Manuscript Number: BBR-D-12-00329R2

Title: Anxiolytic effects of ethanol are partially related to a reduced expression of adenylyl cyclase 5 but not to μ -opioid receptor activation in rat nucleus accumbens.

Article Type: Research Reports

Keywords: cAMP, nucleus accumbens, anxiety, opioid receptors, adenylyl cyclase

Corresponding Author: Dr P. de Gortari,

Corresponding Author's Institution:

First Author: Marcela Morales-Mulia, Ph.D.

Order of Authors: Marcela Morales-Mulia, Ph.D.; Erika Estrada-Camarena, Ph.D.; María Isabel Amaya, Technician; Silvia Mejía-Mauríes, Technician; Isabel Sollozo-Dupont, M.S.; Guadalupe Mengod, Ph.D.; P. de Gortari

Abstract: Anxiolytic effects of alcohol participate in the reinforcing properties of the drug, in which nucleus accumbens (NAcc) is implicated. The opioidergic system in NAcc is considered a main pathway involved in the emotional responses of animals: rats microinjected with morphine in NAcc and the systemic administration of μ -opioid receptors (MOR) agonists yield low anxiety scores in the elevated plus maze (EPM), a behavioral test of anxiety. However, the specific participation of NAcc MOR in the anxiolytic effect of ethanol has not been studied. AC5, a cAMP-synthesizing adenylyl-cyclase, is highly expressed in NAcc; it is negatively coupled to MOR and has been implicated in anxiety levels of animals. We evaluated the anxiolytic effects of an intra-gastric administration of ethanol (2.5g/kg) in animals subjected to EPM at 1, 4, and 8h after drug or water exposure. Locomotion was assayed with the open-field test; we also measured accumbal AC5 and MOR mRNA levels by RT-PCR. After 1h, ethanol-exposed animals showed anxiolytic-like behavior, as well as decreased and increased AC5 and MOR expression in NAcc, respectively. Intra-accumbal injection of β -funaltrexamine (FNA), a MOR antagonist, did not block ethanol-induced anxiolysis, rather it induced a tendency to increase anxiety levels in the water-exposed group. FNA partially decreased accumbal AC5 expression in ethanol-treated rats. We concluded that AC5 in NAcc is participating in the emotional effects of ethanol; that MOR was not mediating the drug-induced AC5 reduction in NAcc nor the ethanol-induced anxiolysis. MOR only might be involved in basal levels of anxiety of animals.

Research Highlights

- After 1h ethanol-administered animals presented anxiolytic behavior
- AC5 expression in nucleus accumbens is reduced only in animals with an acute administration of ethanol.
- MOR expression in nucleus accumbens increased in response to ethanol administration after 1h.
- Injection of MOR antagonist in nucleus accumbens did not reverse anxiolytic behavior of ethanol administered animals.
- FNA (MOR antagonist) partially decreased accumbal AC5 expression in ethanol-treated rats.
- We concluded that AC5 in NAcc is participating in the emotional effects of ethanol.
- MOR was not mediating the drug-induced AC5 reduction in NAcc nor the ethanol-induced anxiolysis.
- MOR only might be involved in basal levels of anxiety of animals.

Anxiolytic effects of ethanol are partially related to a reduced expression of
adenylyl cyclase 5 but not to μ -opioid receptor activation in rat nucleus accumbens.

Morales-Mulia M¹, Estrada-Camarena E², Amaya M.I¹, Mejía-Mauries S², Sollozo-Dupont
I², Mengod G³, de Gortari P^{1*}.

¹ Neurofisiología Molecular, Dirección de Neurociencias, INPRFM. México D.F.

² Neuropsicofarmacología, Dirección de Neurociencias, INPRFM. México D.F.

³ Departamento de Neuroquímica y Neurofarmacología, Institut d'Investigacions,
Biomèdiques de Barcelona, CSIC-IDIBAPS, CIBERNED, Barcelona, España.

*Corresponding author: Patricia de Gortari G. Instituto Nacional de Psiquiatría Ramón de la
Fuente. Calzada México-Xochimilco 101, Col. San Lorenzo Huipulco, México D.F. 14370.

Email: gortari@imp.edu.mx

Tel. 52 (55) 4160-5056.

Fax: 52 (55) 5655-9980

ABSTRACT.

Anxiolytic effects of alcohol participate in the reinforcing properties of the drug, in which nucleus accumbens (NAcc) is implicated. The opioidergic system in NAcc is considered a main pathway involved in the emotional responses of animals: rats microinjected with morphine in NAcc and the systemic administration of μ -opioid receptors (MOR) agonists yield low anxiety scores in the elevated plus maze (EPM), a behavioral test of anxiety. However, the specific participation of NAcc MOR in the anxiolytic effect of ethanol has not been studied. AC5, a cAMP-synthesizing adenylyl-cyclase, is highly expressed in NAcc; it is negatively coupled to MOR and has been implicated in anxiety levels of animals.

We evaluated the anxiolytic effects of an intra-gastric administration of ethanol (2.5g/kg) in animals subjected to EPM at 1, 4, and 8h after drug or water exposure. Locomotion was assayed with the open-field test; we also measured accumbal AC5 and MOR mRNA levels by RT-PCR. After 1h, ethanol-exposed animals showed anxiolytic-like behavior, as well as decreased and increased AC5 and MOR expression in NAcc, respectively. Intra-accumbal injection of β -funaltrexamine (FNA), a MOR antagonist, did not block ethanol-induced anxiolysis, rather it induced a tendency to increase anxiety levels in the water-exposed group. FNA partially decreased accumbal AC5 expression in ethanol-treated rats. We concluded that AC5 in NAcc is participating in the emotional effects of ethanol; that MOR was not mediating the drug-induced AC5 reduction in NAcc nor the ethanol-induced anxiolysis. MOR only might be involved in basal levels of anxiety of animals.

