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Research report

# The effect of constant darkness and circadian resynchronization on the recovery of alcohol hangover

Analía G. Karadayian[a,b,*], Silvia Lores-Arnaiz[b], Rodolfo A. Cutrera[a]

[a] Laboratory of Neurobiology and Rhythms, Department of Physiology, School of Medicine, Universidad de Buenos Aires, Buenos Aires, Argentina
[b] Institute of Biochemistry and Molecular Medicine, School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina

## HIGHLIGHTS

- Darkness is effective to reduce the recovery time for the impairments due to hangover.
- Synchronized clock is involved in the recovery of deleterious effect of hangover.
- Circadian clock was found to be involved in the recovery of hangover symptoms.

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## ABSTRACT

Alcohol hangover (AH) is a particular state after binge-like drinking. AH begins when ethanol is absent in plasma and is characterized by a cluster of physical and psychological symptoms. Alcohol disrupts circadian patterns of behavioral and physiological parameters; however, the involvement of circadian clock on the recovery of AH was not explored. Our aim was to study the effect of continuous darkness and the possible involvement of the circadian clock in the recovery time of neuromuscular impairment and anxiety related-behavior due to AH. Male Swiss mice were habituated to 12:12 L:D or continuous darkness. Each group was injected i.p. either with saline (control group) or with ethanol (3.8 g/kg BW) (hangover group). Motor performance and anxiety phenotype were evaluated at a basal point (ZT0) and every 2 h up to 20 h after blood alcohol levels were close to zero (hangover onset). A third group was subjected to a phase advance during which a hangover episode was induced and behavioral tests were carried out for each group of treatment and resynchronization day. Constant darkness resulted to be in a faster recovery of both motor and anxiety impairments in AH compared with the recovery pattern observed under normal light–dark conditions. Mice suffering from a phase shift exhibited behavioral disruptions due to both AH and phase advance. Results indicated that a synchronized circadian clock is necessary for an adequate recovery of alcohol hangover symptoms.



## 1. Introduction

Excessive alcohol consumption has a variety of serious consequences on health. The different effects are widespread, altering numerous physiological, endocrine and behavioral functions. A particular state after binge-like drinking is defined as alcohol hangover (AH). In this sense, AH is described in humans as a physiological state which involves the unpleasant next-day effect following an evening of excessive alcohol consumption [1]. Hangover begins when ethanol is absent in plasma and is characterized by a cluster of physical and psychological symptoms which include headaches, nausea, diarrhea, fatigue and tremors combined with decreased occupational, cognitive and/or visuospatial skills [2–4] with substantial individual, social and economical consequences [5]. Together with this, previous research work described for experimental animals, hypo-activity [6], fluctuations in body temperature, anxiety-like behavior [7] and reduced wheel running activity [8,9].

We previously reported that hangover induced serious motor and affective impairment which persist several hours (16–20 h)

*Abbreviations:* %FEO, proportion of entrance into open arms; %TSO, proportion of time spent in open arms; AH, alcohol hangover; BD, basal day; CT, circadian time; EMP, elevated-plus maze; LD, light–dark; RD, resynchronization day; TE, total number of entries; ZT, Zeitgeber time.

* Corresponding author at: Institute of Biochemistry and Molecular Medicine, School of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956, C1113AAD Buenos Aires, Argentina. Tel.: +54 11 4964 8244; fax: +54 11 5950 9500.
*E-mail address:* analiakaradayian@conicet.gov.ar (A.G. Karadayian).

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after acute ethanol exposure [10,11]. In addition, we have demonstrated an association between this motor impairments and changes in brain cortex energetic metabolism [12]. Our data join to other research work that demonstrated that hangover symptoms in both humans and animals models are time- and dose-dependent [13–15]. These evidences were obtained by experimental models of AH or binge-like drinking pattern when animals were tested under normal light–dark conditions.

