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Influences of B-Endorphins in Ethanol Consumption Patterns and Aquisition of a Conditioned Taste Aversion Mediated by the Drug.

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Articulo Original

Abstract:.Los efectos reforzantes del etanol están mediados, al menos parcialmente, por el sistema opiáceo. El etanol altera la síntesis y la liberación de β-endorfinas. Ratones genéticamente modificados que no sintetizan β-endorfinas (KO), o bien deficientes en la síntesis del neuropéptido (HT), consumen niveles más elevados de una solución etílica de baja concentración y exhiben una mayor predisposición para autoadministrarse la droga, en comparación con ratones que no presentan esta manipulación genética (WT). El presente trabajo fue realizado con el objeto de: i) extender resultados previos en lo que respecta al análisis de los perfiles de consumo de etanol en ratones deficientes para sintetizar β-endorfinas; y ii) evaluar la adquisición de un condicionamiento aversivo mediado por las propiedades postabsortivas etílicas, en función de las capacidades genéticas de sintetizar el neuropéptido. En el Experimento 1, los ratones fueron evaluados en términos de consumo de una solución de etanol al 7 % en un paradigma de libre elección entre agua y esta solución, durante 8 días. Posteriormente, la concentración de etanol aumentó al 10 % y el consumo voluntario se evaluó por 8 días más. Ratones WT consumieron mayores cantidades de etanol que animales KO. Ratones HT, a su vez, ingirieron mayores cantidades de la droga que ratones KO. En el Experimento 2, ratones (KO, HT y KO) fueron evaluados en un paradigma de aversión condicionada al sabor en el cual una solución de cloruro de sodio (NaCl) fue asociada con una dosis etílica de 2-g/kg. Ratones con genotipo HT o KO expresaron una aversión condicionada hacia el NaCl. Este estudio indica que la deficiencia, o bien, la incapacidad de sintetizar β-endorfinas predispone a los animales a expresar una aversión al etanol lo cual puede modular patrones de autoadministración de la droga.

Palabras claves:
β-endorfinas, aversión condicionada al sabor, consumo de etanol, ratones knockout, sistema opiáceo.

Abstract: Rewarding effects of ethanol may be mediated in part by endogenous opioids. Ethanol alters β-endorphin synthesis and release. β-endorphin heterozygous (HT) and knockout (KO) mice consume higher levels of a low-concentrated alcohol solution and show heightened predisposition to self-administer ethanol in comparison with wild-type (WT) mice (Grisel et al., 1999). This study was conducted in order to: i) re-analyze and extend previous results in terms of ethanol consumption profiles of β-endorphin deficient mice; and ii) analyze conditioned aversive learning mediated by ethanol postabsorptive effects as a function of genetic capabilities to synthesize β-endorphin. In Experiment 1, mice were evaluated in terms of consumption of a low (7%) ethanol solution in a two-bottle free choice paradigm. Ethanol concentration was then increased to 10 % and voluntary intake consumption was tested. WT mice displayed significantly higher consumption levels and ethanol-preference scores than did KO mice, independently from ethanol concentration. HT mice drank more ethanol than did KO mice. In Experiment 2, mice (KO, HT and WT) were tested in a conditioned taste aversion paradigm in which a sodium chloride (NaCl) solution was paired with a 2-g/kg ethanol dose. Only HT and KO displayed a conditioned aversion when using 2-g/kg ethanol as unconditioned stimulus. The present results indicate that total or partial deficiency of β-endorphin synthesis reduces ethanol preference and consumption. Furthermore, this study indicates that the lack of β-endorphin synthesis exacerbates ethanol's aversive postabsorptive effects which can in turn modulate self-administration patterns of the drug.

Key Words:
β-endorphin, conditioned taste aversion, ethanol consumption, knockout mice, opioid system.

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1. Introduction

The endogenous opioid system has been implicated in brain rewarding processes. Indeed, many behaviors

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associated with the processes of positive reinforcement may be controlled by different components of the opioid system. There are three different families of endogenous opioid peptides defined by their precursor molecules: proopiomelanocortin, prenkephalin and prodynorphin. In particular, proopiolmelanocortin (POMC) gives rise to β-endorphins and its biosynthesis mainly occurs in neurons of the arcuate nucleus and a small group of neurons in the nucleus tractus solitari. β-endorphin neurons of the arcuate nucleus project to several brain regions associated with reward, including the ventral tegmental area (VTA), nucleus acumbens (NA), septum, amygdala, hippocampus, frontal cortex and periacueductal gray matter (Khachaturian, Lewis, Schafer, & Watson, 1985).

Ethanol administration has been shown to increase the release of β-endorphin by the pituitary gland, hypothalamus and other brain regions (Rasmusen et al., 1998). Ethanol-mediated increases in β-endorphin release have also been observed in VTA and NA. The activation of μ and δ opioid receptors as a consequence of enhanced β-endorphin release in response to ethanol appears to play an important role in the reinforcing properties of the drug (Gianoulakis, 2004).

It has also been suggested that ethanol activation of the mesolimbic system is mediated by ethanol-dependent activation of the endogenous opioid system at the level of VTA and NA (Spanagel & Weiss, 1999; Kohl, Katner, Chernet, & McBride, 1998; Gonzales & Weiss, 1998).