Keywords: cAMP, nucleus accumbens, anxiety, opioid receptors, adenylyl cyclase.

1. Introduction

One of the signs of alcohol dependence is the increased anxious behavior presented by animals and humans that results from hyperactivation of the central nervous system; this behavior is evident during drug withdrawal [1,2]. Thus, anxiolytic effects of alcohol are involved in the reinforcing properties of the drug: individuals with chronic stress or high anxiety levels may be more sensitive to the anxiolytic effects of ethanol and may show higher predisposition to consume alcohol than unstressed subjects. Accordingly, human and animal studies show that acute exposure from low-to-moderate doses of ethanol induces anxiolytic-like behavior [1-3].

The endogenous opioid system is essential in ethanol reinforcing actions and alcohol drinking behavior, possibly through the activation of μ or δ opioid receptors in the brain, and in particular in the meso-accumbens circuit [4-7]. This circuit is constituted mainly by dopaminergic cells located in the ventral tegmental area (VTA) and nucleus accumbens (NAcc), which receives fibers arising from the VTA. NAcc is implicated in reward and reinforcing properties of ethanol and other drugs of abuse and has crucial roles in motor function and emotional responses [8-10]. In fact, the performance in two animal tests for anxiety-like behavior, the avoidance test and the elevated plus maze (EPM), induces prominent Fos-like immunoreactivity in NAcc, among other areas [11]. Also the microinjection of morphine, GABA_A and GABA_B agonists into the NAcc induces anxiolytic-like effects when animals are tested with the EPM [12,13]. Although anxiolytic effects of ethanol may be mediated through activation of the GABA_A receptor [1,14], other systems may also contribute, such as the endogenous opioid system: systemic administration of agonists of κ or μ -opioid receptors (MOR) produces anxiolytic actions [15,16]. In addition, the role of MOR in anxiolysis has been demonstrated by studying MOR-knockout (MOR-KO) mice, which present decreased basal anxiety-like behavior in several tests [17].

1 The relation between the opioid system and ethanol-induced anxiolytic effects has not been
2 extensively studied. The scarce existing research has been performed in MOR-KO mice.
3
4 Some authors have found that MOR-KO mice do not present ethanol-induced anxiolytic
5 effects, while others show the opposite [18,19]. Discrepancies in emotional responses to
6 ethanol may be explained by differences in the route of administration of alcohol, its time of
7 exposure, and the time elapsed after ethanol administration and the moment animals are
8 subjected to the anxiety test.
9

10
11
12
13
14
15
16
17 Intracellular cAMP levels regulate the phosphorylation of CREB transcription factor
18 (cAMP response element binding protein) by the activity of protein kinase A (PKA). On the
19 other hand, intracellular cAMP levels are controlled in part by its synthesis through adenylyl
20 cyclase activities: adenylyl cyclase 5, AC5, is an isoenzyme highly expressed in dorsal
21 striatum and NAcc [20,21] that mediates MOR signaling [22,23]. MOR is negatively
22 coupled to AC5, and mRNAs of both are simultaneously expressed in NAcc [24]; this
23 suggests that AC5 is an important effector for opioid receptors in this brain region [23]. AC5
24 may play a key role in anxiety-related behaviors given that its genetic deletion in mice
25 produces an anxiolytic phenotype [25], and also because its blockage by siRNAs limited
26 only to the NAcc induces anxiolytic behavior in rodents [25].
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42

43 Alcohol consumption and acute or chronic administration of ethanol is known to affect
44 the cAMP pathway in the NAcc [26,27]. Participation of accumbal CREB in anxiety
45 behavior is suggested by using local viral gene transfer of a dominant negative mutant of
46 CREB, which increases anxiety levels of rodents [28,29], whereas the inhibition of PKA in
47 NAcc of animals does not have any effect on their anxiety-like behavior [26]. Therefore, the
48 purpose of the present study was to try to correlate changes in expression patterns of MOR
49 and AC5 in the NAcc with the anxiolytic-like behavior of animals receiving an acute
50 intragastric (i.g.) dose of ethanol (2.5 g/kg of body weight [b.w.]), followed by the exposure
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

of rats to the elevated plus maze test (EPM) at different times after alcohol administration. To evaluate the participation of accumbal MOR in anxiety behavior of ethanol-exposed animals and its relation to changes in AC5 expression, we injected a selective MOR antagonist (β -funaltrexamine, FNA) into the NAcc of ethanol or water-administered animals.

2. Materials and methods

2.1 Animals and treatment

All experiments were conducted in accordance to the National Institute of Health's *Guide for the Care and Use of Laboratory Animals* (NIH publications No. 80-23, revised 1996). Adult male Wistar rats (250 – 300 g b.w.), fed *ad libitum* (Purina Chow) and maintained in a 12h light-dark cycle, received a single acute dose of ethanol (2.5 g/kg b.w.) or distilled water by oral administration with an intragastric cannula, as previously reported [30]. To diminish stress, animals were habituated to the cannula by a daily oral administration of distilled water (5 ml/kg b.w.) during 7 days previous to the experiment. The ethanol solution (31.5% v/v) was prepared by diluting 2.5 g ethanol in distilled water to a final volume of 10 ml. To evaluate anxiety-like behavior, animals were subjected to the elevated plus maze (EPM) for 10 min, at 1, 4, or 8 h (n=8-10 animals/group) after water or ethanol administration. Locomotion of all rats was assessed by their performance in the open field test for 5 min; immediately after they were decapitated. Brains were removed and frozen on dry ice (-70°C) until analyzed. MOR and AC5 mRNA levels in the NAcc of all animals subjected to EPM after water or ethanol treatment were evaluated by RT-PCR.

