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Acquisition and expression of ethanol-induced conditioned place preference in mice is inhibited by naloxone

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Abstract:
The effects of opioid antagonists on conditioned reward produced by ethanol provide variable and sometimes conflicting results, especially in mice. In the present set of experiments, male C57BL/6 mice received 4 vehicle and 4 ethanol conditionings, and the rewarding effects of ethanol were assessed in an unbiased version of the conditioned place preference (CPP) apparatus and an unbiased stimulus assignment procedure. Intraperitoneal (*ip*) administration of ethanol (2 g/kg, but not 1 g/kg) resulted in the conditioned reward when conditionings lasted for 6 min but not when conditioning lasted for 20 min. Administration of the non-selective opioid receptor antagonist naloxone (1 and 5 mg/kg) before the conditionings attenuated the acquisition of ethanol-induced place preference. Naloxone (1 mg/kg) also inhibited expression of the CPP response, but it did not alter the preference of vehicle-conditioned mice, suggesting the lack of its own motivational effects in this experimental setting. Taken together, the present results suggest that an unbiased version of ethanol-induced CPP in C57BL/6 mice could be a valid model for the study of the motivational effects of ethanol, confirming and expanding previous findings that have demonstrated inhibitory effects of opioid receptor antagonist on alcohol conditioned reward.

Key words:
ethanol, conditioned reward, opioid receptor antagonist, naloxone

Introduction

Alcohol is one of the most commonly abused substances that leads to development of dependence, tolerance and addiction [20]. In the laboratory setting, almost all abused substances produce rewarding and reinforcing effects that can be measured using the conditioned place preference (CPP) paradigm and other tests [7]. However, ethanol-induced CPP is difficult to demonstrate in laboratory rodents. With some exceptions [4, 5, 16, 18], several studies have shown an inability of ethanol to produce CPP in rats (see [23] for an excellent review). In the mouse, the ability of ethanol to produce CPP is also variable and depends on a number of factors, including the time of administration that precedes the conditionings. For example, ethanol-induced CPP was shown when ethanol was given intraperitoneally (*ip*) immediately before the conditionings, but when ethanol was given *ip* after conditionings, a conditioned place aversion (CPA) was observed [10]. The CPP produced by ethanol also depends on the duration of the conditionings [12] and the dose of ethanol used for the conditionings, with ≥ 2 g/kg, *ip* typically producing statistically significant effects [2, 8–10]. However, other authors have found that ethanol produces CPP in mice at doses of 0.5–2 g/kg given immediately before conditionings [15], but others

have failed to demonstrate ethanol-induced CPP at a dose of 2 g/kg [6, 22]. Therefore, the first goal of present work was to establish conditions in which ethanol would produce a reliable CPP in our experimental settings.

Ethanol's addictive effects are at least partially mediated by the activation of the endogenous opioid system. Acute and chronic ethanol administration affects the binding properties of opioid receptors and modulates opioid peptide synthesis and secretion [14]. Similar to most other drugs of abuse, the rewarding properties of ethanol are associated with its ability to increase mesolimbic dopamine release [13]. The ethanol-induced increase in extracellular dopamine is dose-dependently reversed in rats by the administration of naltrexone, a non-selective opioid receptor antagonist [3]. However, the effects of opioid receptor antagonists on the acquisition and expression of ethanol-induced CPP are variable. An intra-ventral tegmental area (VTA) infusion of the opioid receptor antagonist methylnaloxonium [2] and the systemic administration of naltrexone [19] inhibit the expression of ethanol-induced CPP in mice. Similarly, naloxone inhibits the acquisition of ethanol-induced CPP in rats [5, 18]. However, Cunningham et al. [8, 10] indicate that the systemic administration of naloxone is without effects on both the acquisition and the expression of ethanol-induced conditioned reward in mice. However, Kuzmin et al. demonstrate that, although naloxone fails to affect the acquisition of ethanol-induced CPP, it blocks the expression of ethanol-induced conditioned reward in mice; this effect occurs at the dose displaying aversive effects on its own (1 mg/kg) [17]. Therefore, the second goal of the present study was to assess the effects of naloxone on the acquisition and expression of ethanol-induced CPP as well as its own motivational properties. Experiments were performed using an unbiased version of the CPP apparatus and an unbiased stimulus assignment procedure in mice because this appears to determine the intensity of ethanol conditioned reward [9].