The effects of alcohol consumption on circadian rhythms were widely studied. In fact, it is known that the normal circadian patterns of a variety of behavioral and physiological parameters are disrupted by alcohol administration, ingestion, and/or withdrawal syndrome [16]. For instance, in laboratory rats and mice as in human subjects, alcohol administration alters the circadian response related to locomotor activity, body temperature [17], sleep [18], food intake [19] and the secretion of the stress related hormones [20,21]. A low blood alcohol content of 0.1% reached after the consumption of several alcoholic drinks (about 6–8 beverages) could lead to impaired clock synchronization [22]. Also, a previous work demonstrated that acute ethanol can inhibit photic phase shifts in hamsters [23]. Although the effects of alcohol consumption on circadian rhythms and its associated physiological functions were extensively studied, the involvement of circadian clock and the effect of changes in photoperiod on the recovery of AH were not explored. Moreover, it was stated that individual differences in circadian rhythmicity (i.e. circadian typology) and sleep factors should be taken into account as they influence performances and may interact with hangover effects [24]. Furthermore, it was hypothesized that alcohol-induced dysrhythmia could be one of cause for hangover syndrome [25]. Taking all together into account, the aim of this work was to study motor function and affective behavior during AH in order to achieve several goals: (1) to determine the time course of neuromuscular impairment and anxiety related-behavior under continuous darkness; (2) to compare the recovery of AH under normal light–dark conditions between constant darkness and (3) to establish the possible involvement of the circadian clock on the behavioral recovery at the onset of AH.

## 2. Materials and methods

### 2.1. Animals

A total of 150 from six cohorts of male Swiss mice (*Mus musculus*) weighing 30–40 g were acquired from the School of Pharmacy and Biochemistry, Universidad de Buenos Aires, and housed in a soundproof room under conditions of controlled temperature (22 ± 2 °C) and humidity, with a 12-h light/dark cycle. Standard rat chow and tap water were provided ad libitum.

Animal handling, treatment and experimental procedures were reviewed in accordance with the guidelines of the National Institutes of Health (USA) and with Regulation 6344/96 of Argentina's National Drug, Food and Medical Technology Administration (ANMAT). Moreover, the present study had the legal ethical accreditation from Ethics Committee for Laboratory Animal Handling of the School of Medicine from Universidad de Buenos Aires where the protocol was performed. All efforts were made to minimize suffering and reduce the number of animals used.

### 2.2. Experimental procedure

Animals received intraperitoneal (i.p.) injections of 15% EtOH at a dose of 3.8 g/kg. Ethanol dose was previously applied in alcohol-induced hangover animal models [6,9]. Control mice received saline i.p. injections. The onset of alcohol hangover was previously determined being six hours after ethanol administration. This time point matched when blood alcohol concentration was less than or equal to 10% of the maximum value reached for the dose of ethanol used [26]. Three different experiments regarding photoperiod conditions were carried out as follows.

#### 2.2.1. Experiment I: time extension of motor function impairments and anxiety-like behavior during alcohol hangover

A total of 40 animals were habituated to a photoperiod of 12:12 h light/dark cycle. Behavioral tests were carried out at a basal point that matched with lights onset (ZT0) and every 2 h up to 20 h after alcohol hangover onset (ZT3 of the following day) (see Fig. 1, Experiment I). Animals were randomly assigned to saline or ethanol treatment before baseline tests ($n = 10$ per treatment and for each behavioral task). Each subject was tested every two hours in only one behavioral test avoiding multiple tasks for animals groups. Control groups (saline treatment) let observe in- and between-group differences due to time-course (photoperiod), acute treatments and carry-over effects.

#### 2.2.2. Experiment II: effect of constant darkness on the time extension of motor function impairments and anxiety-like phenotype during alcohol hangover

A total of 40 animals were transferred to constant darkness. Following the same schedule for Experiment I, behavioral tests were carried out at a basal point that matched with the subjective lights onset (CT0) and every 2 h up to 20 h after alcohol hangover onset (CT3 of the following day) (see Fig. 1, Experiment II). Animals were randomly assigned to saline or ethanol treatment before baseline tests ($n = 10$ per treatment and for each behavioral task). Each subject was tested every two hours in only one behavioral test avoiding multiple tasks for animals groups. Control groups (saline treatment) let observe in- and between-group differences due to time-course (photoperiod), acute treatments and carry-over effects.