Taking into account that the β-endorphin system appears to play a key role in mediating ethanol's rewarding effects, the development of a β-endorphin deficient mouse line (Rubinstein et al., 1996) provides a useful tool for analyzing the participation of this neuropeptide in ethanol reward processes (Gianoulakis, 1993; 2004).

A previous study evaluated ethanol preference and consumption patterns as a function of the capacity to synthesize β-endorphins (Grisel et al., 1999). The results of this study indicated that β-endorphin lacking (KO), or deficient (HT) mice prefer and consume significantly higher levels of a low-concentrated ethanol solution (7% v/v) when compared with WT mice. Additionally, HT subjects also expressed preferences for a higher ethanol concentration (10% v/v) in relation with intake levels and preference scores showed by sibling WT mice (Grisel et al. 1999). A previous study conducted by Grahame and Cunningham (1998), showed that this β-endorphin knockout mice also exhibited heightened predisposition to self-administer ethanol in comparison with WT animals, in an intravenously self-administration paradigm.

Ethanol postabsorptive effects have been observed to act as effective unconditioned stimuli (US) capable of supporting associative learning that shapes seeking and intake behaviors of this drug (Cordoba, Molina, Basso, & Orsingher, 1990; Cunningham, Fidler, & Hill, 2000; US Department of Health and Human Services, 2000; Chester, Lumeng, Li, & Grahame, 2003; Risinger, Malott, Prather, Niehus, & Cunningham, 1994; Froehlich, Harts, Lumeng, & Li, 1988).

Rodents that consume lower levels of ethanol may do so because they are more sensitive to aversive consequences of the drug. For this reason, they are prone to express higher levels of conditioned taste aversion responses.

In the present study we assessed ethanol consumption patterns of mice genetically deficient in terms of β-endorphin synthesis in order to extend previous results that analyzed the implication of this neuropeptide in ethanol consumption profiles and preference indexes for the drug (Grisel et al., 1999). In a second experiment we analyzed the sensitivity of KO, HT and WT mice to ethanol's postabsorptive effects utilizing an experimental approach analogous to a taste aversion conditioning procedure. Postabsortive drug consequences of ethanol are often evaluated through conditioning procedures to assess rewarding or aversive effects of the drug.

2. *Materials and Methods*

2.1. Subjects

Wild-type, knockout and heterozygous mice for β-endorphin synthesis were employed in the present study. The generation of β-endorphin mutant mice has been previously described by Rubinstein et al. (1993, 1996). In brief, a point mutation was introduced by site-directed mutagenesis into exon 3 of the POMC gene to generate a premature translational stop codon. The resultant truncated prohormone lacks the carboxyl-terminal 31 amino-acids composing β-endorphin, but is expressed at normal levels and correctly processed to adrenocorticotropic hormone, melanocyte stimulating hormones and γ-lipotropic hormone. The mutant allele was originally introduced into 129S2/SvPas-derived D3 embryonic stem cells and subsequently backcrossed for five successive generations to C57BL/6N mice (Simonsen, Gilroy, CA, USA) and then an additional two or four to C57BL/6J mice (The Jackson Laboratories, Bar Harbor, ME, USA). The β-endorphin wild-type, heterozygous and knockout mice used in the present experiments were derived at the N9 generation on a C57BL/6 background from heterozygote mating

pairs.

Mice were born and reared in the breeding colony at the Instituto Ferreyra (Cordoba, Argentina), in a temperature-controlled environment, maintained on a 12-h light/dark cycle, and fed and watered ad libitum. After weaning mice were group housed by sex with three to five animals per cage and were tested at 6-8 weeks of age. All experimental protocols were approved by the appropriate institutional animal care committee at our institute and followed the guidelines in the Public Health Service Guide for the Care and Use of Laboratory Animals. Mice had ad libitum access to food during the entire experimental period.

2.2. Genotyping

All animals employed in the present study were genotyped at approximately 30-days of age. Genotyping was performed on genomic DNA samples obtained from mouse tails by polymerase chain reaction (PCR). For DNA extraction, mice were anesthetized with 2,2,2-tri-bromoetanol (Aldrich, Milwaukee, WI, USA) 300 mg/kg i.p. Tail samples were obtained and incubated with 0.5 ml digestion buffer (50 mM Tris–HCL, pH 8.0; 100 mM EDTA; 0.5% sodium dodecyl sulfate; 0.5 mg/ml proteinase K, at 55 °C overnight (12–14 h). DNA was purified using standard procedures. In the PCR protocol we used a combination of three oligonucleotide primers, 889: 5'-ACCTCCGAGAAGAGCCAG-3' (POMC exon 3), 187: 5'-ACATGTTCATCTCTATACATAC-3' (3' flanking sequence in POMC gene), 186: 5'-GAGGATTGGGAAGACAATAGCA-3' (PGK-neo cassette, specific for the targeted allele; Cibersyn, USA). The set of oligonucleotide 889–187 amplified a 1.4 kb product corresponding to the WT allele and the pair of oligonucleotide 186–187 amplified a 1.2-kb product corresponding to the mutated allele. PCR conditions were hot start 94 °C for 3 min followed by 35 cycles at 94 °C for 45 seconds, 60 °C for 1 min, 72 °C for 1.45 min and an additional elongation cycle at 72 °C for 20 min.

