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The Role of Opioid Receptor Subtypes in the Development of Behavioral Sensitization to Ethanol

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Nonspecific blockade of opioid receptors has been found to prevent development of behavioral sensitization to ethanol. Whether this effect is achieved through a specific opioid receptor subtype, however, is not clear. The present study investigated, for the first time, the role of specific opioid receptor subtypes in the development of ethanol-(2.5 g/kg/day; six sessions) induced locomotor sensitization in mice. We confirmed previous results showing that the nonspecific antagonism of opioid receptors (naltrexone; 0–2 mg/kg) prevented the development of behavioral sensitization to ethanol, an effect attained at doses presumed to occupy only mu opioid receptors. This was confirmed by using the selective mu opioid receptor antagonist CTOP (0–1.5 mg/kg), which also blocked sensitization to ethanol. The selective delta receptor antagonist, naltrindole (0–10 mg/kg), however, did not alter sensitization. We further assessed the role of mu opioid receptors in sensitization to ethanol by exploring the involvement of mu_1, mu_{1+2}, and mu_3 opioid receptor subtypes. Results of these experiments revealed that the blockade of mu_1 (naloxonazine; 0–30 mg/kg) or mu_3 opioid receptors (3-methoxynaltrexone; 0–6 mg/kg) did not prevent locomotor sensitization to ethanol. Using naloxonazine under treatment conditions that block mu_{1+2} opioid receptor subtypes we observed a retarded sensitization. The present data suggest that the concurrent inactivation of all mu opioid receptor subtypes may be required to prevent the neural adaptations underlying the development of behavioral sensitization to ethanol. In addition, these results support previous data suggesting a putative role for the mu opioid receptor endogenous ligand, β-endorphin, and the hypothalamic arcuate nucleus in ethanol sensitization.
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INTRODUCTION

When repeatedly administered, drugs of abuse produce persistent increases in both their psychomotor stimulating effects (Badiani *et al*, 2000; Kalivas *et al*, 1992; Phillips *et al*, 1994) and their incentive motivational properties (Lett, 1989; Piazza *et al*, 1989; Robinson and Berridge, 1993), a phenomenon known as behavioral sensitization. The neural changes that underlie behavioral sensitization to drugs of abuse are thought to contribute to the transfer from moderate consumption to the compulsive patterns of drug-seeking and drug-craving that characterizes addictive behavior (Robinson and Berridge, 1993; Stewart and Badiani, 1993). Elucidating the neurochemistry of drug sensitization, therefore, can provide helpful insights into the understanding of the development of addiction.

In search of a common neurochemical substrate underlying the sensitization process to drugs of abuse, the glutamatergic and the dopaminergic systems have been repeatedly implicated (Robinson and Berridge, 1993; Vanderschuren and Kalivas, 2000; Vezina and Kim, 1999). With respect to ethanol, however, data reveal a controversial role of those systems in explaining the development of behavioral sensitization. It has been proposed that the activation of the *N*-methyl-D-aspartate (NMDA) subclass of glutamate receptors is necessary for the development of ethanol sensitization in mice (Broadbent *et al*, 2003; Camarini *et al*, 2000a). However, when further investigated, it has been reported that this effect is related to the action of NMDA antagonists on acute ethanol-induced locomotor activity (Meyer and Phillips, 2003). In fact, an enhanced, rather than attenuated sensitized response to ethanol has been found using low doses of the NMDA antagonist MK-801, which increased the stimulatory acute effects of ethanol (Meyer and Phillips, 2003). Sensitization to ethanol has also been associated with increases in dopamine (DA) D_2 receptor binding (Souza-Formigoni *et al*, 1999). Deletion of the DA D_2 receptor gene in mice, however, did not alter ethanol-induced sensitization (Palmer *et al*, 2003); additionally, the nonspecific DA receptor antagonist, haloperidol, has been shown to not block this effect of ethanol (Broadbent *et al*, 1995). The involvement of other systems in ethanol sensitization, such as γ-aminobutyric acid (GABA) neurotransmission, has been additionally proposed. However, data indicate that ethanol sensitization

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may not be associated with changes in $GABA_A$ receptor function (Meyer *et al*, 2005).

A critical role of the opioid system in the sensitizing effects of ethanol in mice has recently been proposed (Camarini *et al*, 2000b; Miquel *et al*, 2003). Nonselective antagonism of opioid receptors with naloxone has been shown to block the development of ethanol sensitization (Camarini *et al*, 2000b). While both naloxone and naltrexone are characterized as nonspecific opioid receptor antagonists, they have been found to have a higher affinity for mu than for delta opioid receptors (Takemori and Portoghese, 1984; Williams *et al*, 2001). Low doses of naloxone and naltrexone (eg 1 mg/kg and less) are suggested to be specific for the mu opioid receptor, whereas higher doses may additionally recruit delta opioid receptors (Mhatre and Holloway, 2003; Takemori and Portoghese, 1984). According to this, data from experiments showing a blockade of ethanol sensitization with opioid antagonists suggest that mu opioid receptors may be particularly involved in ethanol sensitization (Camarini *et al*, 2000b). However, the hypothesis that behavioral sensitization to ethanol is specifically achieved through mu opioid receptors needs to be assessed.

Recent data suggest that the acute locomotor stimulating effects of ethanol are mediated by mu, but not delta opioid receptors (Hall *et al*, 2001; Pastor *et al*, 2005; Sanchis-Segura *et al*, 2004). In addition, it has been proposed that acute ethanol stimulation may be selectively mediated by some of the subtypes of the mu opioid receptor. Specifically, the antagonism of mu_{1+2} or mu_3, but not mu_1 receptors, have been found to attenuate behavioral stimulation induced by acute ethanol administration (Pastor *et al*, 2005). Whether the functional relevance of these mu receptor subtypes could be extended to the chronic effects of ethanol on locomotor activity, however, is not known.

One goal of the present study was to test the hypothesis that the development of sensitization to the locomotor stimulatory effects of ethanol is specifically mediated by mu opioid receptors. This was accomplished by comparing the effects of naltrexone on ethanol sensitization to those obtained with D-Pen-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) and naltrindole, selective mu and delta opioid receptor antagonists, respectively. A second goal of the current study was to explore a putative involvement of mu opioid receptor subtypes (mu_1, mu_{1+2}, or mu_3) in the sensitizing effects of ethanol. This objective was addressed by using naloxonazine (which can be used as a mu_1 or a mu_{1+2} antagonist) and 3-methoxynaltrexone (mu_3 antagonist). Although there are many types of behavioral sensitization (ie sensitization to stereotypic behavior), the present article only involves studies of locomotor sensitization, and therefore the term sensitization will hereafter be used to refer specifically to locomotor sensitization.