#### 2.2.3. Experiment III: effect of phase shift on the motor impairment and anxiety-like behavior at the onset of alcohol hangover

A total of 70 animals were habituated to a photoperiod of 12:12 h light/dark cycle. All mice were transferred to a different room where a phase shift was introduced as shown in Fig. 6. Animals experienced a 5 h phase advance which changed the photoperiod conditions to a new 12:12 h light/dark cycle (lights off at 2:00 p.m.) and also resulted in a shortening of the active phase during the night. It was previously reported that resynchronization after a phase shift requires a day for each hour of advance of the LD cycle [27]. Thus, five days of resynchronization was necessary to establish the new circadian rhythm. The involvement of circadian clock on the effect of alcohol hangover was evaluated by testing motor and anxiety-like behavior across the resynchronization period. For that propose, animals were randomly divided in five groups ($n = 14$) and a hangover episode was induced for each group and resynchronization day. Considering this methodology, for each resynchronization day, a group of 14 mice ($n = 7$, controls; $n = 7$, treated with alcohol) were evaluated in two different behavioral test. Thus, animals were divided as RD 1 (resynchronization day 1), RD 2, RD 3, RD 4 and RD 5. In addition, to ensure that basal level of the behavioral parameters were similar to that obtained for Experiment I and II, all animals which were divided in the five groups mentioned above, were previously tested before the phase shift; thus, basal level for each group was obtained (BD 1, BD 2, BD 3, BD 4 and BD 5; being BD, basal day). Behavioral tests were carried out at the onset of alcohol hangover which matched with lights off at 2:00 p.m.

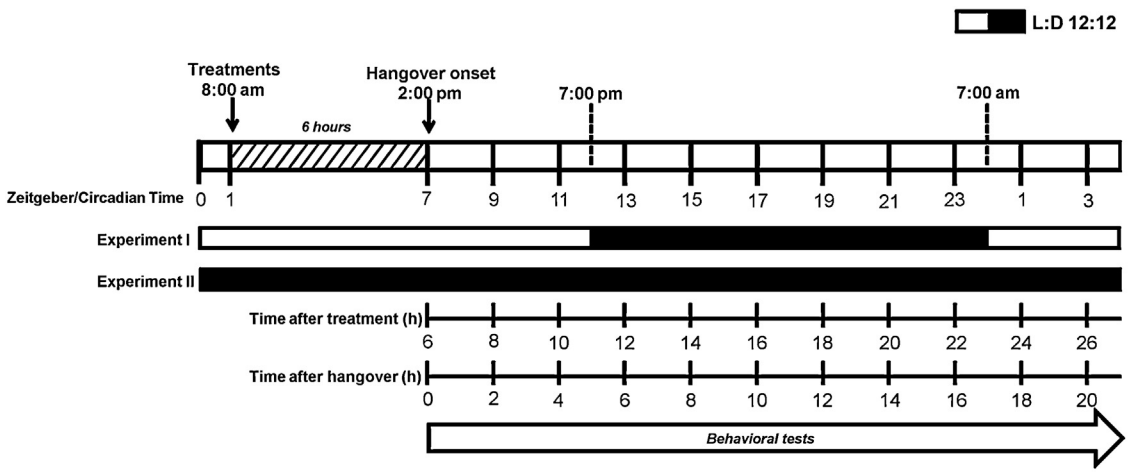


**Fig. 1.** Timeline. Experiments I and II. Male Swiss mice habituated to 12:12 h light/dark cycle (Experiment I, ZT12: 7:00 pm.) or to constant darkness (Experiment II) received intraperitoneal treatment with saline or ethanol at a dose of 3.8 mg/kg or an equivalent of normal saline at 8:00 am. Behavioral tests were performed before and six hours after treatment when alcohol hangover began. L:D, light:dark.

