overnight in rabbit polyclonal antibody anti-c-Fos (Santa Cruz Biotechnology, Santa Cruz, California) (1:1000 in PBS 0.1 M, thimerosal 0.02%, normal goat serum 1%) at 4°C. Sections were then rinsed and incubated for 2 h in goat anti-rabbit biotinylated antibody (Vector Laboratories, Burlingame, California) (1:250 in PBS-T). After being rinsed, sections were incubated for 2 h in avidin-biotinylated horseradish peroxidase complex (1:125, ABC kit, Vector Laboratories). After successive washes in PBS-T and Tris buffer (0.25 M; pH 7.4), the antibody–antigen complex was developed with 0.05% 3,3′-diaminobenzidine (Sigma Aldrich, Saint Louis, Missouri) and 0.015% H_2O_2 in 20 ml Tris buffer 0.1 M. Sections were mounted on gelatin-coated slides, dehydrated, and coverslipped. Controls for the specificity of primary antisera used were carried out by substitution of primary antibody with PBS (Delfino et al., 2004).

Data quantification

For quantitative analysis, cells positive for c-Fos immunoreactivity were identified by the presence of dense immunohistochemical staining within the nuclei, under a light microscope. Digital images of the selected sections were taken at 200× on a Nikon Microscope (Eclipse 55i) equipped with a digital camera (Nikon DS, Control Unit DS-L1). To reach the same levels of brightness and contrast of the photomicrographs included in Figure 2, these values were adjusted by using the Photoshop software.

For every area, the number of Fos-positive nuclei was counted within a grid under ImageJ 1.36 b, provided by National Institutes of Health, USA (public domain software). The counting was performed bilaterally in each brain area by an observer blind to drug treatment. These counts were averaged into a single score for each region of each animal and finally the group mean ± SEM was calculated. Fos-positive cells were quantified in the following brain regions, identified according to the anatomic atlas of Paxinos and Franklin (2004): AcbSh and AcbC, cingulate cortex 1 and 2 (Cg1 and Cg2), CPu, bed nucleus of the stria terminalis (BST), the basolateral amygdaloid nucleus (BLA), central nucleus of the amygdale (CeC), dentate gyrus (DG), CA1, and CA3 areas of the hippocampus.

Statistical analysis

To determine differences between the experimental groups for each brain area, data were analyzed with a two-way analysis of variance (ANOVA) with sex and treatment as the main factors. In all areas, when a significant interaction between the two factors was observed, subsequent one-way ANOVAs and Tukey's *post hoc* test were applied. In all cases, $P < 0.05$ was considered statistically significant.

RESULTS

Two way ANOVA showed a significant interaction between treatment and sex for hippocampal DG and CA3 ($P < 0.001$), while no interactions between treatment and sex were observed for the other brain areas studied. In addition, two-way ANOVA showed no significant effect of neither sex nor treatment in the hippocampus (see Table I).

Hippocampus

Dentate gyrus

One way ANOVA revealed significant differences between groups in the number of c-Fos-positive nuclei ($P < 0.001$) in male but not in female mice. In males, *post hoc* Tukey's test showed a significant decrease in c-Fos expression in the withdrawal group compared to saline control group ($P < 0.01$); no significant differences were observed between dependence, NAL control, BAC control, and saline control groups. BAC pretreatment had no effect on c-Fos expression of MOR withdrawn males. No significant, differences