MATERIALS AND METHODS

Subjects and Housing

Male Swiss (IOPS Orl) albino mice, a strain chosen for its sensitivity to the stimulatory and sensitizing effects of ethanol (Camarini *et al*, 2000b; Miquel *et al*, 2003), were purchased from Janvier Spain, SA (Barcelona, Spain), housed (three per cage) and acclimated to the colony room for at least 2 weeks prior to study initiation. At the time of testing, mice were 6 weeks old. The colony was maintained in a humidity- (50%) and temperature-controlled ($22 \pm 1^{\circ}C$) environment under a 12-h light/dark cycle (lights on at 0800) with standard laboratory rodent chow (Panlab SL, Spain) and tap water available *ad libitum*. Behavioral testing occurred between 1000 and 1400. All experimental procedures complied with the European Community Council Directive (86/609/ECC) for the use of laboratory animal subjects.

Drug Sources and Preparation

Ethanol (Panreac SA, Spain) was diluted to 20% (v/v) in 0.9% physiological saline from 96% solutions. This concentration of ethanol was selected based on previous studies (Pastor *et al*, 2002; Sanchis-Segura *et al*, 2004) showing no signs of toxicity after intraperitoneal (i.p.) injections. Naltrexone, CTOP, naltrindole, naloxonazine, and 3-methoxynaltrexone were obtained from Sigma-Aldrich Química (Spain) and were administered (i.p.) in an injection volume of 10 ml/kg. All solutions were prepared fresh daily in saline except naloxonazine, which was dissolved in a tartaric acid solution (TA; 0.2% w/v) in saline. This concentration of TA was chosen because it did not affect spontaneous locomotor activity (Pastor *et al*, 2005). Saline solution was administered to control groups. However, in experiments involving naloxonazine, control groups were injected with vehicle (0.2 % w/v TA, in saline).

Locomotor Activity Test

All subjects were tested in open-field chambers consisting of cylinders 25 cm in diameter and 30 cm high. Locomotor activity was registered by a computerized video-tracking system (Ethovision 2.0., Noldus, The Netherlands). Movement of the animal within the open-field was automatically recorded and later translated by Ethovision software to horizontal distance traveled (in cm), which served as the dependent variable for the present behavioral studies. The duration of the test was 20 min, however, as previously described (Pastor *et al*, 2002), only the last 15 min were considered for statistical analyses. This interval of time was chosen in order to ensure the absorption and distribution of ethanol within the CNS after its i.p. injection (Quertemont *et al*, 2003) and to minimize a possible masking effect of nonspecific locomotor activation derived from handling, injections, and experimental novelty, which is particularly present in the first minutes of the test (Dudek and Tritto, 1994; Kelley, 1993). We confirmed, moreover, that all main effects found in the present study were also present when the total 20 min test was analyzed. Behavioral testing was conducted in sound-attenuated rooms ($8\,m^2$) with dim illumination (20 W; regular white light) to minimize stress induced by highly illuminated environments.

Behavioral Procedures

Six separate experiments were conducted using a common experimental design (presented in Table 1). Based on previous literature (Meyer and Phillips, 2003; Miquel *et al*,

2003), the sensitization protocol was divided into two phases: treatment and challenge. The treatment phase (days 1–11) involved six trials on alternate days, one trial per day. On each behavioral test day of this phase (days 1, 3, 5, 7, 9, and 11), mice received saline, naltrexone, CTOP, naltrindole, naloxonazine, or 3-methoxynaltrexone prior to a saline or ethanol injection and were individually placed in the open-field chamber immediately after the second injection. On days 2, 4, 6, 8, and 10 mice were left undisturbed in the colony room. The treatment phase allowed us to study the effect of opioid antagonists on the development of sensitization to the locomotor stimulating actions of ethanol. A period of 7 days without any experimentation separated the treatment and the challenge phase. The challenge phase involved two trials: the ethanol challenge (day 18), in which all mice received an injection of ethanol immediately before locomotor activity testing, and the saline challenge (day 19), which paralleled the ethanol challenge except that all mice received an injection of saline instead of ethanol. The aim of the challenge phase was two-fold. First, to assess whether sensitization was impeded by the presence of opioid antagonists and to study whether opioid antagonists altered the duration of sensitization. Second, the saline challenge was conducted to study possible conditioned locomotor effects induced by the testing environment where ethanol-stimulated behavior occurred. Dosage and timing between pretreatment and treatment injections (detailed in Table 1) were undertaken as follows: Naltrexone (0, 1, or 2 mg/kg) was given 30 min before saline or ethanol, naltrindole (0, 5, or 10 mg/kg) was

Table 1 Schedule of Injections for Experimental Groups

	Treatment phase			Challenge phase	
	Days 1, 3, 5, 7, 9, and 11			Day 18	Day 19
Group	**Pretreatment**	**Time between injections**	**Treatment**	**Treatment**	**Treatment**
Exp. 1					
S-S	Saline	30 min	Saline	Ethanol	Saline
S-E	Saline	30 min	Ethanol	Ethanol	Saline
NX-S	Naltrexone	30 min	Saline	Ethanol	Saline
NX-E	Naltrexone	30 min	Ethanol	Ethanol	Saline
Exp. 2b					
S-S	Saline	15 min	Saline	Ethanol	Saline
S-E	Saline	15 min	Ethanol	Ethanol	Saline
CTOP-S	CTOP	15 min	Saline	Ethanol	Saline
CTOP-E	CTOP	15 min	Ethanol	Ethanol	Saline
Exp. 3					
S-S	Saline	15 min	Saline	Ethanol	Saline
S-E	Saline	15 min	Ethanol	Ethanol	Saline
ND-S	Naltrindole	15 min	Saline	Ethanol	Saline
ND-E	Naltrindole	15 min	Ethanol	Ethanol	Saline
Exp. 4 and 5					
V-S	Vehicle	20 h/15 min	Saline	Ethanol	Saline
V-E	Vehicle	20 h/15 mm	Ethanol	Ethanol	Saline
NZ-S	Naloxonazine	20 h/15 min	Saline	Ethanol	Saline
NZ-E	Naloxonazine	20 h/15 min	Ethanol	Ethanol	Saline
Exp. 6					
S-S	Saline	0 min	Saline	Ethanol	Saline
S-E	Saline	0 min	Ethanol	Ethanol	Saline
MTX-S	3-Methoxynaltrexone	0 min	Saline	Ethanol	Saline
MTX-E	3-Methoxynaltrexone	0 min	Ethanol	Ethanol	Saline