Materials and Methods

Subjects

Male C57BL/6 mice (Institute of Pharmacology, PAS, Kraków, Poland) weighing 21–26 g were used in all of the experiments. The animals were housed in groups of 10 in standard plastic cages at a temperature of 21 ± 1°C and humidity of ~50% in a controlled animal colony with an automatic 12 h light/dark cycle (lights on at 07:00, off at 19:00). Food and water were available *ad libitum*. Each experimental group consisted of 6–10 mice per treatment. All mice were used only once. The experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee for Animal Experiments, Institute of Pharmacology.

Drugs

Ethanol 95% v/v was diluted to 20% v/v in sterile 0.9% physiological saline. The dose of ethanol was manipulated by varying the volume of injection of the 20% ethanol/saline mixture (range of 0.125 to 0.3 ml depending on the body weight of mice). The volume of vehicle injection was 0.2 ml saline. Naloxone hydrochloride (Sigma Aldrich, Poznań, Poland) was dissolved in sterile water and given in a volume of 10 ml/kg body weight. Both drugs were given *ip*.

Apparatus

The CPP apparatus consisted of 3 rectangular arms (30 × 15 × 20 cm) spaced at 120° from each other, which were all accessible from a central triangular platform [21]. The apparatus was made of an opaque plastic material (Metaplex), and the three arms differed in distinctive visual, tactile and olfactory cues. The white arm had a black floor with small holes and was marked with a drop of peppermint odor NDC-0395-1913-91 (Humco, TX, USA). The one black arm had a white rough floor and was marked with a drop of anise odor NDC-0395-2015-91 (Humco, TX, USA). Another black arm with a plain black floor had no odor. These distinct cues served as the conditioned stimuli. The use of tactile, texture floor cues allowed the mice to be in direct contact with the conditioned stimuli to experience their conditioned effect during preference testing [1]. The guillotine doors (with and without a passing gateway) were made of a material corresponding to the respective wall colors and were inserted during conditioning sessions and during the pre-tests and post-tests. The ceiling of the arms was made of transparent Plexiglass. During testing, the location of a mouse was monitored through

a closed circuit TV camera positioned directly above the apparatus. The testing room had dim indirect lighting comprised of two 15 W bulbs positioned about 1 m above the apparatus (illumination of the floor was about 14–16 lux). A radio speaker positioned about 1 m above the apparatus delivered white noise. The apparatus was kept free of urine and feces. The floors were washed and dried between testing sessions.

Procedure

The experiment was performed according to an unbiased procedure and consisted of 5 phases conducted on 11 consecutive days: *i)* adaptation (day 1), *ii)* pre-test (day 2), *iii)* conditionings with ethanol (days 3, 5, 7 and 9), *iv)* conditionings with vehicle (days 4, 6, 8 and 10), and *v)* the post-test (day 11).

During the adaptation, mice were carried into the testing room, weighed and handled by the experimenter. This adaptation phase was intended to reduce the novelty and stress associated with handling and injections. During the pre-test, mice were placed individually on the central triangular platform of the apparatus with free access to all 3 arms for 20 min. The time spent in each arm was recorded. For each mouse, the two arms registering the most similar preferences were identified and one such arm was randomly paired with ethanol, and the other arm was paired with vehicle. This was an important step in the experimental procedure that avoided any preference bias before conditioning. During conditionings, mice received ethanol or vehicle immediately before being placed into the respective arm of the apparatus for 6 or 20 min. The post-test was performed similarly to the pre-test with the mice being placed individually on the central triangular platform of the apparatus with free access to all arms for 20 min. The time spent in each arm was recorded. The third arm was visited only during the pre-test and post-test.

Experiment 1:
Relationship between the dose of ethanol and the duration of conditioning on the intensity of the conditioned reward

During conditionings, mice received ethanol (0, 1 or 2 g/kg, *ip*) or vehicle immediately before being placed into the respective arm of the apparatus for 6 or 20 min, to determine the relationship between the dose of ethanol and the duration of the conditioning sessions with the intensity of the conditioned reward.