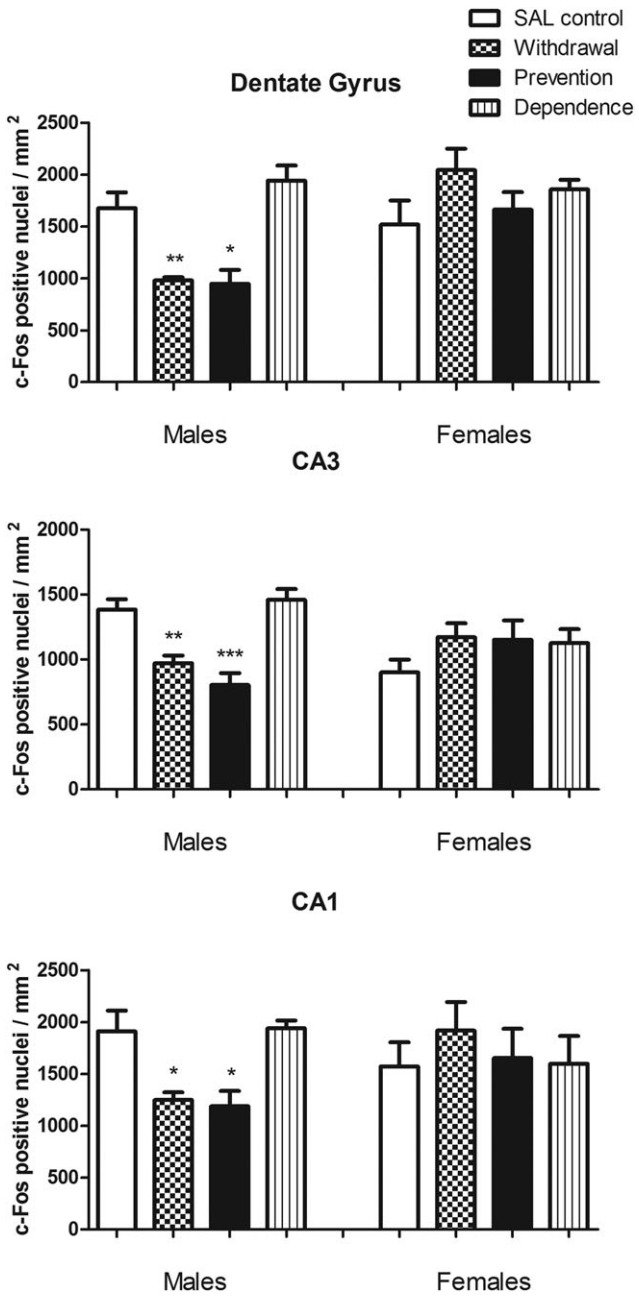

Fig. 1. c-Fos expression in male and female mice following MOR withdrawal, after pretreatment with BAC (prevention group), and after chronic MOR treatment (dependence group) in hippocampal dentate gyrus, CA3 and CA1. Data are expressed as mean ± SEM ($n = 5$ for each group). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared to saline control group (Tukey's test).

were observed between any of the female experimental groups (Figs 1, 2 and Tables I, II).

CA3

One way ANOVA revealed significant differences between groups in the number of c-Fos-positive nuclei ($P < 0.001$) in male but not in female mice. In males, *post hoc* Tukey's test showed a significant decrease in c-Fos expression in the MOR withdrawal group compared to saline control group ($P < 0.01$); no significant differences were observed between dependence, BAC control and saline control groups. Additionally, in this area a diminution in the number of Fos-positive nuclei was found in the NAL control group (Table II). BAC pretreatment had no effect on c-Fos expression of MOR withdrawn males. No significant differences were observed between any of the female experimental groups (Figs 1, 2 and Tables I, II).

CA1

Although there was neither significant main effect nor interaction between treatment and sex for CA1, a tendency toward significant P values was observed for interaction ($P < 0.06$). One way ANOVA revealed significant differences between experimental groups in the number of c-Fos positive nuclei ($P < 0.001$) in male but not in female mice. In males, *post hoc* Tukey's test showed a significant decrease in c-Fos expression in MOR withdrawal group compared to saline control group ($P < 0.05$); no significant differences were observed between dependence, NAL control, BAC control, and saline control groups. BAC pretreatment had no effect on c-Fos expression of MOR-withdrawn males. No significant differences were observed between any of the female experimental groups (Figs 1, 2 and Tables I, II).

No significant differences were observed between experimental groups in any of the other brain areas studied (see Table II).

DISCUSSION

Our experimental results show a decreased c-Fos expression in male mice during NAL-precipitated withdrawal in specific brain regions of the hippocampus, such as DG, CA3, and CA1; however, no changes were observed in females. In addition, the $GABA_B$ agonist BAC was not able to reestablish c-Fos expression modified during MOR withdrawal in any of the affected brain areas.