Ethanol was injected at the dose of 2.5 g/kg. Naltrexone doses: 1 or 2 mg/kg; CTOP doses: 1 or 1.5 mg/kg; Naltrindole: 5 and 10 mg/kg. Naloxonazine doses were 15 or 30 mg/kg when given 20 h before saline or ethanol and 10 or 15 mg/kg when given 15 min before. 3-Methoxynaltrexone doses: 3 or 6 mg/kg. During the treatment phase, mice were left undisturbed on days 2, 4, 6, 8, and 10. No experiments were conducted during the 7-day period between the treatment and the challenge phase. Locomotor activity was measured immediately following the treatment injection.

injected 15 min prior to saline or ethanol, while naloxonazine was given under two different treatment conditions: 15 min or 20 h before saline or ethanol. In this respect, radioligand binding studies have demonstrated that naloxonazine has a long-lasting (20 h after its administration) action as a specific mu_1 opioid receptor antagonist after an immediate transitory blockade of mu_{1+2} receptors (Ling *et al*, 1986). In the present study we compared the effects of naloxonazine on ethanol sensitization when blocking mu_1 to those obtained when antagonizing mu_{1+2} opioid receptors. When naloxonazine was given 20 h before ethanol, doses of naloxonazine were 0, 15, or 30 mg/kg. When given 15 min before ethanol, doses were 0, 10, or 15 mg/kg. 3-Methoxynaltrexone (0, 3, or 6 mg/kg) was administered immediately before saline or ethanol. Doses of naltrexone, naltrindole, naloxonazine, and 3-methoxynaltrexone were selected based on studies involving ethanol-induced behaviors in rodents, including acute ethanol stimulation (Ciccocioppo *et al*, 2002; Honkanen *et al*, 1996; Mhatre and Holloway, 2003; Pastor *et al*, 2005; Sanchis-Segura *et al*, 2004). As there were no previous data showing CTOP effects on ethanol-induced locomotor behaviors, we examined the effect of this compound on acute ethanol stimulation (experiment 2a). Taken from this experiment, doses of CTOP tested for sensitization were 1 and 1.5 mg/kg. The same dose of ethanol (2.5 g/kg) was used for the treatment and the challenge phase; this dose was selected based on previous studies showing that it significantly increases horizontal locomotion in mice (Pastor *et al*, 2002; Sanchis-Segura *et al*, 2004). For all experiments, mice were moved from the colony room to the testing room and left undisturbed at least 30–45 min prior to experimentation to permit acclimation to the testing environment. Group size was 9–12 mice in all experiments: a total of 54–60 animals per experiment were used.

Determination of Blood Ethanol Levels

To study whether naltrexone and CTOP (the two compounds that prevented the development of ethanol sensitization) altered blood ethanol levels (BEL), a separate experiment was conducted ($n = 6$ per group). This experiment paralleled the injection and timing schedule followed for the behavioral study during the treatment phase; however, no locomotor activity testing was undertaken. Doses of naltrexone and CTOP used in this experiment were 2 and 1.5 mg/kg, respectively. On days 1 and 11, tail vein blood samples (20 µl) were collected 20 min after ethanol (2.5 g/kg) administration. Following Boehm *et al* (2000), each blood sample was immediately placed in a microcentrifuge tube containing 50 µl of ice-cold 5% $ZnSO_4$ solution. A 50 µl aliquot of 0.3 N $Ba(OH)_2$ and 300 µl of deionized water were added. After centrifugation at 4°C (5 min, 12000 rpm), the supernatant was removed and BELs were determined using head space gas chromatography (CE Instruments GC 8000, HS 850).

Data Analysis

The dependent variable for all behavioral experiments was distance traveled (in cm) during 15 min. For the treatment phase of the experiments, data were analyzed with repeated measures analysis of variance (ANOVA), with the pretreatment, antagonist dose (saline, naltrexone, CTOP, naltrindole, naloxonazine, or 3-methoxynaltrexone) and the treatment, ethanol dose (0 or 2.5 g/kg) as the between-groups variables and test sessions (days 1–11) as the repeated measure. Data from each challenge day (day 18, ethanol; day 19, saline) were analyzed separately, with only pretreatment and treatment as between-groups variables (two-way ANOVA). When significant interactions were obtained, Tukey's honestly significant difference (HSD) tests were used to determine specific differences between groups. Significance levels were set at $\alpha = 0.05$. BELs were analyzed using two-way ANOVAs with repeated measures (pretreatment × trial). All statistical analyses were conduced with the Statistica 6.1 software package (StatSoft, Inc., Tulsa, OK, USA).

RESULTS

Experiment 1

Figure 1 shows the effect of repeated ethanol exposure on locomotor activity in mice pretreated with naltrexone. Analysis of the treatment phase supported that our protocol was suitable to induce behavioral sensitization to ethanol, as ANOVA revealed a main effect of ethanol dose ($F_{1,46} = 76.16$; $p < 0.01$) and a significant ethanol dose × trial interaction was found to be significant ($F_{5,230} = 7.12$; $p < 0.01$). Naltrexone pretreatment prevented the development of ethanol sensitization but it did not affect activity in saline-treated mice; a significant effect of the naltrexone dose ($F_{2,46} = 37.15$; $p < 0.01$) and an interaction between