### 2.3. Behavioral assessments

Neuromuscular coordination and anxiety-related behavior were evaluated using two different behavioral tests. During experimental procedures, test boxes or the apparatus used for behavioral studies were cleaned with 10% EtOH after every individual test session to prevent the next mouse from being influenced by the odors deposited in the urine and feces of the previous mouse.

#### 2.3.1. Tightrope test

Motor coordination was evaluated with a modified tightrope test [28]. Briefly, the procedure consisted in placing the animal on the middle of a 60 cm long horizontal rope suspended 30 cm above the floor and time was recorded until the animal either reached the end of the rope or fell down during a period of 60 s. A score was assigned accordingly (Table 1): animals reaching the end of the rope in ≤6 s were given 1 point and an additional point was given for every additional 6 s needed to complete the test. Animals that stayed on the rope for 60 s without reaching the end were given 11 points. When mice fell down, while test was running, 11 points were assigned and 1 extra point was added for every 6 s before the test ending time (60 s). The test evaluates the motor performance of the animal as a mean of its intrinsic neuromuscular coordination. In this sense, a lower score is taken as a better motor performance. For the purpose of this work, results were shown as a percentage of the motor performance which was calculated considering the maximum possible score for the test (20 points, being as 100%) and the score reached for each animal.

**Table 1**
Scoring for tightrope test in mice.

| Mice reaching the end of the rope | | Mice not reaching the end of the rope | |
|---|---|---|---|
| Time (s) | Score | Time (s) | Score |
| 6 | 1 | 60 | 11 |
| 12 | 2 | 54 | 12 |
| 18 | 3 | 48 | 13 |
| 24 | 4 | 42 | 14 |
| 30 | 5 | 36 | 15 |
| 36 | 6 | 30 | 16 |
| 42 | 7 | 24 | 17 |
| 48 | 8 | 18 | 18 |
| 54 | 9 | 12 | 19 |
| 60 | 10 | 6 | 20 |

Taken from Boveris and Navarro [28].
It is shown the score assigned for the time used to complete the tightrope test. Mice that do not reach the end of the rope received a higher score. A lower score represents a better motor performance.

#### 2.3.2. Elevated-plus maze

Anxiety-like behavior was evaluated by the elevated-plus maze (EPM). The apparatus (made of Plexiglas) consisted of two open arms (10 cm × 50 cm) alternating at right angles with two closed arms (10 cm × 50 cm × 10 cm), delimiting a central area. The whole maze was elevated 50 cm above the floor. Mice were placed in the central area of the maze, facing one of the closed arms, and were allowed to explore it for 5 min as previously described [29]. The animal's behavior was analyzed by video tracking system ANY Maze (Stoelting Co., Wood Dale, Illinois). The proportion of entrance into open arms (%FEO) and the proportion of time spent in open arms (%TSO) together with the total number of entries (TE) were measured following a four-paw criterion: entry into the arm of the EPM was defined as the animal placing all four paws in that particular part of the maze. The maze's arms were equally illuminated so that the animals did not perceive lighting differences. The elevated-plus maze rests on the conflict between the tendency of mice to explore a novel environment and the aversive properties of a brightly lit, open area. It is considered that anxiety-like behavior is characterized by a decreased in %FEO and %TSO. Also, the parameter of TE provides a built-in control measure for general hyperactivity or sedation.

### 2.4. Statistical analysis

Results are presented as means ± SEM. Before each analysis, test variables were checked for normality, so all data were evaluated by the Kolmogorov–Smirnov test to follow a posterior parametric or nonparametric statistical analysis. Data obtain from behavioral test from Experiment I and II were analyzed using the unpaired independent Student's *t*-test to analyze the significance of differences between hangover and control groups; also, in-group differences were examined by repeated-measures two-factor ANOVA. For Experiment III, three statistical comparisons were made: unpaired independent Student's *t*-test to analyze differences between hangover and control groups and differences between same groups of treatment across the resynchronization process (e.g. hangover group RD 1 vs. hangover group RD 2). In addition, paired sample Student's *t*-test was applied to analyze differences due to the phase shift (e.g. hangover group BD 1 vs. hangover group RD 1). SPSS