There are no reports about sex differences in the effects of chronic MOR treatment and MOR withdrawal syndrome on c-Fos expression. The sexual dimorphism observed herein confirms the greater sensitivity of males to morphine withdrawal, compared to females. As reported in previous studies from our laboratory, male mice developed MOR abstinence syndrome in three of the six parameters measured (wet-dog shakes, liquid feces, and sniffing), while females only showed the withdrawal syndrome in two parameters (wet-dog shakes and liquid feces). In addition, the expression of these withdrawal signs was more intense in male than in female mice (Diaz et al., 2001). We have also found that the levels of DA and its metabolite (DOPAC) and the density of μ-opioid receptor was modified in male mice during MOR with-

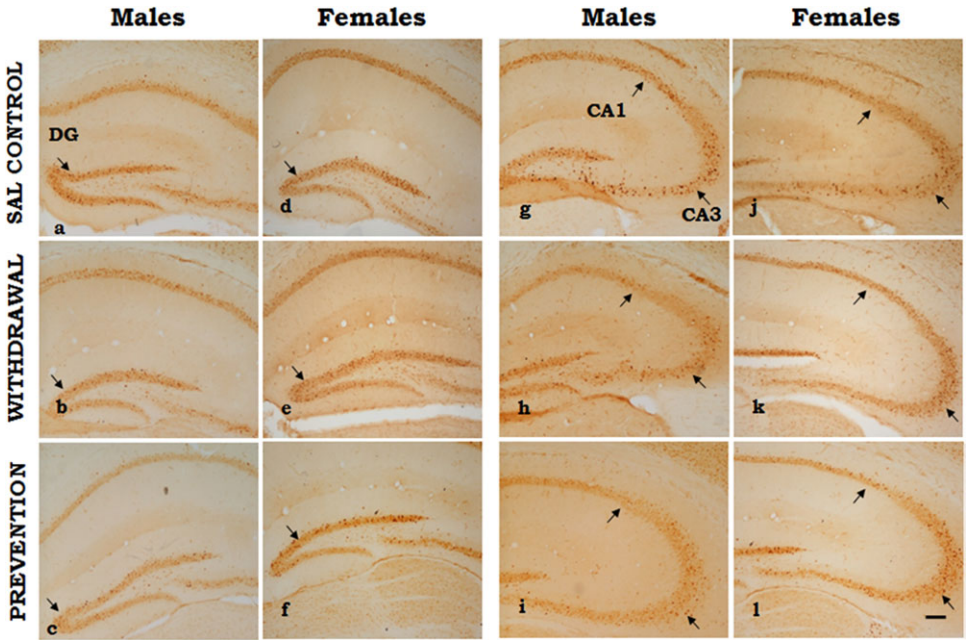

Fig. 2. c-Fos immunoreactivity in mice of saline (SAL) control, withdrawal, and prevention groups. The first and second columns show the dentate gyrus in sections cut at the level of the hippocampus (bregma −2.18 mm) in males (a, b, and c) and females (d, e, and f). The third and fourth columns show CA3 and CA1 in sections cut at the level of the hippocampus (bregma −2.18 mm) in males (g, h, and i) and females (j, k, and l). Scale bar: 100 μm.

drawal but not in females (Diaz et al., 2003, 2004, 2005, 2006). Furthermore, these sex differences in NAL-precipitated MOR withdrawal could not be attributed to pharmacokinetic factors (Diaz et al., 2007). Sex hormones like estrogens could increase the expression of c-Fos in the hippocampus (Rudick and Woolley, 2000). The use of prepubertal mice (indicated by vaginal smears) allows us to suggest in view of our results, that factors other than cyclical hormonal regulation may be affecting c-Fos expression in mice. In summary, the present results support previous knowledge asserting that male and female animals differ in their behavioral, neurological, and pharmacological responses to drugs.

In the present study, mice received a chronic treatment (9 days) with a low dose of MOR (2 mg/kg), a protocol that induces more physiologic conditions for the animals, i.e. mice develop a MOR dependence without altering its behavior. Although, we have not increased the dose during the 9 days, we could demonstrate that mice were rendered dependent by injecting NAL and verifying the abstinence syndrome (Diaz et al., 2001). Even though we could positively induce MOR dependence, we did not observe changes in c-Fos expression during the dependent state in any of the brain areas investigated, for either sex. These results are in agreement with previous studies, where MOR dependent mice showed no differences in c-Fos expression (Liu et al., 2009; Sharf et al., 2008) compared to control animals.