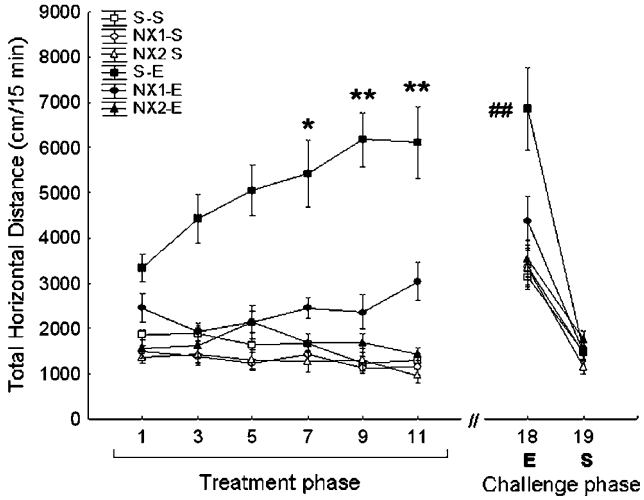

Figure 1 Effects of naltrexone on ethanol sensitization. Group means (±SEM) for the last 15 min of a 20-min test are presented. Numbers in the abscise axis refer to the day of the experiment. On days 1–11 mice received, every other day, the treatments indicated in the legend (S, saline; E, ethanol; NX1, naltrexone 1 mg/kg; NX2, naltrexone 2 mg/kg). On days 18 and 19 all mice received ethanol and saline, respectively. Through all the experiment the ethanol dose was 2.5 g/kg. Asterisks indicate significant increases in locomotor activity compared with day 1 of the same group (*$p < 0.05$, **$p < 0.01$). Number signs indicate significant differences to the rest of the groups tested on day 18 ($^{\#\#}p < 0.01$).

naltrexone dose and ethanol dose ($F_{2,46} = 25.29$; $p < 0.01$) were obtained. The triple interaction (naltrexone dose × ethanol dose × trials) was found significative ($F_{10,230} = 3.98$; $p < 0.01$). Tukey's *post hoc* comparisons showed that ethanol induced a significant increase in locomotor activity on day 1; the saline–ethanol group was different from the rest of the groups ($p < 0.05$) except the naltrexone 1 mg/kg-ethanol group. In the saline–ethanol group, it was found an increased response to ethanol on days 7 ($p < 0.05$), 9, or 11 ($p < 0.01$) relative to the acute response to ethanol; however, there were not differences between days 7 and 11, suggesting a ceiling effect of ethanol. All groups, except the saline–ethanol group, were found to not increase (or decrease) their locomotor activity scores across the six test sessions conducted during the treatment phase. With respect to the ethanol challenge, only the saline–ethanol group showed a sensitized response to ethanol. A two-way ANOVA revealed a significant effect of the naltrexone dose ($F_{2,46} = 4.7$; $p < 0.05$), ethanol dose ($F_{1,46} = 14.22$; $p < 0.01$) and a significant interaction ($F_{2,46} = 7.13$; $p < 0.01$), confirming the differences among groups observed during the treatment phase. The saline challenge showed that the treatments applied before this challenge did not affect the locomotor response to a saline injection. With respect to BELs, we tested the effects of 2 mg/kg of naltrexone on BELs on days 1 and 11, and no effects of this pretreatment were found (day 1, 60 ± 3.6 mM for the saline–ethanol group, and 63 ± 4.5 mM for the naltrexone–ethanol group; day 11, 69 ± 4.9 mM for the saline–ethanol group, and 72 ± 3.9 mM for the naltrexone–ethanol group).

Experiment 2a and 2b

Acute ethanol stimulation was tested in animals pretreated with several doses of CTOP (Figure 2a). The stimulant response to ethanol was dose-dependently attenuated by CTOP, an observation supported by the results of a two-way ANOVA that revealed a significant effect of the ethanol treatment ($F_{1,80} = 16.34$; $p < 0.01$), the pretreatment with CTOP ($F_{4,80} = 14.05$; $p < 0.01$) and the interaction between the two factors ($F_{4,80} = 4.08$; $p < 0.01$). Tukey's *post hoc* tests indicated that mice treated with 1 or 1.5 (but not 0.1 or 0.5) mg/kg of CTOP in combination with ethanol exhibit no stimulation of locomotor behavior ($p < 0.05$). These doses of CTOP (1 and 1.5 mg/kg), which were found to not affect spontaneous locomotor activity, were used to assess the effect of mu opioid receptor blockade on ethanol sensitization (Figure 2b). For the sensitization experiment, ANOVA analyses of the treatment phase revealed a significant main effect of CTOP dose ($F_{2,52} = 91.72$; $p < 0.01$), ethanol dose ($F_{1,52} = 150.58$; $p < 0.01$) and their interaction ($F_{2,52} = 63.30$; $p < 0.01$). The repeated tests (trial factor) were found to interact with ethanol ($F_{5,260} = 3.48$; $p < 0.05$) and CTOP dose ($F_{10,260} = 3.72$; $p < 0.05$). The triple interaction was also found statistically significant ($F_{10,260} = 3.28$; $p < 0.05$). Tukey's tests confirmed that both CTOP doses attenuated acute ethanol stimulation ($p < 0.05$). *Post hoc* tests comparing within-group values showed that, across the six trials of the treatment phase, no differences in locomotor activity (increases or decreases) were found except for the saline–ethanol group, which displayed an increased response to ethanol on days 7, 9 ($p < 0.05$), and 11 ($p < 0.01$) as compared to day 1. The prevention of sensitization by CTOP was found only at the dose of 1.5 mg/kg ($p < 0.01$ compared to saline–ethanol), as data from the ethanol challenge displayed a sensitized response to ethanol in the group pretreated with 1 mg/kg of CTOP and treated with ethanol. Analyses of this challenge revealed a significant main effect of the pretreatment ($F_{2,52} = 8.07$; $p < 0.01$), treatment ($F_{1,52} = 33.51$; $p < 0.01$), and a significant interaction ($F_{2,52} = 3.34$; $p < 0.05$). The locomotor activity measured after a saline injection (day 19) was found not