2.2. Elevated plus maze (EPM)

The EPM has been described in detail elsewhere [31]. Briefly, the experimental device consists of an elevated (40 cm above the floor) plus-shaped maze placed in a room

1 illuminated by a 40-W red light bulb. The maze comprises four perpendicular arms, 50 cm
2 long and 10 cm wide. Two opposing arms are surrounded by 40-cm-high, white, opaque,
3 plastic walls (closed arms), while the open arms lack walls. The animal was placed in the
4 center of the maze facing a closed arm. An entry into an arm was determined when the
5 animal placed all four paws inside this part. The cumulative time spent in the open arms, the
6 number of entries made into the open arms, and the total number of crossings was video-
7 recorded over a 10-min session by a digital camera. Data are expressed as the percentage of
8 the total time spent in the open arms, total number of entries into the open arms (these two
9 parameters reflect anxiety levels), and total arm entries. The latter is a measure of the
10 exploratory behavior of animals.
11
12
13
14
15
16
17
18
19
20
21
22
23
24

25 *2.3. Open-field test*

26
27
28 In order to evaluate non-specific actions of drug treatment on general activity,
29 locomotion was tested using the open-field test in all animals previously subjected to the
30 EPM. The apparatus consists of an opaque-Plexiglas box (40 x 30 x 20 cm) with the floor
31 divided into 12 equal squares (11 x 11 cm). Animals were placed in a corner of the box and
32 an observer, blind to the pharmacological treatments, registered the number of times the
33 animal crossed squares during a 5-min session [32].
34
35
36
37
38
39
40
41
42
43

44 *2.4. Stereotaxic surgery for MOR antagonist injection*

45
46 For the bilateral infusion of β -funaltrexamine (FNA: a MOR-antagonist; 4 μ g/0.3 μ l)
47 into the NAcc, rats were anesthetized with ketamine (0.45 ml/kg) and xylazine (0.2 ml/kg)
48 and placed in a stereotaxic instrument (TSE, Systems Germany). Coordinates for the NAcc
49 were: AP=1.4 mm from lambda, L=+/-1.5 mm with respect to the midline and H=-6.9 mm
50 from the skull surface [33]. Vehicle or FNA administration was applied through a cannula
51 attached to a Hamilton syringe (0.1 μ l/min), the cannula was left for 3 min before removal to
52
53
54
55
56
57
58
59
60
61
62
63
64
65

prevent drug or vehicle to be drawn back by capillarity. The drill hole was covered with bone wax after the administration of antibiotic powder (sulfatiazol®), and the skin was sutured.

FNA was prepared in 15% cyclodextran-0.9% saline (vehicle) solution. Dose and latency were taken from studies showing that FNA reduces MOR binding by 49% at 24 hours, and that the decrease persists for up to 11 days [34]. This FNA dose in the NAcc is also able to block feeding effects induced by DAMGO, a MOR agonist [35]; this agonist can induce an anxiolytic-like effect by intra-amygdalar administration [36]. Five days after the injection of FNA or vehicle (V), animals received the i.g. administration of water: FNA+W and V+W groups or ethanol (2.5 mg/Kg of b.w.): FNA+Et and V+Et groups. After 1h, all the animals were subjected to the EPM and then decapitated. Brains were excised to evaluate AC5 mRNA levels in NAcc.

2.5. Tissue collection and sample preparation

Frozen brains were coronally sliced by hand between 2.3 and 1.3 mm from bregma, according to Paxinos and Watson [33]. Subsequently, a square of tissue (1 mm²) containing the anterior commissure was obtained from the right hemisphere to extract the NAcc, including the shell and core sub-regions, and maintained at -70°C until MOR and AC5 mRNA levels were determined by RT-PCR. A total of 4-8 animals/group/time of sacrifice (1, 4 and 8h) that performed the EPM behavioral test were used and their brains were kept frozen until biochemical determinations.

2.6. Semi-quantification by RT-PCR

Frozen brain regions were homogenized in 4M guanidine thiocyanate (ICN, Aurora, OH, USA) and total RNA was extracted as described elsewhere [37]. MOR and AC5 mRNAs were quantified by reverse transcriptase-polymerase chain reaction (RT-PCR) using

two different control transcripts: glyceraldehyde 3-phosphate dehydrogenase (G₃PDH) and cyclophilin. Analysis of gene expression was performed using 4 µl of cDNA and G₃PDH mRNA as a control, as previously reported [38,39], except that 50 pmol per tube of G₃PDH primers (sense: 5' TGAAGGTCGGTGTCAACGGATTTGGC and antisense: CATGTAGGCCATGAGGTCCACCAC) and 21 cycles were used. A 257 bp fragment corresponding to nucleotides 165-422 of cyclophilin cDNA was also amplified for 21 cycles using 50 pmol per tube of primers, whose sequences were: 5' GGGGAGAAAGGATTTGGCTA (sense) and 5'ACATGCTTGCCATCCAGCC (antisense). The sequences of MOR primers were: 5'TAATGGCTGTGACCATGGAA (sense) and 5'CGACTGCTCAGACCCCTTAG (antisense). The sequences of AC5 primers were: 5'CGAGGTCTCCCAGAGACAAG (sense) and 5'TCAGCAAACAGGATGCTCAC (antisense). Final PCR conditions were 31 cycles for MOR and 32 cycles for AC5. Each cycle consisted of 1 min at 95°C followed by 1 min at 64°C, and 1 min at 72°C. All cDNAs had a final extension step of 10 min at 72°C. The resulting cDNAs were quantified from the same RT reaction.