The decrease in c-Fos expression in DG, CA3, and CA1 of withdrawn male mice observed in the present study is in accordance with previous reports, where a diminution of this parameter was also observed in locus coeruleus, in the nucleus of the solitary tract and in the BLA during naltrexone-precipitated MOR withdrawal in rats (Mannelli et al., 2004). On the contrary, Frenois et al., (2002) found that MOR withdrawal increased *c-fos* mRNA in hippocampal CA1 and CA3 in rats. Regarding our present study, c-Fos expression was not modified in AcbSh, AcbC, Cg1, Cg2, CPu, BST, BLA, and CeC during MOR withdrawal, in our experimental conditions. Taken together, these studies demonstrate that MOR withdrawal could cause inductions, but also inhibitions on *c-fos* mRNA, Fos-LIR, and c-Fos expression (Frenois et al., 2002). These differences could be related to the experimental protocol used, the dependence regimens, their resulting latency and especially to the method of inducing withdrawal and its severity (Georges et al., 2000). Other studies have reported different protocols to develop MOR dependence, using high doses of MOR administered i.p. (Beckmann et al., 1995) or using subcutaneous MOR pellets (Frenois et al., 2002; Hayward et al., 1990; Veinante et al., 2003). Taken together our results might be reflecting a specific c-Fos induction pattern corresponding to the protocol used in the present study to develop dependence and NAL precipitated MOR withdrawal. Another factor to be considered is that mechanisms

TABLE I. Statistical analysis of c-Fos expression in different brain regions

	Two-way ANOVA						One-way ANOVA			
	Treatment		Sex		Interaction		Male		Female	
	F	*P*-value	F	*P*-value	F	*P*-value	F	*P*-value	F	*P*-value
Dentate gyrus	$F_{(5, 57)} = 2.16$	NS	$F_{(1, 57)} = 8.06$	NS	$F_{(5, 57)} = 5.45$	<0.001	$F_{(5, 32)} = 12.84$	<0.001	$F_{(5, 36)} = 1.75$	NS
CA3	$F_{(5, 56)} = 2.34$	NS	$F_{(1, 56)} = 0.22$	NS	$F_{(5,56)} = 5.53$	<0.001	$F_{(5, 31)} = 14.42$	<0.001	$F_{(5, 35)} = 0.77$	NS
CA1	$F_{(5, 56)} = 2.34$	NS	$F_{(1, 56)} = 11.63$	0.001	$F_{(5, 56)} = 1.88$	NS	-		-	
Nucleus accumbens core	$F_{(5, 56)} = 0.29$	NS	$F_{(1, 56)} = 11.47$	0.001	$F_{(5, 56)} = 0.40$	NS	-		-	
Nucleus accumbens shell	$F_{(5, 56)} = 0.80$	NS	$F_{(1, 56)} = 6.18$	0.016	$F_{(5, 56)} = 0.63$	NS	-		-	
Caudate putamen	$F_{(5, 56)} = 1.19$	NS	$F_{(1, 56)} = 0.73$	NS	$F_{(5, 56)} = 1.05$	NS	-		-	
Cingulate cortex	$F_{(5, 56)} = 1.92$	NS	$F_{(1. 52)} = 50.39$	<0.001	$F_{(5, 52)} = 1.88$	NS	-		-	
Basolateral amygdala	$F_{(5, 52)} = 2.09$	NS	$F_{(1, 50)} = 33.69$	<0.001	$F_{(5, 50)} = 1.27$	NS	-		-	
Central amygdala	$F_{(5, 50)} = 1.99$	NS	$F_{(1, 51)} = 0.47$	NS	$F_{(5, 51)} = 0.57$	NS	-		-	
Bed nucleus stria terminalis	$F_{(5, 51)} = 1.41$									

Two-way ANOVA with chronic treatment and sex as main factors. In all cases, when a significant interaction between the two factors was observed, subsequent one-way ANOVA was applied. See Materials and methods for details.

that lead to c-Fos activation may be different between rats and mice (Veinante et al., 2003).

The fact that we have only observed alterations in c-Fos expression in the hippocampus could be related with the expression of wet-dog shakes during MOR withdrawal. Indeed, in previous studies from our laboratory we observed that wet-dog shakes were more frequent than other behavioral signs of withdrawal (Diaz et al., 2001). The hippocampus might have a role in both the motivational and the somatic components of MOR withdrawal. In particular, hippocampal CA3 has been linked to the somatic component of MOR withdrawal like wet-dog shakes (Araki et al., 1989; Isaacson and Lanthorn, 1981). In addition, changes in *c-fos* mRNA expression in CA1 have been linked to the motivational component of MOR withdrawal (Frenois et al., 2002). In conclusion, the hippocampal circuit could modulate morphine withdrawal (Isaacson and Lanthorn, 1981).