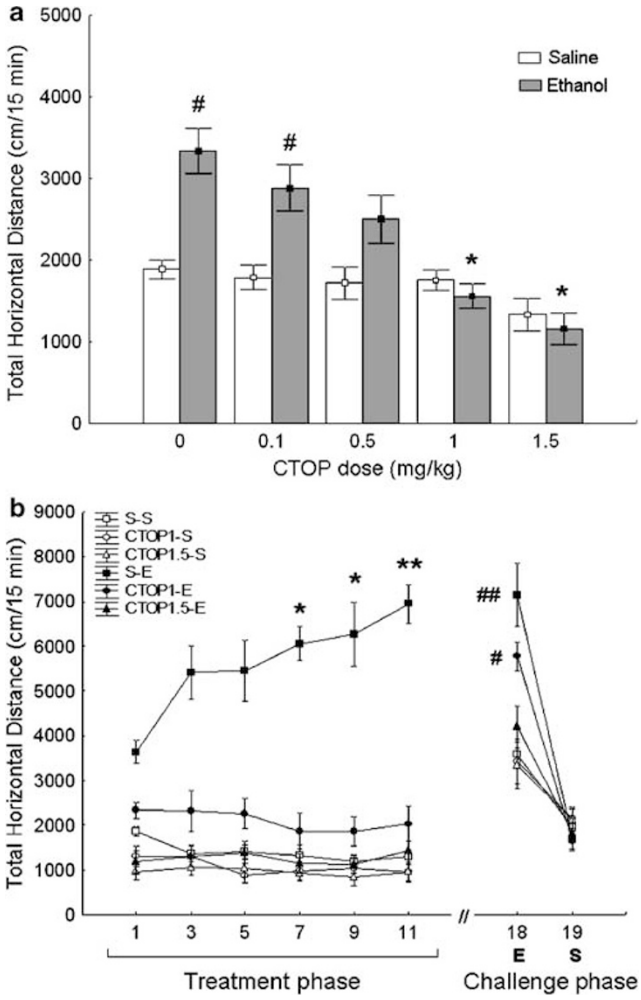

Figure 2 (a) Effects of CTOP on acute ethanol stimulation. Group means (±SEM) for the last 15 min of a 20-min test are presented. Animals were pretreated with CTOP (0, 0.1, 0.5, 1 or 1.5 mg/kg) and challenged with ethanol (0 or 2.5 g/kg) 15 min after CTOP administration (*$p < 0.05$ significantly different from the saline–ethanol group; #$p < 0.05$ significantly different from the saline–saline group). (b) Effects of CTOP on ethanol sensitization. Group means (±SEM) for the last 15 min of a 20-min test are presented. Numbers in the abscise axis refer to the day of the experiment. On days 1–11 mice received, every other day, the treatments indicated in the legend. (S, saline; E, ethanol; CTOP1, CTOP 1 mg/kg; CTOP1.5, CTOP 1.5 mg/kg). On days 18 and 19 all mice received ethanol and saline, respectively. Ethanol dose was 2.5 g/kg. Asterisks indicate significant increases in locomotor activity compared with day 1 of the same group (*$p < 0.05$, **$p < 0.01$). Number signs indicate significant differences to groups also tested on day 18. # indicates different from the rest of the groups except S-E ($p < 0.05$). ## indicates different from the rest of the groups except CTOP1-E ($p < 0.01$).

different among groups. On days 1 and 11, BELs were determined in animals pretreated with 1.5 mg/kg of CTOP; no effects of this pretreatment were found. Data were 64 ± 4.9 mM for the saline–ethanol group and 67 ± 5.8 mM for the CTOP–ethanol group on day 1 and 73 ± 4.5 mM for the saline–ethanol group and 71 ± 4.7 mM for the CTOP–ethanol group on day 11.

Experiment 3

As shown in Figure 3, the selective delta opioid receptor antagonist naltrindole did not affect ethanol sensitization. Ethanol induced a robust acute locomotor stimulant effect that significantly increased across test sessions, indicating behavioral sensitization; this effect was obtained regardless of the naltrindole dose. These conclusions were supported by the results of the ANOVA, which showed a significant effect of ethanol ($F_{1,48} = 420.74$; $p < 0.01$) and an ethanol × trial interaction ($F_{5,240} = 18.95$; $p < 0.01$). The trial factor was also found statistically significant ($F_{5,240} = 12.82$; $p < 0.01$). The lack of an effect of naltrindole pretreatment was also present when tested a week after the treatment phase. A two-way ANOVA for the ethanol challenge revealed that all groups repeatedly treated with ethanol during the treatment phase, regardless of naltrindole dose, displayed a sensitized response to ethanol, which was manifested as a main effect of ethanol dose ($F_{1,48} = 46.89$; $p < 0.01$), but not naltrindole dose was found. In addition, no interaction between the two factors was found. With respect to the saline challenge, as occurred in experiments 1 and 2b, experimental groups did not differ in the locomotor response to saline.

Experiments 4 and 5

The effects of naloxonazine on ethanol sensitization are presented in Figures 4 and 5, as this opioid antagonist was administered under two different treatment conditions, 20 h (Figure 4) or 15 min (Figure 5) before ethanol. When naloxonazine was given 20 h before ethanol, it did not alter the development of sensitization to ethanol. During the treatment phase, the ethanol ($F_{1,50} = 464.32$; $p < 0.01$) and trial factors ($F_{5,250} = 11.71$; $p < 0.01$) were found significant, but naloxonazine dose lacked effect and did not interact with any other factor. The ethanol challenge revealed a greater response to ethanol ($F_{1,50} = 45.32$; $p < 0.01$) in all

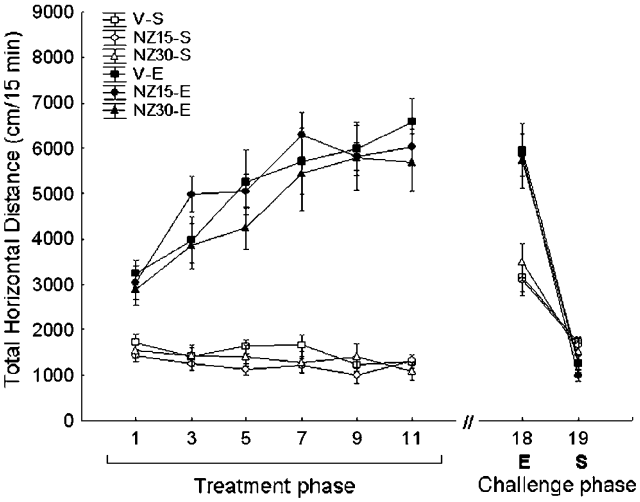

Figure 4 Effects of naloxonazine, given 20 h before, on ethanol sensitization. Group means (±SEM) for the last 15 min of a 20-min test are presented. Numbers in the abscise axis refer to the day of the experiment. On days 1–11 mice received, every other day, the treatments indicated in the legend. (V, vehicle; E, ethanol; NZ15, naloxonazine 15 mg/kg; NZ30, naloxonazine 30 mg/kg). On days 18 and 19 all mice received ethanol and saline, respectively. Through all the experiment ethanol was injected at the dose of 2.5 g/kg.