Experiment 2:
Effects of naloxone on the acquisition of ethanol-induced CPP

a) Effects of naloxone on the acquisition of ethanol-induced CPP: The effects of naloxone, 1 or 5 mg/kg (*ip*), administration on the acquisition of ethanol-induced CPP were investigated. Naloxone was given 5 min before the injections of ethanol and vehicle, and the mice were conditioned for 6 min.
b) Motivational effects of naloxone: As a control, another group of mice received injections of 1 mg/kg naloxone and vehicle 5 min and immediately prior to naloxone conditionings, respectively, and two vehicle injections prior to the vehicle conditionings. Conditionings lasted for 6 min and were done in arms that did not differ in initial preference.

Experiment 3:
Effects of naloxone on the expression of ethanol-induced CPP

Groups of vehicle- or ethanol-conditioned mice received 1 mg/kg naloxone 5 min before the post-conditioning test to assess its effect on the expression of ethanol-induced CPP.

Data presentation and statistics

Data are calculated as the raw pre- and post-conditioning test times and as Δ CPP times (the difference between the post-conditioning and pre-conditioning test preferences in the drug-associated arm of the apparatus) in seconds. The raw data were assessed using a mixed-design ANOVA (with treatment and pre- or post-tests as between and repeated factors, respectively). The Δ CPP data were assessed with a one-way ANOVA. Duncan's test was used for *post-hoc* analyses. The data fulfilled criteria of a normal distribution, and as revealed by three various approaches, the variances were homogeneous. The α value was set at $p < 0.05$. Statistical analyses were performed with Statistica 8.0 for Windows.

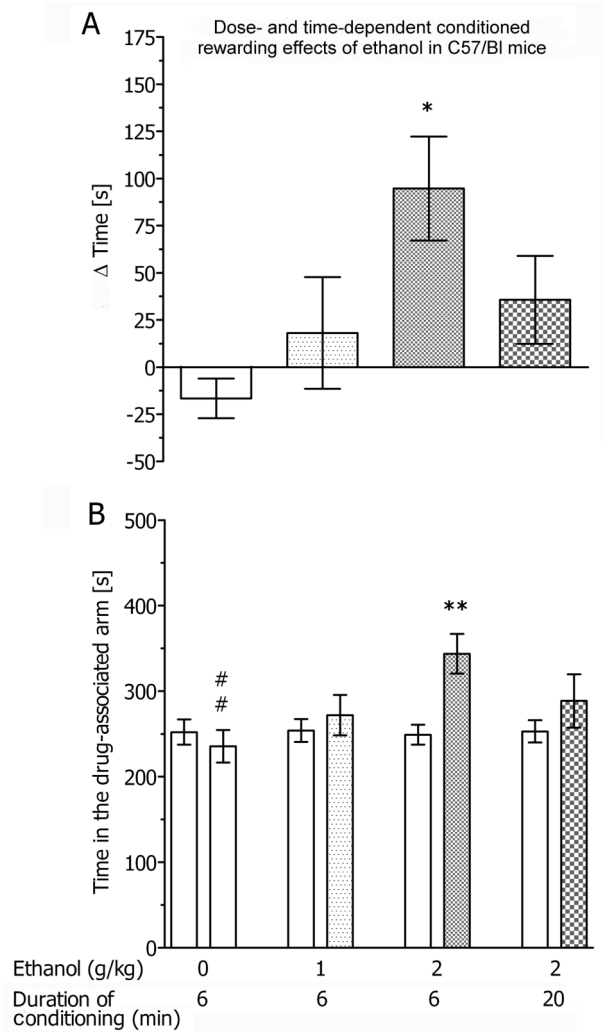

Fig. 1. The intensity of conditioned ethanol reward depends on the duration of the conditionings and the dose of ethanol. Mice were conditioned with 0, 1 or 2 g/kg of ethanol for 6 or 20 min. (**A**) The data presented are the Δ CPP times (post-test – pre-test) ± SEM spent in the ethanol paired arms of the apparatus. Symbols: * $p < 0.05$ *vs.* vehicle group. (**B**) The raw data of the pre-test (left bars) and the post-test (right bars). Symbols: ** $p < 0.01$ *vs.* the respective pre-test data; ## $p < 0.01$ *vs.* post-test results of ethanol 2 g/kg conditioning 6 min group