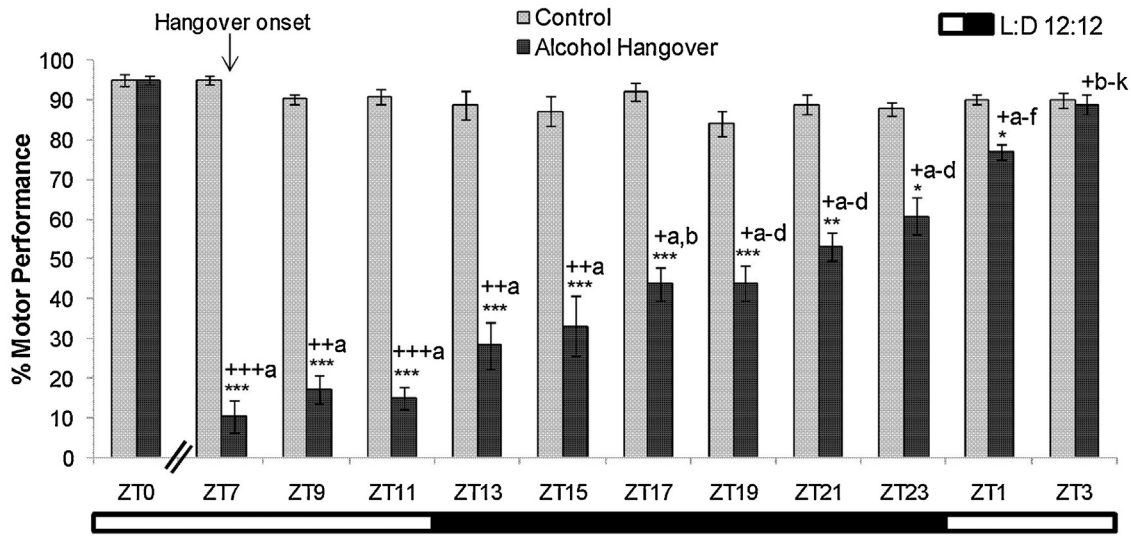


**Fig. 2.** Motor performance on the tightrope test during alcohol hangover. Values are expressed as mean ± SEM ($n = 10$ each group). ZT: Zeitgeber time; L:D, light:dark. Unpaired independent samples Student's *t*-test was used ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) for intergroup differences. Repeated-measures two-factor ANOVA was used ($^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$) for in-group difference. Letters indicate the time point of the comparison as follows: a: ZT0, b: ZT7, c: ZT9, d: ZT11, e: ZT13, f: ZT15, g: ZT17, h: ZT19, i: ZT21, j: ZT23 and k: ZT1. Bars shading indicate group: gray, control; black, alcohol hangover.

(version 13.0) statistical software was used and a difference was considered statistically significant when $p < 0.05$.

## 3. Results

### 3.1. Time extension of changes in motor performance and affective behavior due to alcohol hangover

Motor performance was evaluated by the tightrope test at different timetables before and after the onset of alcohol hangover. Results obtained are shown in Fig. 2. At the beginning of hangover (ZT7), mice average motor performance was almost 85% lower compared either with controls ($p < 0.001$) or its basal point (95% at ZT0) ($p < 0.001$). During the next 18 h, hangover mice exhibited a reduced neuromuscular coordination compared with controls ($p < 0.001$ from ZT9 to ZT19; $p < 0.01$ for ZT21; $p < 0.05$ for ZT23 and ZT1). Alcohol-treated animals showed a progressive improvement in their neuromuscular coordination as time goes on. Motor function in hangover mice was not different from controls or from its basal point at ZT3 (20 h after hangover onset). Control mice did not show any significant differences in motor performance during the evaluation period (20 h).