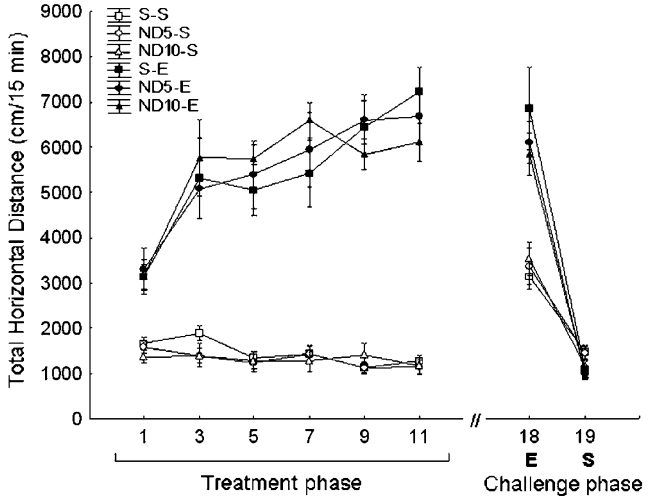

Figure 3 Effects of naltrindole on ethanol sensitization. Group means (±SEM) for the last 15 min of a 20-min test are presented. Numbers in the abscise axis refer to the day of the experiment. On days 1–11 mice received, every other day, the treatments indicated in the legend (S, saline; E, ethanol; ND5, naltrindole 5 mg/kg; ND10, naltrindole 10 mg/kg). On days 18 and 19 all mice received ethanol and saline, respectively. Through all the experiment ethanol was injected at the dose of 2.5 g/kg.

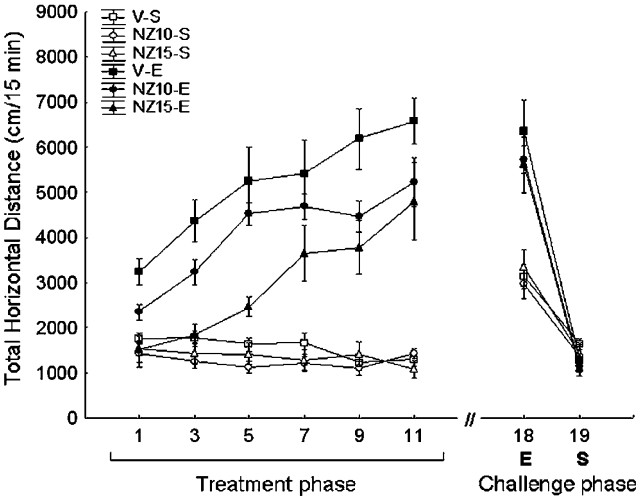

Figure 5 Effects of naloxonazine, given 15 min before, on ethanol sensitization. Group means (±SEM) for the last 15 min of a 20-min test are presented. Numbers in the abscise axis refer to the day of the experiment. On days 1–11 mice received, every other day, the treatments indicated in the legend. (V, vehicle; E, ethanol; NZ10, naloxonazine 10 mg/kg; NZ15, naloxonazine 15 mg/kg). On days 18 and 19 all mice received ethanol and saline, respectively. Through all the experiment ethanol was injected at the dose of 2.5 g/kg.

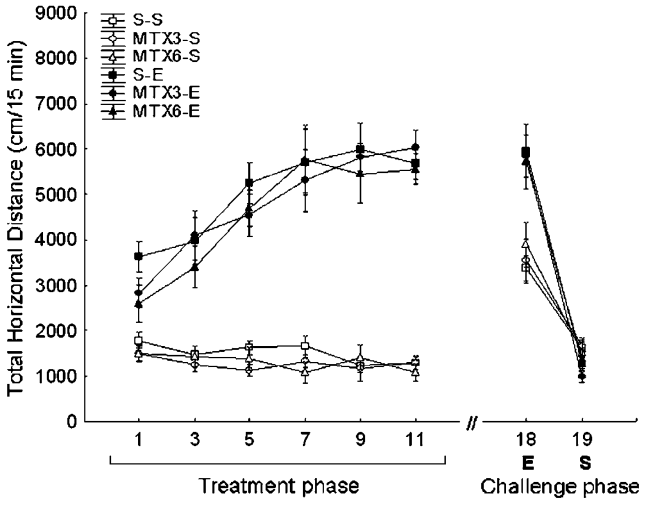

Figure 6 Effects of 3-methoxynaltrexone on ethanol sensitization. Group means (±SEM) for the last 15 min of a 20-min test are presented. Numbers in the abscise axis refer to the day of the experiment. On days 1–11 mice received, every other day, the treatments indicated in the legend (S, saline; E, ethanol; MTX3, 3-methoxynaltrexone 3 mg/kg; MTX6, 3-methoxynaltrexone 6 mg/kg). On days 18 and 19 all mice received ethanol and saline, respectively. Through all the experiment ethanol was injected at the dose of 2.5 g/kg.

groups that received ethanol during the treatment phase regardless of the pretreatment. No differences among groups were found on day 19 (saline challenge). When naloxonazine was given 15 min before ethanol, the development of sensitization was significantly altered. ANOVA analysis for the treatment phase revealed a main effect of naloxonazine dose ($F_{2,49} = 14.26$; $p < 0.01$) and a naloxonazine dose × ethanol dose interaction ($F_{2,49} = 9.68$; $p < 0.01$). Also, the effect of ethanol ($F_{1,49} = 225.58$; $p < 0.01$) and the ethanol × trial interaction ($F_{5,245} = 13.77$; $p < 0.01$) were found to be statistically significant; however, the naloxonazine dose × ethanol dose × trial interaction was not significant. The ethanol challenge, however, revealed that regardless of the pretreatment, all groups treated with ethanol during the treatment phase displayed a sensitized response to ethanol as only the treatment factor was found significant ($F_{1,49} = 42.80$; $p < 0.01$). There were no differences among groups on day 19 (ie the saline challenge day).

Experiment 6

Administration of 3-methoxyanaltrexone (Figure 6) did not alter the ability of ethanol to induce sensitization to its locomotor stimulating effects. As a lack of the antagonist dose × ethanol dose × trial interaction was not found, we did not analyze the effects of this compound on acute ethanol stimulation, although a modestly reduced response to ethanol was present on day 1. ANOVA for the treatment phase showed only a significant effect of ethanol dose ($F_{1,50} = 381.99$; $p < 0.01$), trial factor ($F_{5,250} = 12.79$; $p < 0.01$), and ethanol dose × trial interaction ($F_{5,250} = 19.19$; $p < 0.01$). This lack of effect of 3-methoxyanaltrexone was also found on day 18 (ie the ethanol challenge). In fact, a sensitized response to ethanol was found in all groups that previously received ethanol (ethanol treatment: $F_{1,50} = 29.98$; $p < 0.01$) despite that some of them were administered with this mu_3 antagonist. The saline challenge did not reveal any significant difference among groups.