PCR products (10 µl of MOR and AC5) and 4 µl G₃PDH or 4 µl cyclophilin were separated by electrophoresis in a 2% agarose (Ultra-pure Bio-Rad, Hercules, CA, USA) gel stained with ethidium bromide (1mg/L). Densities were measured with the Advanced American Biotech Imaging software (American-Applied Biotechnology, Fullerton, CA, USA). The relative amounts of MOR and AC5 mRNAs were calculated as the ratio between MOR or AC5 and that of its control amplicon.

2.7. Statistical analysis

A two-way ANOVA was performed to evaluate behavioral and gene expression data at different times of ethanol or water administration; this test was also used to analyze the

1 results of anxiety-like behavior and AC5 mRNA levels in animals injected with MOR
2 antagonist (FNA) or vehicle in the NAcc. Significant differences between groups were
3
4 analyzed by the post-hoc Student Newman Keul's test in the case of the behavioral data.
5
6 Specific paired comparison was performed with a Student's *t*-test when necessary.
7
8 Significance was considered at $p < 0.05$.
9
10

11 **3. Results**

12 *3.1 Behavioral tests.*

13
14 We analyzed the anxiolytic effect after acute ethanol (2.5 g/kg i.g.) administration in
15
16 animals performing the EPM test. A *t*-test indicated a significant increase in the time spent
17
18 by animals in the open arms after 1h ($t=3.18$, $df=15$, $p < 0.006$) of ethanol administration
19
20 when compared to controls (Fig. 1A). A decrease in the time spent in the closed arms
21
22 ($t=3.36$, $df=15$, $p=0.01$) (Fig. 1B) was observed, as well as an increase in the total number of
23
24 entries ($t=-2.28$, $df=15$, $p < 0.05$) (Fig. 1C). No anxiolytic-like effects were observed at other
25
26 evaluation times after ethanol administration.
27
28
29
30
31
32
33
34
35

36
37 Acute ethanol administration did not alter motor activity of treated animals subjected to
38
39 the open field test at any time (Table 1). These results indicate that the increased time spent
40
41 in the open arms by ethanol-treated animals was not due to increased locomotor activity.
42
43
44

45 *3.2 MOR and AC5 mRNA levels in nucleus accumbens.*

46
47 Statistical analysis of MOR mRNA levels showed a significant main effect of time
48
49 [$F_{(2,46)}=39.425$, $p < 0.0001$], treatment [$F_{(1,46)}=16.668$, $p < 0.0001$], and interaction between
50
51 these factors [$F_{(2,46)}=42.612$, $p < 0.0001$]. Ethanol increased MOR mRNA levels by $33 \pm 5\%$,
52
53 1h after administration, followed by decreases of $28 \pm 6\%$ (4h) and $53 \pm 8\%$ (8h) (Fig. 2A).
54
55 A two-way ANOVA of AC5 mRNA expression showed a significant main effect of time
56
57
58
59
60
61
62
63
64
65

[$F_{(2,48)}=92.825$, $p<0.0001$], treatment [$F_{(1,48)}=25.029$, $p<0.0001$] and time x treatment interaction [$F_{(2,48)}=92.825$, $p<0.0001$]. AC5 mRNA levels decreased by $54 \pm 2\%$ at 1h, followed by an increase of $87 \pm 11\%$ at 4h and an increase of $30 \pm 2\%$ at 8h after ethanol exposure (Fig. 2B).

3.3. Anxiety-like behavior of ethanol-administered animals injected with MOR antagonist in the NAcc.

Vehicle-injected animals in the NAcc and administered with ethanol (V+Et) showed longer time spent in the open arms in the EPM test ($52 \pm 10\%$) when compared to water-administered rats, V+W group ($20 \pm 7\%$, $p<0.001$). These results confirmed the anxiolytic effect of ethanol treatment after 1h. Animals injected with FNA in the NAcc and administered with ethanol (FNA+Et) also showed an increase in the time spent in the open arms when compared to their controls (FNA+W). These results showed that MOR antagonist was not able to reverse the anxiolytic effect induced by ethanol (Fig 3A). Although FNA injection seemed to reduce the time spent in the open arms of the FNA+W group in comparison to V+W animals, the decrease was not significant. Thus, MOR blockade showed a tendency to induce anxiety at basal level. A two-way ANOVA showed main effect of ethanol administration [$F_{(1,34)}=18.79$, $p<0.001$], but not of FNA injection [$F_{(1,34)}=1.07$, ns] or of the interaction between ethanol and FNA treatment [$F_{(1,34)}=0.49$, ns].

The time spent in the closed arms in the EPM test was reduced in ethanol-administered groups in comparison with their controls: V+Et = $45 \pm 11\%$ vs. V+W = $77 \pm 7\%$, $p<0.05$, and FNA+Et = $45 \pm 9\%$ vs. FNA+W = 90 ± 5 , $p<0.05$. A two-way ANOVA showed a significant effect of ethanol treatment [$F_{(1,34)}=18.93$, $p<0.001$], but not of FNA [$F_{(1,34)}=0.6$, ns], or of the interaction between variables [$F_{(1,34)}=0.47$, ns] (Fig 3B).