2.3. Evaluation of ethanol intake patterns

During the first day of the experiment mice were weighed and individually housed in pine-shaving bedding lined cages with wire lids. Two 25-ml graduated tubes (± 0.1 ml) containing tap water were placed on each cage in order to allow animals to adapt to these drinking tubes. During the following four consecutive days, the volume of both tubes was recorded and were refilled, as necessary. During the adaptation period, as well as, during the experimental phase, tube readings occurred daily between 11:00 and 13:00 h, and after each reading tube positions were switched to avoid the development of side preferences. Following these four water-drinking days, one of the tubes was filled with a 7% v/v ethanol solution while the second tube contained water. This experimental phase lasted eight days. Ethanol solution was changed daily and the position of tubes was counterbalanced for side across cages. At the end of this period, and during eight additional days, mice had continuous access to 10% v/v ethanol and water. Home-cage bedding was changed every eight days and mice were re-weighed. Consumption scores of ethanol and water were daily assessed. Daily ethanol intake scores were calculated in terms of absolute grams of ethanol per kilogram of body weight (g/kg) as well as in terms of percent ethanol preference [(mls drank from the ethanol tube/ (mls drank from the ethanol tube + mls drank from the water tube) x 100)].

A total of 23 mice, representative of the three genotypes were evaluated in the present experiment (WT-male: 5; WT-female: 2; HT-male: 6; HT-female: 2; KO-male: 5 and KO-female: 3). Water consumption scores during the adaptation session of the experiment were subjected to a two-way mixed ANOVA (genetic line x days). Ethanol intake scores (g/kg and percent preference values) were analyzed through a three-way mixed ANOVA defined by genotype (WT, HT or KO), as between factor and, ethanol solution (7% or 10% v/v) and day of evaluation (1 to 8) as repeated measures. All data were collapsed across gender factor because preliminar analysis indicated that this variable failed to interact with the remaining factors under consideration. The loci of significant differences were further analyzed via Least Standard Deviation (LSD) Fisher's post-hoc tests ($p < 0.05$).

2.4. Ethanol-mediated conditioned taste aversion

A total number of 67 mice (males and females), representative from the three different genetic lines, were employed in the present experiment. The number of animals representative of the three genotypes and both sex evaluated, has been summarized in Table 1. Before training began, mice were adapted to a 2-hr/day water restriction schedule. Food was always available. During the taste conditioning phase, a sodium chloride solution [0.20M (1.2 % w/v) of NaCl in distilled water] was presented during the 2 hr drinking period, as the unique liquid source. Exposure to NaCl solution was immediately followed by an intraperitoneal (i.p.) administration of a 2-g/kg of ethanol dose (0.015 ml of body weight of a 17.1% v/v ethanol solution; EtOH group) or a similar volume of water (Control group). Mice received two conditioning trials separated by a 48 hr interval. During the days between conditioning, mice

had access to tap water for two hours, to allow rehydration. Water and NaCl solution were presented at room temperature in 25-ml graduated glass-tubes (± 0.1 ml). Forty eight hours following the second conditioning trial mice were evaluated in terms of consumption of NaCl. The test lasted 2 hrs and NaCl intake scores (mls / 10 g of body weight) were recorded at 10, 30, 60 and 120 min; these recording bins ensured supervision of possible measurement error arising from liquid spillage. Mixed-ANOVAs served to analyze water consumption scores during the adaptation phase of the experiment; total NaCl intake levels during conditioning and training, and consumption scores displayed during the first 10 minutes to evaluate the initial responsiveness to this sapid cue. Genotype and pharmacological treatment were included as independent factors. Days of evaluation were included as repeated measures in the respective analysis. Because preliminary analysis failed to exhibit significant interactions between sex and the remaining factors, all data were collapsed across this variable. The loci of significant differences were further analyzed via Least Standard Deviation (LSD) Fisher's post-hoc tests ($p< 0.05$).

Table 1.
Number of mice representative of the three genotypes and sex evaluated at different pharmacological treatments.

	Pharmacological treatment: EtOH		Pharmacological treatment: Water	
	Males	Females	Males	Females
WT	6	6	6	6
HT	6	5	6	5
KO	6	5	4	6

3. *Results*

3.1. Ethanol intake patterns

As stated, mice representative of the three genetic lines were tested in terms of consumption of two different ethanol concentrations (7 and 10% v/v). A three-way mixed ANOVA was conducted to compare body weights of mice as a function of sex and genotype, as independent factors, and day as repeated measure. The ANOVA showed main effects of sex and day {$F (1, 17) = 42.74$ and $F (2, 34) = 10.77$, respectively; both ps'< 0.01}. As can be expected male's body weights were significantly higher than those corresponding to female mice. Additionally, body weights increased as a function of the passage of time. A significant interaction between genotype and day of evaluation also achieved significance {$F (4, 34) = 4.46$; $p< 0.01$}. Sex failed to interact with the remaining variables under consideration. Fisher post hoc tests indicated that KO mice were heavier than WT mice across days. They also exhibited higher body weights when compared with HT animals during the two last days in which this variable was measured. Furthermore, WT mice exhibited lower body weights relative to HT animals but only during the first day of test.