DISCUSSION

The findings of the present study provide new insights into the involvement of the endogenous opioid system in the behavioral effects of ethanol. The current studies demonstrated that the neural processes underlying development of sensitization to the locomotor stimulating effects of ethanol require the activation of mu, but not delta opioid receptors. The role of specific subtypes of the mu opioid receptor (ie mu_{1+2} or mu_3) in ethanol sensitization, however, appears to be limited; as we found that the functional relevance of these subtypes is restricted to the acute locomotor effects of ethanol.

Data demonstrated robust sensitization to the locomotor activating effects of ethanol, as has previously been reported for Swiss mice (Camarini *et al*, 2000b; Miquel *et al*, 2003). Ethanol (2.5 g/kg) produced a significant increase in locomotor activity when acutely administered. This effect increased progressively across the sessions and ended up reaching a plateau, indicating that our protocol led to the development of a ceiling effect on the locomotor stimulation produced by repeated ethanol administration. The sensitized response to ethanol was also evident when mice were tested following an abstinence period of 7 days, suggesting, as previously reported (Lessov and Phillips, 1998), persistent neural changes induced by ethanol. Interestingly, it has been postulated that such persistence could be associated with potentially permanent adaptations in neural pathways important for the motivational properties of drugs of abuse (Kalivas *et al*, 1993; Robinson and Berridge, 1993). We also observed that mice sensitized to ethanol did not show a conditioned locomotor response to the environment where testing with ethanol occurred. On the last day of the experiments, mice treated with repeated ethanol or saline did not differ in their locomotor response to a saline injection. The absence of any conditioned effect is critical to interpret the effect of pharmacological compounds that prevent drug-induced sensitization. If a conditioned effect were present, it would be difficult to discern whether the prevention of sensitization is due to a blockade of the mechanisms that underlie sensitization or whether this blockade is actually achieved by disrupting the associative memories that account for the conditioned locomotor effect (Cunningham and Noble, 1992).

Our results showed that naltrexone, at doses of 1 and 2 mg/kg, prevented the development of ethanol sensitization; an effect that parallels previous data using naloxone (Camarini *et al*, 2000b). Across the treatment phase, mice that were injected with naltrexone displayed an attenuated locomotor response to ethanol, but an unaffected response to saline. Interestingly, when challenged with ethanol (in absence of naltrexone), those groups previously treated with naltrexone and ethanol displayed a magnitude of locomotor stimulation not different from that exhibited by mice that experienced the effects of ethanol for the first time. Both our results and those reported by Camarini *et al* (2000b) are

obtained using doses of naltrexone and naloxone (close to 1 mg/kg) that are supposed to act selectively at mu opioid receptors (Takemori and Portoghese, 1984; Williams *et al*, 2001), suggesting that these particular opioid receptors may be critically involved in ethanol sensitization. This hypothesis seems to be convincing as we found that the selective mu opioid receptor antagonist CTOP (1.5 mg/kg), prevented ethanol sensitization without affecting behavior of saline-treated mice. As there are no precedent data using specific mu opioid receptor antagonists on ethanol-induced locomotor behaviors, it is also noteworthy that CTOP dose-dependently reduced the acute stimulatory effects of ethanol.

Our results indicated that delta opioid receptors are not involved in the development of behavioral sensitization to ethanol, as we found that naltrindole, an antagonist selective for delta opioid receptors (Portoghese *et al*, 1988) failed to prevent the development of ethanol sensitization. It seems unlikely that the lack of effect of naltrindole on ethanol sensitization could be explained by an insufficient dose, as we used doses similar or even higher than those reported to be effective in reducing, for instance, voluntary ethanol consumption or the conditioned reinstatement of ethanol-seeking behavior (Ciccocioppo *et al*, 2002; Hyytia and Kiianmaa, 2001; Kim *et al*, 2000). The current results suggest, therefore, that the neural adaptations responsible for the development of sensitization to ethanol require the activation of mu but not delta opioid receptors.

Although a single gene encoding the mu opioid receptor (MOR-1) has been cloned, several forms of the mu receptor mRNA arising from alternative mRNA slicing have been reported (Pan *et al*, 2001; Pasternak, 2001a, b). Based on a differential antagonism of the analgesic effects of several opiate drugs as well as on radioligand binding studies, compounds that can functionally differentiate three subtypes of mu opioid receptors (mu_1, mu_2, and mu_3) have been identified (Pasternak, 2001a, b; Cadet *et al*, 2003). Interestingly, these subtypes of mu receptor have been implicated in some of the behavioral effects of ethanol. For instance, the antagonism of mu_1 opioid receptors has been found to reduce conditioned ethanol-seeking behavior (Ciccocioppo *et al*, 2002) but only modestly decreased voluntary ethanol intake (Honkanen *et al*, 1996; Franck *et al*, 1998). The blockade of both of mu_{1+2} receptors resulted in a reduction in ethanol consumption as well as a decrease in the discriminative effects of ethanol (Mhatre and Holloway, 2003). Recently, mu_{1+2} and mu_3, but not mu_1 opioid receptors, have been found to mediate the acute locomotor stimulating actions of ethanol (Pastor *et al*, 2005).

In the present study, however, we observed that neither the antagonism of mu_1 (naloxonazine, 20 h before ethanol) nor mu_3 opioid receptors (3-methoxynaltrexone) affected sensitization to ethanol, despite a modest decrease in acute ethanol-induced stimulation that was found with 3-methoxynaltrexone. The blockade of mu_{1+2} receptors (naloxonazine, 15 min before ethanol) attenuated the acute locomotor stimulating effects of ethanol and delayed, but did not prevent sensitization to ethanol. It has to be noted that currently there are no pharmacological tools to specifically block mu_2 receptors. However, there is little evidence supporting a hypothesis that mu_2 receptors may underlie ethanol sensitization, as antagonism of mu_{1+2} opioid receptors lacked effect at blocking sensitization. Other possibilities, such as the concurrent blockade of mu_{1+3} or mu_{2+3} opioid receptors, can be hypothesized as putative mechanisms underlying sensitization to ethanol. However, the antagonism of mu_1 or mu_3 receptors, when tested independently, had no effect on ethanol sensitization; it seems very unlikely, thus, that the concurrent inactivation of mu_{1+3} opioid receptors could result in a blockade of sensitization. Also, limited evidence supports an involvement of mu_{2+3} opioid receptors, as we found that the blockade of mu_3 or mu_{1+2} opioid receptors lacked effect at preventing sensitization to ethanol. Altogether, we tentatively propose that a concurrent blockade of all mu opioid receptor subtypes may be required to block the development of behavioral sensitization to ethanol.