Affective behavior was studied by measuring anxiety-like phenotype on EPM in a particular schedule as the same way for motor performance test. Results are shown in Fig. 3. A very significant decrease in the frequency of entrance into the open arms (%FEO; Fig. 3A) was observed in hangover mice from ZT7 to ZT11 ($p < 0.001$, compared with controls). As expected, during the dark phase, control and hangover mice showed a gradually significant increase in %FEO ($p < 0.05$, compared with same group from ZT0 to ZT11) that was persistent up to ZT23 when %FEO levels dropped being similar to basal levels at ZT1 and ZT3. No significant differences were observed between control and hangover mice from ZT23 to the end of the experiment or between same groups with its basal levels (ZT0). Similar data was obtained for the proportion of time spent into the open arms (%TSO; Fig. 3B). A significant reduction in %TSO was observed in hangover mice from ZT7 to ZT11 ($p < 0.01$, compared with controls). As observed for motor performance, during the dark period, control and hangover mice displayed a significant increase in %TSO ($p < 0.05$, compared with same group from ZT0 to ZT11). Control and hangover groups returned to baseline at ZT3 when no significant differences were found between both of them. Additionally, the number of total entries (TE; Fig. 3C) as an indicator of general activity was measure in the EPM. Hangover mice displayed a significant decreased in TE from ZT7 to ZT 11 ($p < 0.001$, compared with controls). Both control and hangover groups exhibited a significant increase in TE during the dark period ($p < 0.05$, compared with same groups from ZT0 to ZT11). No significant differences between control and hangover mice were observed at ZT1 (18 h after hangover onset).

### 3.2. Effect of constant darkness on the time extension of motor function impairment and anxiety-like phenotype during alcohol hangover

As the same for Experiment I, motor performance (Fig. 4) and anxiety-like behavior (Fig. 5) was evaluated in control and hangover mice; however, in this case, all animals were subjective to continuous darkness. Hangover mice showed a 25% decrease in motor performance from CT7 to CT11 ($p < 0.001$, compared with controls or same group at CT0). At CT13, hangover mice exhibited 18% reduction in motor coordination ($p < 0.05$, compared with control). From CT15 to the end of the experiment, no significant differences were found between control and hangover mice. Motor coordination was 80–90% along that period of time both for control and ethanol-treated groups.

Anxiety-like behavior was observed in hangover mice by a reduction in %TSO, %FEO and TE from the beginning of alcohol hangover (CT7) to CT9 ($p < 0.001$, for %FEO and TE compared with controls) and to CT11 ($p < 0.01$, for %TSO compared with controls). Both experimental groups did not show significant differences for the three measured parameters from CT11 to the last time points test.

### 3.3. Effect of phase shift and resynchronization on the motor impairment and anxiety-like behavior at the onset of alcohol hangover

To analyze the possible influence of circadian clock in the recovery of alcohol hangover, a phase shift was set up to mice and behavioral changes in each resynchronization day was studied in control and alcohol-treated mice (see Section 2.2.3).