Water consumption scores during the adaptation period were analyzed using a two-way mixed ANOVA defined by genotype (WT, HT or KO) and day of water exposure. The analysis only showed a significant main effect of day {$F (3, 60) = 31.77$; $p< 0.01$}. As can be observed in Figure 1, animals rapidly adapted to consume water from these tubes. Consumption scores during the second adaptation session were significantly higher than those recorded during the first session. It is important to note that water consumption was not affected by the genotype factor. Additionally, mice failed to display position preferences for the tubes presented at the adaptation phase.

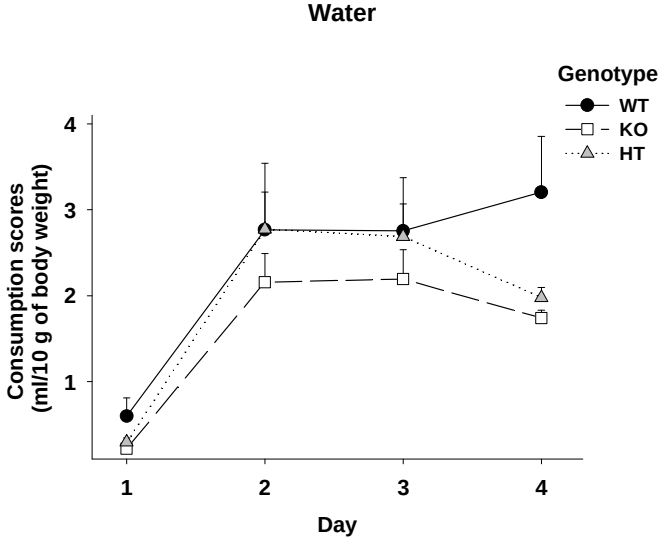

Figure 1: Consumption scores of water (ml/10g of animal body weight) during the adaptation period, as a function of genotype (WT, HT and KO) and day of evaluation (1-4). Values are represented as mean ± SEM.

When considering ethanol preference ratios, the three-way mixed ANOVA defined by genotype (WT, HT or KO), as between factor; ethanol solution (7% or 10% v/v) and day of evaluation (1 to 8), as within factors, showed a significant main effect of ethanol concentration {$F (1, 20) = 5.11$, $p< 0.05$}. A clear reduction of ethanol preference scores was evident when increasing ethanol concentration. Ethanol concentration significantly interacted with day of evaluation {$F (7, 140) = 5.07$, $p< 0.01$}. The triple interaction between genotype, concentration of ethanol and day, also achieved significance in the present

analysis {F (14, 140) = 4.61, p< 0.01}. Sequential two-way ANOVAs, separated by ethanol concentration presented, were conducted to better understand the loci of significant differences aroused from this triple interaction.

A two-way mixed ANOVA was conducted to analyze percent ethanol preference scores when utilizing a 7% v/v ethanol solution. This ANOVA was defined by genotype (WT, HT or KO), as a between factor while days of evaluation were considered as repeated measures (days 1 to 8). This analysis showed a significant main effect of day of evaluation {F (7, 140) = 3.58; p< 0.01}. The interaction between genotype and day of test also reached significance {F (14, 140) = 2.76; p< 0.01}. LSD Fisher's post hoc tests indicated that WT mice exhibited significantly higher ethanol preference scores when compared with KO subjects throughout most of the test phase (days 1-6 and day 8). Heterozygous mice also exhibited higher preference scores when compared with KO subjects. Significant differences between these genotypes were encountered during days 2, 4 and 6. In turn, ethanol preference scores of WT animals were significantly higher than those displayed by HT mice at days of evaluation 1, 7 and 8.

A similar profile of results was observed when considering percent preference scores when utilizing a 10% v/v ethanol solution. The corresponding ANOVA indicated a significant main effect of evaluation day and a significant interaction comprising genotype and day [F (7, 140) = 3.63 and F (14, 140) = 3.57, respectively; both p's< 0.01]. Post hoc tests indicated that KO mice showed significantly lower ethanol preference scores when compared with WT mice. This difference was observed across all days of testing. HT animals showed intermediate ethanol preference patterns when compared with WT and KO mice; during days 1, 2, 4 and 6, HT mice exhibited significantly lower preference scores when compared with WT subjects; while during days 3, 5 and 7, HT animals were found to exhibit higher preference scores when compared with KO mice. These results have been depicted in Figures 2a and 2b.

Absolute amount of ethanol consumption as expressed in terms of grams of ethanol per kg of body weight (g/kg) were considerably lower than those reported by Grisel et al. (1999) in the same strain of mouse. These authors reported that mice consumed between 8 to 14 g/kg of ethanol in a given day of test. In the present study absolute levels of ethanol consumption rarely exceeded 3 g/kg (see Figures 4 and 5).