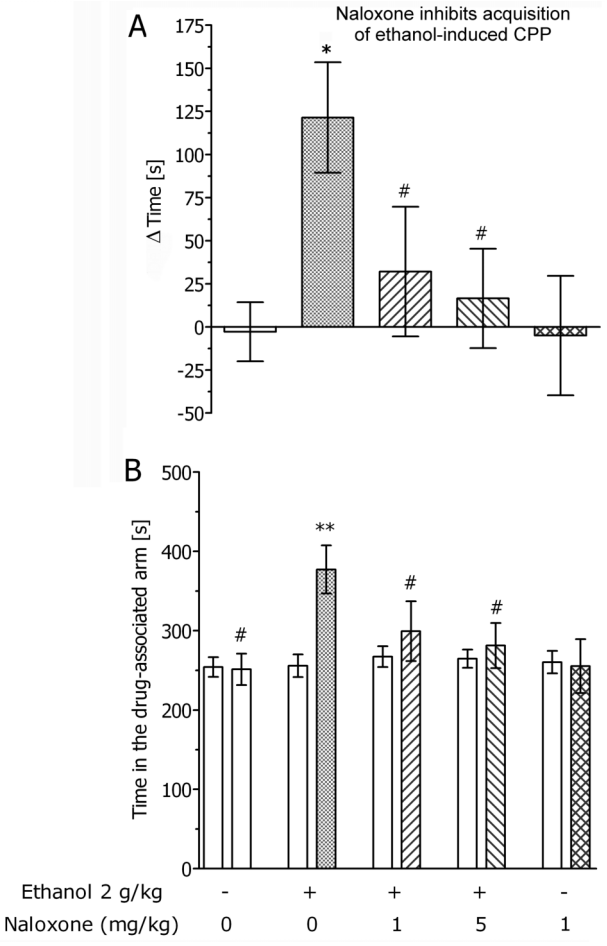

Fig. 2. Naloxone inhibits the acquisition of the conditioned ethanol reward. Mice received vehicle or naloxone injections 5 min prior to each conditioning plus vehicle or ethanol immediately before drug conditionings. On alternating days, mice were conditioned to the effects of naloxone plus vehicle. The last bars represent a control group that was conditioned to the effects of naloxone instead of ethanol. (**A**) The data presented are the ΔCPP (mean ± SEM) times spent in the ethanol paired arms of the apparatus. Symbols: * $p < 0.05$ *vs.* vehicle group; # $p < 0.05$ *vs.* ethanol-conditioned group. (**B**) The raw data of the pre-test (left bars) and the post-test (right bars). Symbols: ** $p < 0.01$ *vs.* the respective pre-test data; # $p < 0.05$ *vs.* post-test results of ethanol 2 g/kg conditioning 6 min group

Results

Experiment 1

Conditioning with 2 g/kg, but not 1 g/kg, of ethanol produced significant CPP in mice when the duration of conditionings was 6 but not 20 min. This effect was observed for the data expressed as Δ CPP [$F(3,35) = 3.86$, $p < 0.05$, Fig. 1A] and for the raw data (mixed-design ANOVA [$F(3,35) = 2.03$, NS]; [$F(1,35) = 7.54$, $p < 0.01$] and [$F(3,35) = 3.86$, $p < 0.05$] for the treatment, pre-test/post-test and interaction, respectively, Fig. 1B).

Experiment 2

Similarly to Experiment 1, ethanol produced a significant conditioned reward at 2 g/kg with conditionings that lasted 6 min. Naloxone given prior to conditionings at 1 mg/kg did not change CPP; however, at