As proposed elsewhere (Meyer and Phillips, 2003), the interpretation of the effects of compounds that prevent ethanol sensitization is challenged by the fact that these drugs also block the acute stimulant effects of ethanol. It might be that in order for sensitization to develop animals require the experience of the acute stimulatory effects of ethanol (Meyer and Phillips, 2003). In the present study, however, we observed that animals treated with 1 mg/kg of CTOP did not show acute stimulation or development of sensitization during the treatment phase. Nevertheless, when challenged with ethanol (in absence of CTOP) 7 days after the end of the treatment phase, they displayed a sensitized response to ethanol. Thus, while the debate on whether or not development of sensitization to ethanol requires exposure to its stimulant effects remains interesting, we consider that the prevention of sensitization that we obtained with naltrexone and CTOP may be achieved by blocking the neural mechanism underlying sensitization. This conclusion is additionally supported by the fact that changes in the pharmacokinetics of ethanol cannot be explaining the effect of these compounds on ethanol sensitization, as we found that blood ethanol concentrations were not modified by naltrexone or CTOP.

The hypothesis that ethanol can interfere the coupling of mu opioid receptors to G proteins has been suggested as an explanation for the involvement of mu receptors in the behavioral effects of ethanol (Chen and Lawrence, 2000; Saland *et al*, 2004). To our understanding, however, the most convincing proposal to comprehend the neural mechanism by which these receptors participate in ethanol sensitization may be related to the actions of the mu opioid receptor endogenous ligand, β-endorphin. Ethanol administration produces an increase in β-endorphin release, as measured by *in vivo* microdialysis in the NAcb (Olive *et al*, 2001; Marinelli *et al*, 2003). Also, acute administration of ethanol increased β-endorphin neurotransmission in the ventral tegmental area (VTA) (Rasmussen *et al*, 1998). The brain sites of synthesis of the β-endorphin precursor, proopiomelanocortin (POMc) primarily include the hypothalamic arcuate nucleus (ArcN) (Khatchaturian *et al*, 1985). Interestingly, lesions of the ArcN prevented the development of behavioral sensitization to ethanol (Miquel *et al*, 2003). The parallelism between the effects of an ArcN lesion and the antagonism of mu opioid receptors on ethanol strongly suggests that the involvement of the opioid

system in ethanol sensitization could depend on the activation of mu opioid receptors by ethanol-induced release of β-endorphin.

It is well established that the VTA and the NAcb, as well as the prefrontal cortex, are critical contributors to drug-induced behavioral sensitization (Pierce and Kalivas, 1997; Robinson and Kolb, 1997; Vanderschuren and Kalivas, 2000). The POMc projections arising from the ventrolateral part of the ArcN project to the VTA and the NAcb (Chronwall, 1985), and have been suggested to control basal DA release in the NAcb (Spanagel *et al*, 1992). The release of DA in the NAcb seems to be evoked indirectly by the activation of mu opioid receptors located on VTA GABAergic interneurons (Leone *et al*, 1991; Longoni *et al*, 1991; Spanagel *et al*, 1992). This neurochemical pathway has also been proposed to explain ethanol-induced increases in DA release in the NAcb, as opioid receptor antagonists prevented ethanol-induced activation of VTA DA neurons (Gonzales and Weiss, 1998; Tanda and Di Chiara, 1998). Altogether, these data support the hypothesis that mesolimbic DA pathways could be of critical importance to explain ethanol-induced sensitization. However, it is currently difficult to reach clear conclusions on the involvement of DA in sensitization to ethanol. It has been demonstrated that chronic administration of ethanol sensitized DA neurons in the VTA (Brodie, 2002) and provoked long-term changes in the DAergic terminals of the NAcb and the dorsal striatum (Nestby *et al*, 1997). Also, increases in DA D_2 receptor binding have been described in mice sensitized to ethanol (Souza-Formigoni *et al*, 1999). However, mice lacking DA D_2 receptors did not show attenuated ethanol-induced sensitization (Palmer *et al*, 2003) and the nonspecific DA receptor antagonist haloperidol failed at preventing sensitization to ethanol (Broadbent *et al*, 1995).

Although the role of DA in ethanol sensitization requires further research, it might be possible that opioid receptors others than those located in brain structures that modulate DA activity could be involved in ethanol sensitization. For instance, it has been demonstrated that mu opioid receptors in the ventral pallidum (VP) support morphine-induced behavioral sensitization, as injections of the mu receptor antagonist CTOP into the VP blocked behavioral sensitization to morphine (Johnson and Napier, 2000). We hypothesize that this may be also explaining why naltrexone and CTOP blocked ethanol sensitization. Data indicate that the VP is involved in both the motor (Austin and Kalivas, 1990; Hoffman *et al*, 1991) and the incentive (Hiroi and White, 1993; McFarland and Kalivas, 2001) properties of psychomotor stimulants and opiates. At this respect, it is not presently known whether the ArcN sends endorphinic projections to the VP. It is clear, though, that the VP serves as a primary output for the NAcb (Chrobak and Napier, 1993; Groenewegen and Russchen, 1984). Because an ArcN-NAcb projection has been described (Chronwall, 1985), it could be possible that the changes in the VP that may be necessary for the development of ethanol sensitization could be prevented by blocking the action of β-endorphins at the level of the NAcb and/or by blocking mu receptors in the VP.

In summary, these findings extend our understanding of the neurochemistry that underlie ethanol-induced behavioral sensitization, a characteristic sequelae of repeated ethanol exposure with critical implications for addiction. Future experiments will be devoted to explore all these above proposed hypotheses. First, direct evidence on the role of β-endorphins on ethanol sensitization is required. Second, a precise neuroanatomic analysis of the importance of mu opioid repectors in the mesopallidal system in ethanol sensitization would be enlightening.

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