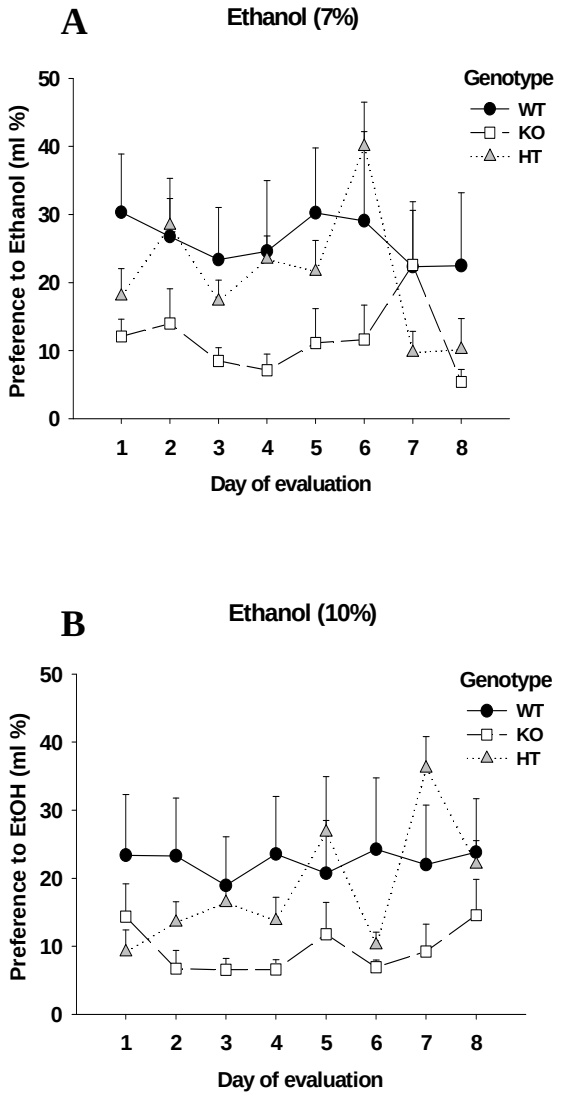

Figure 2: Percentage of preference scores (%) in a two-bottle choice test for a 7% v/v (A) and a 10 % v/v (B) ethanol solution, as a function of genotype (WT, HT and KO) and day of evaluation (1-8). Values are represented as mean ± SEM.

Despite this observation, the profile of ethanol ingestion was different across genotypes. A three-way mixed ANOVA defined by genotype (WT, HT or KO) as between factor; and ethanol concentration (7% or 10% v/v) and day of evaluation (1 to 8) as repeated measures, was conducted to compare absolute amounts of ethanol ingestion. This analysis showed a significant main effect of ethanol solution concentration {F (1, 20) = 6.27, p< 0.025}, ethanol ingestion significantly decreased when animals were evaluated to a higher ethanol concentration. Day of evaluation, also achieved

significance {F (7, 140) = 4.91, p< 0.01}. The interactions between ethanol concentration and day {F (7, 140) = 6.76, p< 0.01}, and between genotype, concentration of ethanol and day of evaluation {F (14, 140) = 3.46, p< 0.01}, were significant.

Sequential two-way mixed ANOVAs defined by genotype and day of evaluation were employed to compare amounts of ethanol (g/kg) consumed when a solution of 7% or 10% v/v ethanol was available for the animals. When animals were exposed to a 7% ethanol solution, the ANOVA showed a main effect of day of evaluation {F (7, 140) = 5.60; p< 0.01} and the interaction between genotype and day also achieved significance {F (14, 140) = 2.34; p< 0.01}. Post hoc tests indicated significant differences in ethanol consumption, particularly after five days of exposure to ethanol. At day of evaluation 6, HT animals consumed significantly higher levels of ethanol than did WT and KO subjects. WT mice also significantly increased ethanol consumption, in comparison with KO mice. A similar profile of ethanol consumption was observed when animals were exposed to a 10% ethanol solution. The ANOVA indicated a main effect of day of test {F (7, 140) = 6.84; p< 0.01}. The interaction between genotype and day also achieved significance {F (14, 140) = 2.81; p< 0.01}. Increases in ethanol intake were observed after six days of experience with a 10%-ethanol solution. HT mice consumed significantly higher ethanol levels than those recorded in WT and KO mice. Additionally, WT mice consumed more ethanol than KO subjects. The results obtained when exposing mice to a 7% or a 10% ethanol solution have been summarized in Figures 3a and 3b, respectively.

3.2. Ethanol-mediated conditioned taste aversion

As previously described mice of both sexes representative of the three different genetic strains were employed. Body weights were recorded at commencement of the study, during both conditioning trials and during test. A three-way mixed ANOVA defined by genotype and sex (independent factors) and days (repeated measures) showed a significant main effect of sex {F (1, 61) = 78.93; p< 0.01}. In addition, a significant interaction between sex and day was also evident {F (3, 183) = 4.91; p< 0.01}. Post hoc comparisons indicated that males exhibited always higher weights than females. Increases in body weights of males were more evident during the last two days of recording. In females, increases in body weights were noticeable during the second day of assessment.