The total number of entries to both compartments of the maze for ethanol-administered animals was not affected by FNA injection (Fig. 3C): (V+Et) = $7 \pm 2\%$ vs. (V+W) = $3.2 \pm 0.7\%$, $p < 0.05$, and (FNA+Et) = $6.4 \pm 1.6\%$ vs. (FNA+W) = $1.7 \pm 0.7\%$, $p < 0.01$. A two-way ANOVA showed an effect of ethanol treatment [$F_{(1,34)}=7.82$, $p < 0.01$], but no effect of FNA treatment [$F_{(1,34)}=0.50$, ns], or the interaction between ethanol and FNA treatment [$F_{(1,34)}=0.06$, ns].

3.4 AC5 mRNA levels in response to the injection of MOR antagonist in nucleus accumbens.

A two-way ANOVA of AC5 mRNA levels in the NAcc of V+W or V+Et animals and of groups injected with vehicle or FNA (FNA+W, FNA+Et) indicated a significant effect of the antagonist injection [$F_{(1,34)}=15.500$, $p < 0.001$] and of ethanol administration [$F_{(1,34)}=29.191$, $p < 0.001$], but not of the interaction between ethanol and FNA treatment [$F_{(1,34)}=1.191$, ns]. Ethanol decreased AC5 mRNA levels to $33 \pm 3\%$, 1h after administration, in V+Et vs. V+W group (100%)(Fig. 4), which confirmed the observed decrease in enzyme expression in the first trial (kinetics). AC5 mRNA levels in FNA+Et animals decreased 19% vs. FNA+W group. FNA increased AC5 mRNA expression (28%) in FNA+Et animals when compared to the V+Et group. Thus, MOR blockage was not enough to reverse the decrease induced by ethanol on enzyme synthesis.

4. Discussion

Ethanol administration reduced anxiety levels only in animals tested 1h after drug exposure. This might correspond to the increased ethanol content in blood (518 mg/l) at 30 min that is maintained elevated (370 mg/l) up to 2h, and undetectable after 8h, as has been described [30]. Thus, it was not surprising that groups of ethanol-administered animals after 4 and 8h had similar anxiety levels than those administered with water. Also, the total number of arm entries was increased in ethanol-treated animals; these data could be

1 interpreted as an increase in their overall activity instead of an anxiolytic-like effect [40].
2
3 However, when we evaluated the locomotion of animals in the open field test, we found no
4
5 differences between groups at any time. Moreover, the increase in the time spent in the open
6
7 arms of the EPM, 1h after drug administration, corresponded to the short time spent by
8
9 animals in the closed arms at the same moment.
10

11 **Expression of MOR and AC5 in NAcc of ethanol-administered animals.**

12
13
14 We observed that MOR mRNA levels increased, whereas those of AC5 decreased in the
15
16 NAcc of the group of ethanol-administered animals that presented anxiolytic behavior (1h
17
18 post-treatment). We interpret MOR up-regulation as the activation of the receptors, given
19
20 that an opioid agonist administration increases MOR mRNA levels [41]. An active release
21
22 of endogenous opioid peptides (β -endorphin and methionin-enkephalin) in the NAcc
23
24 induced by ethanol, which has been well documented [42-44], might be involved in the
25
26 increase in MOR mRNA levels. Our results suggest that the anxiolytic-like behavior of
27
28 ethanol-exposed animals might be related to MOR stimulation in the NAcc. This would be
29
30 important since the participation of accumbal opioidergic system in anxiolysis has been only
31
32 proposed by the injection of non-specific agonists of opioids receptors [13], but the role of
33
34 MOR in the NAcc has not been evaluated. Thus, in a second experiment we tested the role
35
36 of accumbal MOR in emotional actions of alcohol by injecting FNA, a specific MOR
37
38 antagonist, into the NAcc of animals.
39
40
41
42
43
44
45
46
47
48
49

50
51 AC5 mRNA levels were decreased in ethanol-treated animals which presented
52
53 anxiolytic behavior (1h). Our data are in agreement with studies showing that the blockage
54
55 of AC5 expression in NAcc by injecting siRNA-AC5 induces lower anxiety scores than
56
57 controls, when animals perform the EPM [25]. The decreased expression in MOR along
58
59 with high AC5 mRNA levels found after 4-8h post-ethanol (when animals showed normal
60
61
62
63
64
65

1 anxiety levels) suggest that the drug might have affected AC5 expression through the
2 stimulation of the opiodergic system in NAcc and that those changes may be involved in
3 ethanol-induced anxiolysis. Further experiments invalidated this hypothesis.
4
5
6
7
8

9 **MOR antagonist injection into the NAcc.**

10
11 To analyze whether ethanol-induced anxiolytic-like effect in animals after 1h post-
12 treatment was indeed related to the reduced AC5 expression through MOR activation, we
13 injected a MOR specific antagonist (FNA) into the NAcc of water- and ethanol-administered
14 animals. We found that blocking MOR in NAcc was not able to reverse the anxiolytic effect
15 of ethanol. However, control animals injected with FNA (FNA+W) had a tendency to reduce
16 their entrance to the open arms of the maze, which suggested that MOR of NAcc was
17 required to regulate basal anxiety levels as has been proposed [17], but were not related to
18 the anxiolytic effects of ethanol.
19
20
21
22
23
24
25
26
27
28
29
30