The dose of NAL (6 mg/kg) administered in the present study was selected after testing increasing doses of NAL in order to precipitate MOR withdrawal in mice chronically treated with MOR (2 mg/kg) twice daily. This dose of NAL was not able to precipitate behavioral signs of the abstinence syndrome in nondependent animals (Diaz et al., 2001). Therefore, the behavioral changes reported in MOR-abstinent mice depend only on the combination of the MOR-dependent state plus the administration of NAL. However, in the present study, c-Fos expression in the CA3 of male indicates that NAL could have an effect *per se*, given the fact that groups receiving NAL (i.e., NAL control, MOR withdrawal, and prevention) suffered a decrease in c-Fos expression in this particular brain area. Other studies have also shown that high doses of NAL were able to alter Fos expression in certain brain regions in placebo rats (Georges et al., 2000; Gestreau et al., 2000; Hamlin et al., 2004; Le Guen et al., 2001) but, as in our experiments, these alterations in c-Fos expression were less significant than the changes in c-Fos expression induced in withdrawn animals. Even though this NAL effect is difficult to explain, it has to be taken into account that pharmacological properties may vary significantly from one brain region to another (Sadée et al., 2005).

Considering that opioids have been shown to reduce both excitatory and inhibitory synaptic transmission (Hayard and Guyenet, 1998; Jolas and Aghajanian, 1997), neurons that receive equivalent excitatory and inhibitory inputs and are tonically influenced by the endogenous opioid system would have their activation blocked by NAL (Gestreau et al., 2000). This could account for the decreased c-Fos expression in our experimental conditions. In addition, in neurons that constitute the targets of a majority of inhibitory inputs, NAL administration would result in a greater inhibitory effect; hence, it would not induce c-Fos expression. Therefore, the fact that no changes in c-Fos expression were evidenced does

TABLE II. c-Fos expression (c-Fos positive nuclei/area, mm_2) in the Dependence, Withdrawal and Prevention groups, and their respective controls, in different brain regions of male and female mice

Brain Region	SAL control	Withdrawal	Prevention	Dependence	BAC control	NAL control
Dentate gyrus						
Males	1678.0 ± 150.7	979.5 ± 31.7 **	1445.6 ± 157.4 *	1943.1 ± 143.3	2286.7 ± 205.8	2451.8 ± 319.0
Females	1518.6 ± 233.3	2044.3 ± 206.7	1661.4 ± 169.2	1856.3 ± 94.6	2205.9 ± 239.3	2290.1 ± 356.6
CA3						
Males	1386.5 ± 78.3	971.9 ± 58.8 **	803.5 ± 92.5 ***	1459.6 ± 82.6	1242.2 ± 116.7	1119.9 ± 77.1**
Females	901.4 ± 97.1	1172.6 ± 105.8	1152.3 ± 149.8	1126.3 ± 108.1	1034.8 ± 145.1	1151.3 ± 94.4
CA1						
Males	1911.5 ± 200.0	1248.4 ± 72.7 *	1457.1 ± 341.3 *	1938.2 ± 77.2	1947.8 ± 154.6	1644.2 ± 305.9
Females	1572.5 ± 232.7	1917.1 ± 275.5	1653.4 ± 281.8	1598.1 ± 268.1	1730.1 ± 218.2	1394.4 ± 180.1
Nucleus accumbens core						
Males	974.6 ± 57.7	1126.4 ± 128.6	1110.7 ± 53.2	843.5 ± 109.2	1020.7 ± 98.0	767.4 ± 76.0
Females	1178.1 ± 214.2	1024.3 ± 127.1	1136.2 ± 217.6	1396.1 ± 114.8	1307.5 ± 100.8	1302.5 ± 143.8
Nucleus accumbens shell						
Males	1146.9 ± 137.8	1347.4 ± 299.1	1408.7 ± 129.2	1329.0 ± 131.2	1436.9 ± 150.2	1257.4 ± 65.9
Females	1787.3 ± 228.9	1816.6 ± 342.9	1691.9 ± 371.1	1591.9 ± 307.2	2232.9 ± 151.0	1613.5 ± 259.0
Caudate putamen						
Males	491.8 ± 108.2	319.9 ± 54.7	300.8 ± 30.5	478.5 ± 56.6	358.7 ± 85.0	263.4 ± 62.9
Females	492.1 ± 77.3	550.0 ± 121.9	366.6 ± 92.0	540.0 ± 93.9	499.2 ± 86.3	493.0 ± 87.2
Cingulate cortex						
Males	484.2 ± 62.5	601.8 ± 56.3	551.6 ± 58.1	600.0 ± 77.7	532.5 ± 61.4	424.4 ± 72.2
Females	768.7 ± 122.3	651.2 ± 103.5	493.8 ± 80.9	599.1 ± 111.7	532.3 ± 88.2	398.5 ± 58.0
Basolateral amygdala						
Males	126.1± 28.0	143.0 ± 48.7	218.1 ± 36.0	171.9 ± 40.9	207.5 ± 35.4	182.1 ± 34.1
Females	316.5 ± 36.0	312.1 ± 39.1	371.9 ± 74.7	481.8 ± 45.7	259.6 ± 45.7	388.9 ± 33.6
Central amygdala						
Males	183.8 ± 24.0	311.2 ± 79.0	294.2 ± 59.4	235.5 ± 34.1	262.0 ± 53.6	248.3 ± 8.7
Females	397.8 ± 83.0	477.4 ± 65.3	618.2 ± 94.6	611.6 ± 67.5	384.2 ± 60.9	400.4 ± 19.7
Bed nucleus stria terminalis						
Males	584.2 ± 38.8	579.2 ± 26.5	504.4 ± 81.1	430.5 ± 64.6	531.1 ± 73.3	407.9 ± 101.6
Females	635.4 ± 113.7	561.4 ± 98.1	655.0 ± 121.5	433.3 ± 85.3	428.6 ± 72.5	530.6 ± 72.8