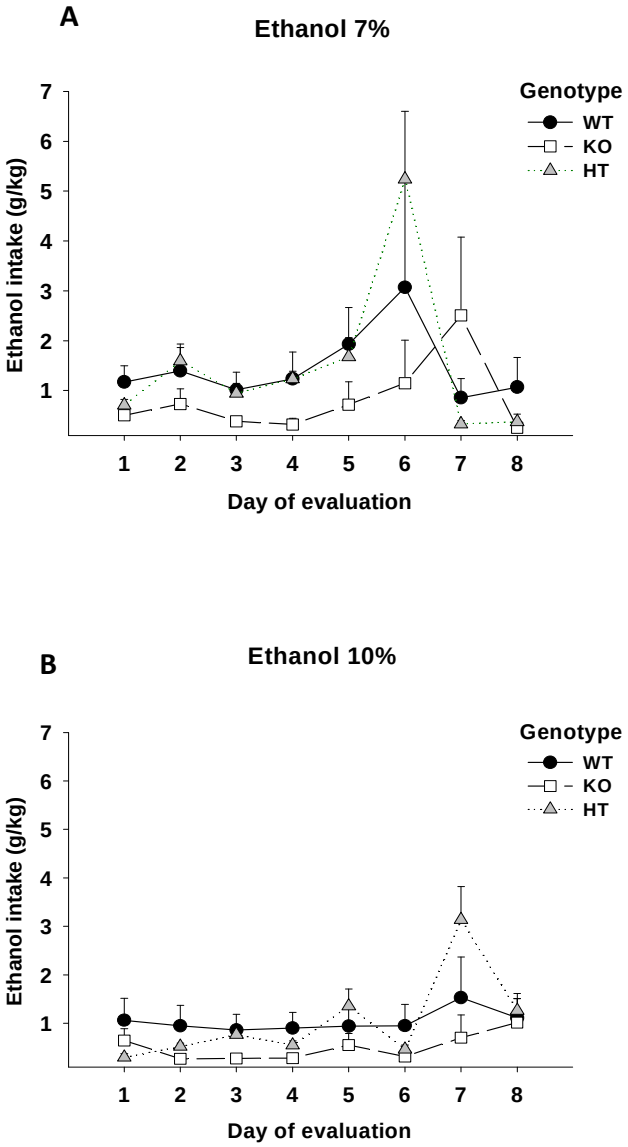

Figure 3: Ethanol consumption levels of a 7% v/v (A) and a 10 % v/v (B) ethanol solution (g/kg) in a two-bottle choice evaluation, as a function of genotype (WT, HT and KO) and day of evaluation (1-8). Values are represented as mean ± SEM.

A three-way mixed ANOVA (genotype x sex x days) was performed to analyze water consumption during the 4-days adaptation period. The ANOVA showed significant main effects of sex and days {F (1, 61) = 29.96 and F (3, 183) = 26.68, respectively; both ps'< 0.01}; post hoc tests indicated that water consumption levels increased between the first and the second day of water adaptation, indicating that animals adapt to drink from a tube, during the 2-hours daily-restricted period of liquid availability. In addition, females exhibited significantly higher levels of water consumption than those displayed by males, during these 4 days of adaptation.

A three-way mixed ANOVA was conducted to analyze the acquisition curve of a conditioned taste aversion to a NaCl solution mediated by interoceptive effects of ethanol. This analysis was defined by genotype (WT, HT and KO) and pharmacological treatment (water or EtOH), as between factors. Three days of NaCl consumption (conditioning days 1 and 2 and testing day) were included, as repeated measures. The ANOVA indicated that total amounts of NaCl consumed (120 min) during conditioning and testing, was significantly affected by the pharmacological treatment {F (1, 61) = 12.68, p< 0.01}, and day {F (2, 122) = 25.96, p <0.01}. The interaction between these factors, also achieved significance {F (2, 122) = 8.94, p< 0.01}. Post hoc Fisher's test indicated that NaCl consumption scores were similar during the first day of conditioning. When animals were injected with ethanol, immediately after consuming this sapid solution during conditioning days, significant reductions in intake scores were observed in comparison with levels of consumption exhibited by water-treated mice. Within-group comparisons indicated that ethanol-treated mice significantly reduced consumption of NaCl across the second day of conditioning and evaluation, when comparing with the first conditioning day. However, water-control mice failed to modify consumption levels of NaCl during conditioning and test. Genotype failed to exert significant effect, as well as, this factor did not interact with the remaining variables under consideration.

If considering the initial reactivity displayed towards NaCl (first 10 min. from a 2-hour period of exposure), the ANOVA showed significant main effects of genotype, pharmacological treatment and day of evaluation {F (2, 61) = 3.81; F (1, 61) = 33.41 and F (2, 122) = 70.61, respectively; all p's< 0.05}. Additionally, significant interactions between pharmacological treatment and day of evaluation; and between genotype and pharmacological treatment were observed {F (2, 122) = 6.99, F (2, 61) = 3.74, respectively; both ps' < 0.01}. Post hoc tests indicated that ethanol-treated mice showed significant decrements in NaCl consumption during the second conditioning trial and at test relative to the scores registered during the first conditioning trial. Moreover, mice that received ethanol as an unconditioned stimulus paired with NaCl were found to exhibit significantly lower intake scores of the flavored conditioned stimulus during the second conditioning trial and at test when compared with counterparts experiencing NaCl associated with water i.g. administration. The overall effects of conditioning have been illustrated in Figure 4. The significant interaction between genotype and pharmacological treatment during conditioning has been illustrated in Figure 5. As can be observed and supported by post-hoc comparisons, KO and HT mice conditioned with ethanol, exhibited significantly lower NaCl intake scores when compared with WT mice treated with this drug or when compared with any of the remaining groups exposed to NaCl-water pairings. No other significant differences between groups were encountered.