31 It is important to consider that our results are not comparable to those performed in
32 MOR KO animals that show insensitivity to the anxiolytic-like effects of ethanol [19], since
33 we are blocking MOR specifically in the NAcc. Other brain regions such as the extended
34 amygdala are important for anxiety-like behavior [45,46]. This superstructure extends from
35 the central nucleus of the amygdala (CeA) to the bed nucleus of the stria terminalis (BNST).
36 Thus, our results suggest that MOR may play a relevant role in the anxiolytic actions of
37 ethanol in other regions of the extended amygdala, as has been shown [36]. MOR in the
38 NAcc, in contrast, may be participating in behaviors other than anxiety that are altered by
39 ethanol, such as locomotion, reward, or analgesia [23].
40
41
42
43
44
45
46
47
48
49
50
51
52

53 Injection of the MOR antagonist partially blocked the reduction of AC5 induced by
54 ethanol exposure, given that enzyme mRNA levels were still decreased in the FNA+Et
55 group in comparison to that of its own control (FNA+W). These results suggest that ethanol
56
57
58
59
60
61
62
63
64
65

1 was able to reduce AC5 expression in NAcc not only through MOR, but also through the
2 activation of other receptors of neurotransmitters or peptides that are negatively coupled to
3 AC5, such as dopamine D2. Animals exposed to ethanol either injected or not with FNA in
4 the NAcc, presented lower anxiety scores and reduced AC5 expression vs. their respective
5 controls. Thus, we cannot discard the participation of AC5 in the anxiolytic actions of the
6 drug, although it would not be related to MOR activation.
7
8
9
10
11
12
13
14

15 We conclude that AC5 expression in the NAcc is a target of acute ethanol effects. Our
16 results suggest that MOR in NAcc is participating in basal levels of anxiety of animals. In
17 contrast, we observed that the anxiolytic effect of ethanol was not due to MOR activation,
18 nor to MOR-induced AC5 reduction in NAcc. However, AC5 in NAcc is participating in the
19 emotional effects of the drug. It is also possible that the observed changes in AC5 and MOR
20 expression by ethanol administration in the NAcc may be participating in different behaviors
21 other than anxiety, such as reward or analgesia, that are triggered by the drug in this brain
22 region [23].
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38

39 **Acknowledgements**

40
41
42 This work was made possible by the support of CONACyT to P.de Gortari (project 128316),
43 M. Morales (project 42095-M), E. Estrada (project 104659) and FIS PI10-01874 grant to G.
44 Mengod.
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

References

- [1] Eckardt MJ, File SE, Gessa GL, Grant KA, Guerri C, Hoffman PL, Kalant H, Koob GF, Li T-K, Tabakoff B. Effects of moderate alcohol consumption on the central nervous system. *Alcohol Clin Exp Res* 1998;22:998-1040.
- [2] Kushner MG, Abrams K, Borchardt C. The relationship between anxiety disorders and alcohol use disorders: a review of major perspectives and findings. *Clin Psychol Rev* 2000;20:149-71.
- [3] Spanagel R, Montkowski A, Allingham K, Stöhr T, Shoaib M, Holsboer F, Landgraf R. Anxiety: a potential predictor of vulnerability to the initiation of ethanol self-administration in rats. *Psychopharmacology* 1995;122:369-373.
- [4] Morgane PJ, Galler JR, Mokler DJ. A review of systems and networks of the limbic forebrain/limbic midbrain. *Prog Neurobiol* 2005;75:143-60.
- [5] Herz A. Endogenous opioid systems and alcohol addiction. *Psychopharmacology* 1997;12:99-111.
- [6] Koob GF. Neuronal mechanisms of drug reinforcement. *Ann NY Acad Sci USA* 1992;654:171-91.
- [7] Wise RA, Bozarth MA. Action of drug of abuse on brain reward systems: an update with specific attention to opiates. *Pharmacol Biochem Behav* 1982;17:239-243.
- [8] Imperato A, Angelucci L, Casoloni P, Zocchi A, Puglisi-Allegra S. Repeated stressful experiences differently affect limbic dopamine release during and following stress. *Brain Res* 1992;577:194-9.
- [9] Finlay JM, Zigmond MJ. The effects of stress on central dopamine neurons: possible clinical implications. *Neurochem Res* 1997;22:1387-94.
- [10] Saal D, Dong Y, Bonci A, Malenka RC. Drugs of abuse and stress trigger a common synaptic adaptation in dopamine neurons. *Neuron* 2003;37:577-82.