Values are expressed as mean ± SEM of 4 to 5 animals per experimental group.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with SAL control group (One-way ANOVA; Tukey's test).

not necessarily indicate an absence of tonic opioid input (Gestreau et al., 2000), or that the hippocampus is not sensitive to MOR withdrawal (Beckmann et al., 1995).

It is well known that opiates acutely inhibit the functional activity of the cAMP pathway (indicated by cellular levels of cAMP and cAMP-dependent protein phosphorylation). With continued opiate exposure, functional activity of the cAMP pathway gradually recovers, and increases far above control levels following NAL precipitated morphine withdrawal. Increased intracellular cAMP acts as the second messenger, activating protein-kinase A, which then translocates to the nucleus where it phosphorylates the CREB (cAMP-responsive-element binding protein) family of transcription factors (Nestler, 2004). CREB has many target genes, including c-Fos and brain derived neurotrophic factor (Carlezon Jr et al., 2005); an increment in this transcription factor has been the mechanism proposed by other researchers to explain the increased expression of c-Fos during MOR withdrawal, a result frequently observed (Frenois et al., 2002; Gracy et al., 2001; Veinante et al., 2003). However, in accordance with our results, other researchers have also found evidence of a decreased expression of c-Fos during MOR withdrawal (Frenois et al., 2002; Mannelli et al., 2004). Therefore, the fact that in our study we observed a decrease in c-Fos expression might reflect an inhibition of the cAMP pathway leading to changes in CREB expression. Nevertheless, additional experiments would be required to explore this hypothesis.

In the present study, BAC pretreatment did not modify c-Fos expression in MOR withdrawn animals. Previous reports demonstrated that BAC is able to prevent the expression of certain NAL-precipitated withdrawal signs (Belozertseva and Andreev, 2000; Bexis et al., 2001; Zarrindast and Mousa-Amadi, 1999). In line with this, we have also shown that BAC prevented the expression of MOR withdrawal syndrome in male as well as female mice (Diaz et al., 2001) and also it was able to reestablish activity of dopaminergic neurons (Diaz et al., 2003) and μ-opioid receptor levels, altered during NAL-precipitated withdrawal (Diaz et al., 2004, 2006). It is known that hippocampal inhibitory neurons possess multiple Ca^{2+} channel subtypes, including N-type, L-type and, at least two other types of high-threshold channels, and that the activation of $GABA_B$ receptors preferentially inhibits N-type channels in these neurons (Lambert and Wilson, 1996). The inability of BAC to reestablish c-Fos expression in morphine withdrawn animals could be partially explained by a reduction of this N-type Ca^{2+} channels in abstinent animals. Further studies would be required to determine whether chronic morphine treatment and the abstinence state differentially alter the expression of N-type Ca^{2+} channels.

In conclusion, the present findings show for the first time sex differences in c-Fos expression during MOR withdrawal syndrome. Further studies about the neural basis of the involvement of sexual dimorphism over addictive behaviors may aid in the development of different treatments targeting opioid addiction for males and females.

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