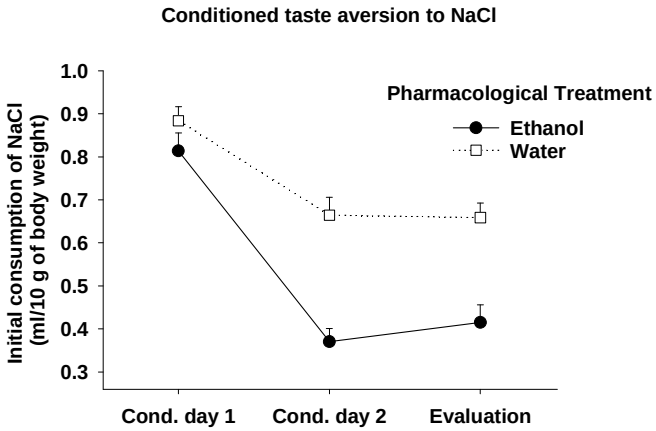

Figure 4: Initial consumption scores (ml/10g of animal body weight) of a 1.2 w/v NaCl solution as a function of pharmacological treatment -2.0 g/kg ethanol (i.p.) or water- and day of test (conditioning days 1 and 2 and evaluation day). Values are represented as mean ± SEM.

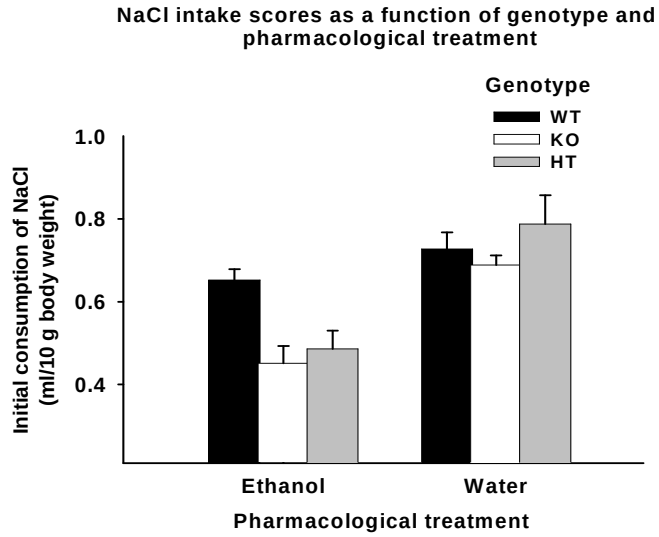

Figure 5: Consumption scores (ml/10g of animal body weight) of a 1.2 w/v NaCl solution as a function of pharmacological treatment -2.0 g/kg ethanol (i.p.) or water- and genotype (WT, HT or KO). Values are represented as mean ± SEM.

4. *Discussion*

The results derived from these experiments indicate that the endogenous opioid system plays an important role modulating voluntary ethanol consumption patterns. Similarly, the predisposition to acquire a conditioned taste aversion mediated by ethanol postabsorptive effects appears to be mediated by the capacity to synthesize β-endorphins. As previously reported by different authors, the endogenous opioid system, particularly β-endorphin-mediated processes, is intimately involved in modulating ethanol ingestive profiles as well as the sensitivity to ethanol's postabsorptive consequences (Kieffer, & Gavériaux-Ruff, 2002; Gianoulakis, 2001; 2004; Oswald, & Wand, 2004).

Numerous and diverse studies show that endogenous opioid system plays a role in ethanol reinforcement. Genetically approaches have demonstrated that μ-opioid receptor knockout mice failed to exhibit oral self-administration of ethanol in comparison with WT C57BL/J mice (Roberts et al., 2000). Similar results have been obtained when analyzing κ-opioid receptor KO mice (Kovacs et al., 2005). Pharmacological studies suggest that ethanol-induced activation of the endorphin and enkephalin systems may serve to reduce aversion to ethanol and hence increase the probability of subsequent ethanol drinking. Ethanol-induced endogenous opioid release may attenuate the aversive properties of the drug. When the action of endogenous opioid system is blocked, administering ethanol in conjunction with naltrexone, the postabsorptive effects of ethanol are more aversive and animals significantly reduced ethanol intake levels (Froehlich, Badia-Elder, Zink, McCullough, & Portoghese, 1998).

The results here described are in accordance with previous studies in which, pharmacological or genetic manipulation of the endogenous opioid system promotes decrements in ethanol preference patterns and/or reduces its reinforcing properties.

Grisel et al. (1999) showed that β-endorphin KO mice and heterozygous mice, exhibit considerably higher levels of preference for a 7 % v/v ethanol solution, in comparison with wild-type animals. Across varying EtOH concentration (10 % v/v) only the heterozygous mice were found to consistently drink more than wild-type mice. KO mice have also been observed to exhibit heightened predisposition to intravenously self-administer ethanol when compared with WT mice (Grahame, Low, & Cunningham, 1998). The results here described do not coincide with those previously reported when taking into account ethanol preference scores and absolute consumption of ethanol exhibited by the genotypes under consideration. In this study, WT and HT mice showed higher ethanol preference ratios when compared with KO mice. When analyzing g/kg of ethanol ingested, HT mice displayed considerably higher levels of ethanol consumption than WT and KO counterparts. Additionally, WT animals significantly expressed higher consumption levels of the drug than KO mice. This pattern of ethanol consumption was similar between both ethanol concentrations under consideration. These results are also in opposite direction those reported by Grisel et al. (1999).