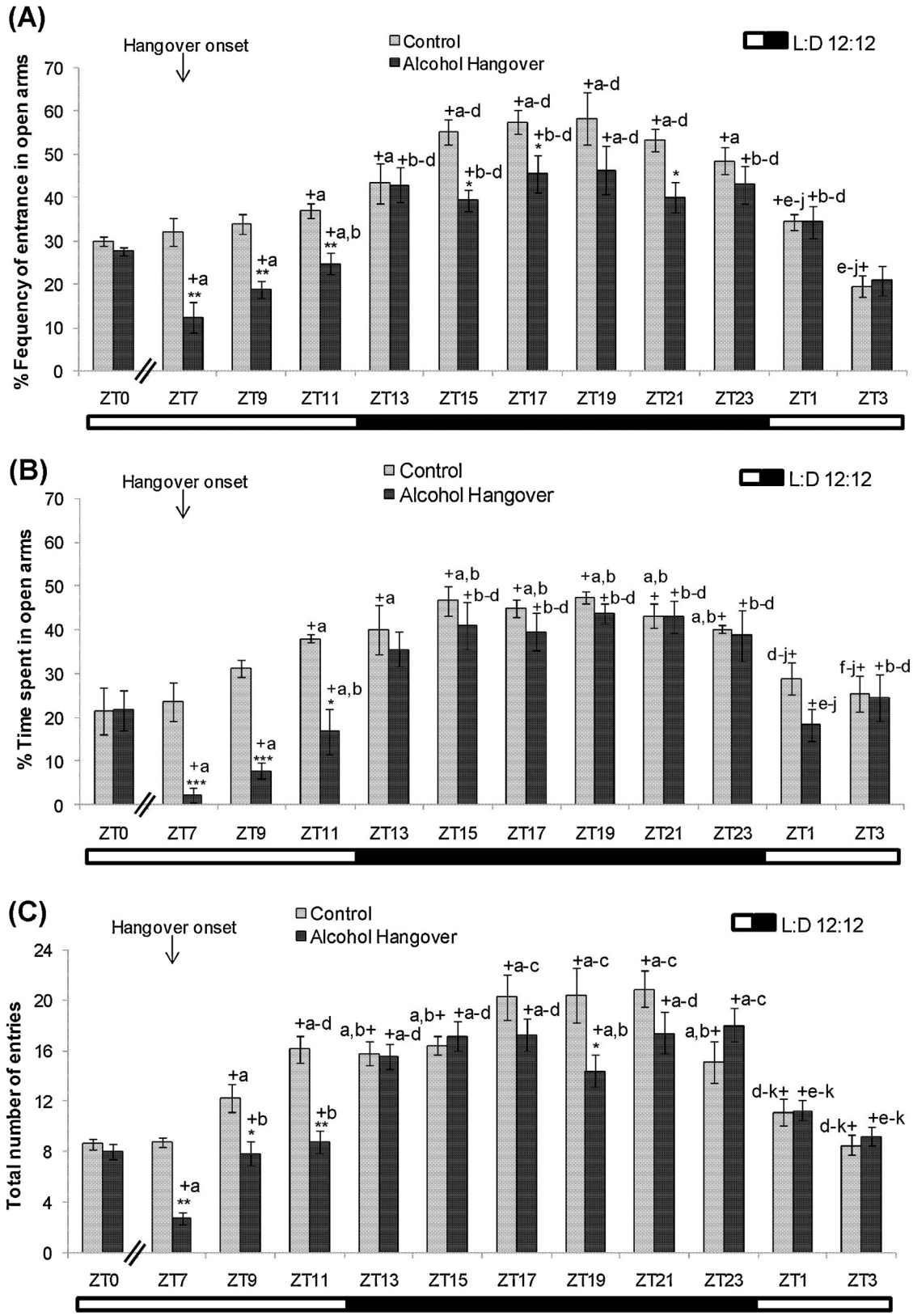


**Fig. 3.** Anxiety-like behavior on the elevated-plus maze during alcohol hangover. Values are expressed as mean ± SEM ($n = 10$ each group). ZT: Zeitgeber time; L:D, light:dark. Unpaired independent samples Student's $t$-test was used ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) for intergroup differences. Repeated-measures two-factor ANOVA was used ($^{+}p < 0.05$) for in-group difference. (A) Frequency (%) of entrance into open arms, (B) proportion (%) of time spent in open arms and (C) total number of entries. Letters indicate the time point of the comparison as follows: a: ZT0, b: ZT7, c: ZT9, d: ZT11, e: ZT13, f: ZT15, g: ZT17, h: ZT19, i: ZT21, j: ZT23 and k: ZT1. Bars shading indicate group: gray, control; black, alcohol hangover.