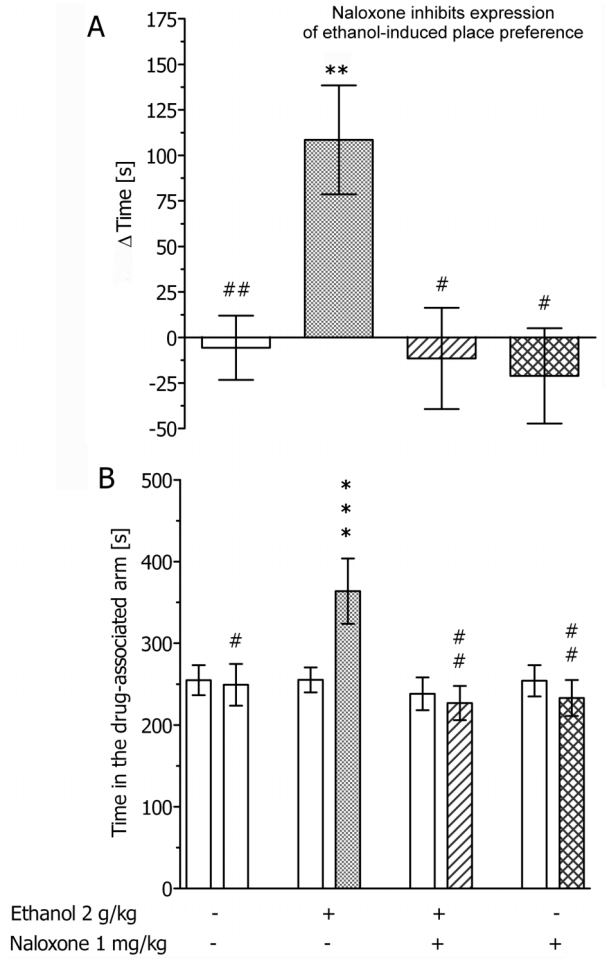

Fig. 3. Naloxone inhibits the expression of conditioned ethanol reward. Five min before the post-test, the vehicle or 2 g/kg ethanol-conditioned mice received an injection of naloxone. (**A**) The data presented are the ΔCPP (mean ± SEM) times spent in the ethanol-paired arms of the apparatus. Symbols: ** $p < 0.01$ *vs.* vehicle group; # $p < 0.05$, ## $p < 0.01$ *vs.* ethanol group. (**B**) The raw data of the pre-test (left bars) and the post-test (right bars). Symbols: *** $p < 0.001$ *vs.* the respective pre-test data; # $p < 0.05$, ## $p < 0.01$ *vs.* post-test result of ethanol 2 g/kg conditioning 6 min group

1 and 5 mg/kg, it inhibited the acquisition of ethanol-induced CPP (Fig. 2). This effect was observed for the data expressed as Δ CPP [$F(4,42) = 2.67$, $p < 0.05$, Fig. 2A] and for the raw data (mixed-design ANOVA: [$F(4,42) = 1.92$, NS]; [$F(1,42) = 5.41$, $p < 0.05$] and [$F(4,42) = 2.67$, $p < 0.05$] for treatment, pre-test/post-test and interaction, respectively, Fig. 2B).

Experiment 3

Naloxone (1 mg/kg) did not affect CPP when given immediately before the post-test in vehicle controls; however, it inhibited the expression of ethanol-induced CPP (Fig. 3). This effect was observed for the data expressed as Δ CPP [$F(3,26) = 5.042$, $p < 0.01$, Fig. 3A] and for the raw data (mixed-design ANOVA [$F(3,26) = 3.086$, $p < 0.05$]; [$F(1,26) = 1.687$, NS] and [$F(3,26) = 5.042$, $p < 0.01$] for treatment, pre-test/post-test and interaction, respectively, Fig. 3B).

Discussion

The present data indicate that ethanol elicited a dose-dependent place preference in male C57BL/6 mice in an unbiased version of the CPP apparatus and an unbiased stimulus assignment procedure. The intensity of the ethanol-conditioned reward was greater when the conditioning trials lasted for 6 min than when they lasted for 20 min. The administration of the non-selective opioid receptor antagonist naloxone before the conditionings attenuated the acquisition of ethanol-induced CPP. When naloxone was given before the post-conditioning test, it blocked the expression of the acquired association, but it did not change the preference of vehicle-conditioned controls and did not appear to produce any motivational properties when tested alone.