- [11] Duncan GE, Knapp DJ, Breese GR. Neuroanatomical characterization of Fos induction in rat behavioral models of anxiety. *Brain Res* 1996;713:79-91.
- [12] Lopes AP, da Cunha IC, Steffens SM, Ferraz A, Vargas JC, de Lima TC, Neto JM, Faria MS, Paschoalini MA. GABAA and GABAB agonist microinjections into medial accumbens shell increase feeding and induce anxiolysis in an animal model of anxiety. *Behav Brain Res* 2007;184:142–149.
- [13] Zarriandast MR, Babapoor-Farrokhran S, Babapoor-Farrokhran S, Rezayof A. Involvement of opioidergic system of the ventral hippocampus, the nucleus accumbens or the central amygdale in anxiety-related behavior. *Life Sci* 2008;82:1175-1181.
- [14] Koob GF. A role for GABA mechanism in the motivational effects of alcohol. *Biochem Pharmacol* 2004;68:1515-25.
- [15] Asakawa A, Inui A, Momose K, Ueno N, Fujino MA, Kasuga M. Endomorphins have orexigenic and anxiolytic activities in mice. *Neuroreport* 1998;9:2265-2267.
- [16] Privette TH, Terrian DM. Kappa opioid agonists produce anxiolytic-like behavior on the elevated plus-maze. *Psychopharmacology* 1995;118:444-450.
- [17] Filliol D, Ghozland S, Chluba J, Martin M, Matthes HW, Simonin F, et al. Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses. *Nat Genet* 2000;25:195-200.
- [18] LaBuda CJ, Fuchs PN. The anxiolytic effect of acute ethanol or diazepam exposure is unaltered in μ -opioid receptor knockout mice. *Brain Res Bull* 2001;55:755-760.
- [19] Ghozland S, Chu K, Kieffer BL, Roberts AJ. Lack of stimulant and anxiolytic-like effects of ethanol and accelerated development of ethanol dependence in mu-opioid receptor knockout mice. *Neuropharmacology* 2005;49:493-501.
- [20] Glatt CE, Snyder SH. Cloning and expression of an adenylyl cyclase localized to the corpus striatum. *Nature* 1993;361:536-8.
- [21] Sanabra C, Mengod G. Neuroanatomical distribution and neurochemical characterization of cells expressing adenylyl cyclase isoforms in mouse and rat brain. *J Chem Neuroanatomy* 2011;41:43-54.

- [22] Lee KW, Hong JH, Choi IY, Yongzhe C, Ja-Kyeong L, Sung-Don Y, et al. Impaired D2 dopamine receptor function in mice lacking type 5 adenylyl cyclase. *J Neurosci* 2002;22:7931-40.
- [23] Kim KS, Lee KW, Lee KW, Im JY, Yoo JY, Kim SW, et al. Adenylyl cyclase type 5 (AC5) is an essential mediator of morphine action. *Proc Natl Acad Sci USA* 2006;103:3908-13.
- [24] de Gortari P, Mengod G. Dopamine D1, D2 and mu-opioid receptors are co-expressed with adenylyl cyclase 5 and phosphodiesterase 7B mRNAs in striatal rat cells. *Brain Res* 2010;1310:37-45.
- [25] Kim KS, Lee KW, Baek IS, Lim CM, Krishnan V, Lee JK, et al. Adenylyl cyclase-5 activity in the nucleus accumbens regulates anxiety-related behavior. *J Neurochem* 2008;107:105-15.
- [26] Misra K, Pandey SC. The decreased cyclic-AMP dependence-protein kinase A function in the nucleus accumbens: a role in alcohol drinking but not in anxiety-like behavior in rats. *Neuropsychopharmacology* 2006;31:1406-1419.
- [27] Li J, Li YH, Yuan XR. Changes of phosphorylation of cAMP response element binding protein in rat nucleus accumbens after chronic ethanol intake: naloxone reversal. *Acta Pharmacol Sin* 2003;24:930-936.
- [28] Barrot M, Olivier JD, Perrotti LI, DiLeone RJ, Berton O, Eisch AJ, et al. CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. *Proc Natl Acad Sci USA* 2002;99:11435-40.
- [29] Barrot M, Wallace DL, Bolaños CA, Graham DL, Perrotti LI, Neve RL, et al. Regulation of anxiety and initiation of sexual behavior by CREB in the nucleus accumbens. *Proc Natl Acad Sci USA* 2005;102:8357-62.
- [30] Méndez M, Leriche M, Calva JC. Acute ethanol administration differentially modulates μ opioid receptors in the rat mesoaccumbens and mesocortical pathways. *Mol Brain Res* 2001 94:148-56.

- [31] File SE. Behavioral detection of anxiolytic action. In: Elliott JM, Heal DJ, Marsden CA, editors. Experimental approaches to anxiety and depression, Wiley and Sons Ltd, London. 1992. p. 25-44.
- [32] Estrada-Camarena E, Fernández-Guasti A, López-Rubalcava C. Antidepressant-like action of different estrogenic compounds in the forced swimming test. *Neuropsychopharmacology* 2003;28:830-8.
- [33] Paxinos G, Watson C. The rat brain in stereotaxic coordinates, 4th edition, Academic Press, San Diego, CA; 1998.
- [34] Meilandt WJ, Barea-Rodriguez E, Harvey SA, Martinez JL Jr. Role of hippocampal CA3 mu opioid receptors in spatial learning and memory. *J Neurosci* 2004;24:2953-2962.
- [35] Ragnauth A, Moroz M, Bodnar RJ. Multiple opioid receptors mediate feeding elicited by mu and delta opioid receptor subtype agonists in the nucleus accumbens shell in rats. *Brain Res* 2000;876:76-87.
- [36] Wilson M, Junor L. The role of amygdalar mu opioid receptors in anxiety-related responses in two rat models. *Neuropsychopharmacology* 2008;33:2957-2968.
- [37] Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-9.
- [38] de Gortari P, Romero F, Cisneros M, Joseph-Bravo P. Acute administration of alcohol modulates pyroglutamyl amino peptidase II activity and mRNA levels in rat limbic regions. *Neurochem Int* 2004;46:347-56.
- [39] Vargas MA, Bourdais J, Sánchez A, Urióstegui B, Moreno E, Joseph-Bravo P, Charli JL. Multiple hypothalamic factors regulate pyroglutamyl peptidase II in cultures of adenohypophyseal cells: role of the cAMP pathway. *J Neuroendocrinol* 1998;10:199-206.
- [40] Wilson MA, Burghardt PR, Ford KA, Wilkinson MB, Primeaux SD. Anxiolytic effects of diazepam and ethanol in two behavioral models: comparison of males and females. *Pharmacol Biochem Behav* 2004;78:445-58.