A previous study conducted by these authors indicated that, as was the case of present results, β-endorphin knockout mice on the original F2 hybrid (129, C57) genetic background exhibited decreased consumption levels and preference scores for ethanol, in a similar two-bottle free choice paradigm (Grisel, Grahame, Mogil, Belknap, & Low, 1995). The authors proposed that differences in ethanol consumption profiles are due to the influence of strain 129-derived alleles at an unknown locus, and not the mutated Pomc gene (Grisel et al., 1999). It seems that, after nine backcrossing generations of C57 mouse strain the influence of 129-derived alleles are not present (Grisel et al., 1999). In addition to these differences in ethanol ingestive profiles, recent experiments indicate that β-endorphin KO mice failed to exhibit locomotor sensitization to chronic ethanol experiences, in comparison with WT counterparts (Allen & Grisel, 2005). If considering that increases in locomotive response has been suggested as one of the mechanisms by which ethanol exerts positive reinforcement (Cunningham, Niehus, & Noble, 1994; Risinger et al., 1994), it could be possible to speculate that KO mice are less sensitive to activating properties of the drug and, consequently, they would be less capable to perceive reinforcing properties of the drug. Grisel, Bartels, Allen, and Turgeon (2008) have recently conducted a work to determine the influence of β-endorphins on the stress response by evaluating basal measures of anxiety as well as on EtOH-induced anxiolytic behavior in β-endorphins transgenic mice. They found a direct relationship between β-endorphin levels and the percentage of entries into open arms of the Plus Maze as well as the time spent in the light compartment of the Light-Dark box during basal conditions, suggesting that this peptide normally inhibits anxious behavior. However, mice lacking β-endorphins demonstrated an exaggerated anxiolytic

response to EtOH in these assays.

A recent study has evaluated ethanol preference patterns in β-endorphin KO mice under baseline conditions and after stress exposure (Racz et al., 2008). They found that ethanol consumption was significantly reduced in the absence of β-endorphins. In addition, stress exposure resulted in increased ethanol consumption in wild-type mice but did not influence ethanol-drinking in β-endorphin knockouts.

The lack of concordance between results obtained by different, either experimental protocols or environmental animal facilities, determines the necessity of increasing the analysis of the mechanisms which involve β-endorphins in the regulation of ethanol intake and preference patterns. Experiments in which KO mice are supplemented with the administration of the neuropeptide, could be conducted to analyze if patterns of ethanol consumption and behavioral responsiveness are similar those exhibited by wild-type siblings.

In the present study, consumption scores of ethanol exhibited by animals derived from the C57BL/6J strain (WT) were considerably lower than those commonly reported for this genetic line of mouse (Bachmanov et al., 2002; Gabriel, & Cunnigham, 2005; Gianoulakis, Krishnan, & Thavundayil, 1996; Grahame, Mosemiller, Low, & Froehlich, 2000; Roberts et al., 2000). Once again, different intervening variables; environmental as well as genetic, could be responsible for the above mentioned changes concerning ethanol intake patterns in this mouse strain. Expression or silence of some genes can be modified when increasing the number of generation backcrosses (Opsahl et al., 2002); this could be a potential explanation for this opposite results if considering that the animals here employed were reared and maintained without outcrossing for a considerable number of generations.

According to the present results, the acquisition of an aversive conditioned response mediated by ethanol's unconditioned capabilities also seems to be mediated by the capacity to synthesize β-endorphin. Animals with decreased β-endorphin circulating levels (KO and HT) exhibited reliable conditioned taste aversions following NaCl-ethanol pairings; a phenomenon which was less evident in WT mice.

Different studies suggest that ethanol self-administration patterns are intrinsically related with the predisposition of animals to develop conditioned taste aversion mediated by the drug's postabsorptive effects (Chester et al., 2003; Grahame, Chester, Rodd-Henricks, Li, & Lumeng, 2001). It is interesting to note that in the present study, KO mice relative to WT animals, not only showed lower predisposition to self-administer different ethanol concentrations but were also more likely to express conditioned taste aversions mediated by ethanol's postadministration effects.

Genetic correlations between ethanol consumption levels and the propensity to develop ethanol-mediated CTA have been widely reported in previous literature. Rat and mice strains bred for ethanol avoidance, are more sensitive to express ethanol CTA than animals bred for ethanol preferences (Chester et al., 2003; Froehlich et al., 1998; Quintanilla, Callejas, & Tampier, 2001). Additionally, rats selectively bred for their capacity to develop CTA mediated by different unconditioned stimuli (ethanol being one of them) exhibit lower ethanol preferences than do counterparts selectively bred for their resistance to develop CTA (Elkins, Walters, & Orr, 1992). Several authors have also reported that exist genetic correlations between ethanol CTA acquisition and the sensitivity to withdrawal effects of the drug (Broadbent, Muccino, & Cunningham, 2002; Crabbe, Kosobud, & Young, 1983). As a function of the results here described, β-endorphins synthesis seems participate in attenuation of aversive postabsorptive ethanol effects. Consequently, animals unable to synthesize the endogenous neuropeptide do not express compensatory mechanisms that mitigate negative consequences of ethanol intoxication.

Finally, the present study suggests that β-endorphins mediate responsiveness to ethanol, not only in terms of consummatory patterns of the drug but also in terms of the sensitivity of the organism to ethanol's aversive unconditioned capabilities.

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