The rewarding effects of 2 g/kg ethanol given *ip* immediately before conditionings produce CPP in most studies in mice (see the Introduction section and [6, 22] for negative data). The present results indicate that the intensity of conditioned ethanol reward was inversely related to the length of the conditioning trials (Fig. 1) because the conditionings that lasted for 6 min, but not 20 min, produced significant CPP, confirming the results of Cunningham and Prather in DBA mice [12]. These authors reported that the magnitude of ethanol-induced CPP was greatest at the shortest (5 min) conditioning trial duration and diminished with increasing conditioning trial duration to 15 or 30 min; however, at all conditioning times, significant CPP was observed. This relatively short-lived rewarding action of ethanol could be due to the pharmacokinetics. In C57BL/6 mice, the blood concentration of ethanol diminished rapidly from 163 ± 8 mg% to 113 ± 14 mg% at 5 and 20 min, respectively, after administration at 1.75 g/kg [19].

Naloxone, a non-selective opioid receptor antagonist, inhibited the acquisition of ethanol-induced CPP

at 1 and 5 mg/kg (Fig. 2). Similar inhibitory effects of naloxone and selective μ- and δ-opioid receptor antagonists on the acquisition of ethanol-induced CPP have been reported in rats [5, 18]. However, experiments performed in mice have indicated an inhibitory effect on acquisition only at a dose as high as 10 mg/kg [17], and, according to the authors, this effect could be non-specific because naloxone given alone produced significant place aversion. Furthermore, Cunningham et al. [8] reported that naloxone (1.5 and 10 mg/kg) failed to affect the acquisition of ethanol-induced CPP, although it produced CPA on its own. The inhibitory effects of naloxone on the acquisition of CPP could be explained by its purported aversive effect, but in case of present study, such an interpretation is unlikely because the dose of naloxone (1 mg/kg) that effectively decreased the conditioned effects of ethanol did not produce motivational properties on its own (Fig. 2). Similar results revealing a lack of CPA induced by naloxone (1 and 3 mg/kg) and β-funaltrexamine, naltrindole and nor-binaltorphimine have been reported by Matsuzawa et al. [18]. Given the complexity of ethanol-induced CPP and the variable effect of opioid receptor antagonists on the acquisition of ethanol rewarding effects, the present data suggest that the aversive properties of naloxone were neither responsible for nor sufficient to attenuate the conditioned rewarding effects of ethanol.

Naloxone also blocked the expression of the acquired conditioned ethanol reward, but it did not change the preference of the vehicle-conditioned group (Fig. 3). Cunningham et al. [8, 10] have demonstrated that naloxone (1.5, 3 and 10 mg/kg) inhibits the expression of ethanol-induced CPP in DBA mice, but this effect is restricted to the last 30 min of a 60 min preference test. These authors have interpreted this effect as a facilitation of the extinction of the conditioned ethanol reward. However, other studies have demonstrated that opioid receptor antagonists inhibit the expression of ethanol-induced CPP in mice after an intra-VTA infusion of methylnaloxonium [2] and after the systemic administration of naltrexone [19] and naloxone [17]. The purported aversive properties of opiate antagonists appear unlikely because in the present study, the dose of naloxone that effectively attenuated the expression of ethanol-induced CPP produced no aversion. Additionally, Cunningham et al. [10] demonstrated that the administration of the highly aversive compound – lithium chloride, instead of naloxone did not affect the expression of conditioned ethanol reward.

In the present experimental setup, it was possible to demonstrate the inhibitory effects of naloxone both on the acquisition and the expression of ethanol-induced place preference in mice at a dose that lacked its own motivational properties. The outcome of the present experiments could be the result of using the mouse strain C57BL/6, which is less sensitive to the rewarding effects of ethanol but highly sensitive to the rewarding effects of opioids and therefore, most likely, opioid antagonist actions [11]. However, we used an unbiased version of the CPP apparatus and an unbiased stimulus assignment procedure, factors which appear to increase the sensitivity of mice to ethanol conditioned reward [9]. Overall, the present data indicate that ethanol-induced CPP in C57BL/6 mice could be a sensitive model for the study of the motivational effects of ethanol and the compounds that modify them. The effectiveness of opioid receptor antagonists in the reduction of the actions of ethanol is in accordance with clinical studies that have demonstrated a naltrexone-induced reduction of drinking behavior and alcohol craving and a prevention from relapse in alcohol-dependent humans [20].

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