- 1 [41] Yu X, Mao X, Blake AD, Li WX, Chang SL. Morphine and endomorphins
2 differentially regulate micro-opioid receptor mRNA in SHSY-5Y human neuroblastoma
3 cells. J Pharmacol Exp Ther 2003;306:447-454.
4
5
6 [42] Marinelli PW, Quirion R, Gianoulakis C. A microdialysis profile of β -endorphin and
7 catecholamines in the rat nucleus accumbens following alcohol administration.
8 Psychopharmacology 2003;169:60-7.
9
10
11 [43] Marinelli PW, Bai L, Quirion R, Gianoulakis C. A microdialysis profile of Met-
12 enkephalin release in the rat nucleus accumbens following alcohol administration.
13 Alcohol Clin Exp Res 2005;29:1821-28.
14
15
16 [44] Méndez M, Barbosa-Luna IG, Pérez-Luna JM, Cupo A, Oikawa J. Effects of acute
17 ethanol administration on methionine-enkephalin expression and release in regions of the
18 rat brain. Neuropeptides 2010;44:413-20.
19
20
21 [45] LeDoux JE, Iwata J, Cicchetti P, Reis DJ. Different projections of the central
22 amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear J
23 Neurosci 1988;8:2517-2529.
24
25
26 [46] Walker DL, Davis M. Double dissociation between the involvement of the bed nucleus
27 of the stria terminalis and the central nucleus of the amygdale in startle increases
28 produced by conditioned versus unconditioned fear. J Neurosci 1997;17:9375-9383.
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Figure legends

Figure 1. Effect of acute ethanol administration on anxiety-related behavior of animals subjected to the elevated plus maze test. Male Wistar rats received a single acute dose of ethanol (2.5 g/kg body weight i.g.) or distilled water and were subjected to the behavioral test 1, 4, or, 8 h after treatment. White bars represent control animal parameters and black bars represent ethanol-treated animal parameters. A) Percentage of time spent in the open arms (controls=100%). B) Percentage of time spent in the closed arms (controls=100%). C) Number of total entries/10 min. Data are expressed as the percentage of time spent in the open arms or in closed arms vs. control (total time spent in both compartments=100%); data are the mean $\pm$ SEM of 8 animals. * p <0.050, ** p <0.010.

Table 1. Effect of ethanol administration on the locomotor activity. Locomotor activity was counted after a single acute dose of ethanol (2.5 g/kg i.g.) or distilled water in Wistar rats. Locomotor activity was evaluated with the open-field test. Data represent the mean $\pm$ S.E.M. of 8 animals. The apparatus registers the number of times the animal crosses squares during a 5-min session.

Figure 2. Effect of acute ethanol administration on gene expression in the nucleus accumbens. Animal treatment was performed as described in Fig.1. Rats were sacrificed 1:15, 4:15 and 8:15 h after ethanol or water administration and their brains removed. Gene expression was performed by RT-PCR. White and black bars represent control and ethanol-treated animal values, respectively. A) MOR mRNA levels and B) AC5 mRNA levels. Data are the ratio between each cDNA (MOR or AC5) and cyclophilin cDNA and expressed as the mean $\pm$ SEM calculated as the percentage of control values (C=100%) of 9 animals. * p <0.01, ** p <0.0001 vs. values of water-administered animals. Significant differences were also observed between ethanol-treated animals in the nucleus accumbens: # significantly

different vs. ethanol-treated animals after 1h; + significantly different vs. ethanol-treated animals after 4h.

Figure 3. Effect of funaltrexamine injected bilaterally into the nucleus accumbens of rats that showed an anxiolytic-like effect 1h after acute ethanol treatment. Animals were subjected to the elevated plus maze test after receiving a single acute dose of ethanol (2.5 g/kg body weight i.g.) or distilled water (1h post-treatment). White bars represent control animal parameters and black bars represent ethanol-treated animal parameters. A) Percentage of time spent in the open arms (controls=100%). B) Percentage of time spent in the closed arms (controls=100%). C) Number of total entries/10 min. Data are expressed as the percentage of time spent in the open arms or in closed arms vs. control (total time spent in both compartments=100%); data are the mean $\pm$ SEM of 9-10 animals. * p <0.050, ** p <0.010, *** p <0.001.

Figure 4. Effect of funaltrexamine on AC5 mRNA levels in the nucleus accumbens in animals that showed anxiolytic-like effect 1h after acute ethanol treatment. Animal treatment was performed as described in materials and methods. Rats were sacrificed 1:15, after ethanol or water administration and their brains removed. Gene expression was performed by RT-PCR. White and black bars represent control and ethanol-treated animal values, respectively. Data are the ratio between AC5 cDNA and cyclophilin cDNA and expressed as the mean $\pm$ SEM calculated as the percentage of control values (C=100%) of 9-10 animals. * p <0.0001 vs. values of water-administered animals.

Figure(s)

Figure(s)

Figure(s)

Figure(s)

Table(s)

Treatments	Time	# Squares/5 min
Control	1 h	50.0 ± 11.3
Ethanol	1 h	50.8 ± 7.0
Control	4 h	32.2 ± 3.1
Ethanol	4 h	36.0 ± 3.1
Control	8 h	39.0 ± 4.1
Ethanol	8 h	42.6 ± 4.4