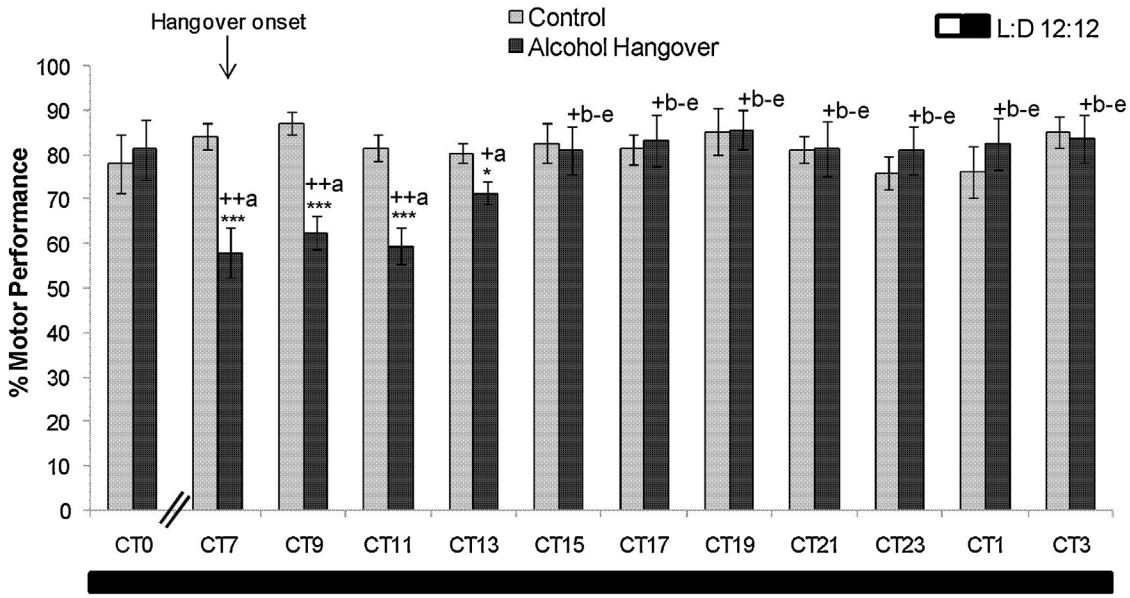


**Fig. 4.** Effect of constant darkness on the time extension of motor function impairment during alcohol hangover. Values are expressed as mean ± SEM ($n = 10$ each group). CT: circadian time; L:D, light:dark. Unpaired independent samples Student's *t*-test was used ($^{*}p < 0.05$, $^{***}p < 0.001$) for intergroup differences. Repeated-measures two-factor ANOVA was used ($^{+}p < 0.05$) for in-group difference. Letters indicate the time point of the comparison as follows: a: ZT0, b: ZT7, c: ZT9, d: ZT11, e: ZT13, f: ZT15, g: ZT17, h: ZT19, i: ZT21, j: ZT23 and k: ZT1. Bars shading indicate group: gray, control; black, alcohol hangover.

Phase shift induced changes in motor performance from control mice at RD 1 ($p < 0.001$), RD 2 ($p < 0.01$) and RD 3 ($p < 0.01$) compared with BD 1, BD 2 and BD 3 respectively (Fig. 7). A very significant decrease in motor performance was observed in hangover animals at RD 1, RD 2 and RD 3 ($p < 0.001$, compared with controls or same group compared with its according BD). Motor impairment was still evidenced at RD 4 and RD 5 when neuromuscular coordination was around 65% ($p < 0.05$, compared with controls or between resynchronization days). Thus, no recovery was observed at the end of the resynchronization process at the onset of alcohol hangover.

When anxiety-like behavior was evaluated after phase shift (Fig. 8A–C), control mice exhibited a decreased in %TSO at RD 1 and RD 3 ($p < 0.05$ and $p < 0.01$, compared with BD 1 and BD 3 respectively). Also, a significant increment in the number of total entries at RD 1, RD 2, RD 3 and RD 4 ($p < 0.001$, compared with BD 1; $p < 0.01$, compared with BD 2 and BD 3; $p < 0.05$, compared with BD 4 respectively); however, no differences were observed in %FEO which indicates signs of general anxiety by an increment of the activity into close arms due to the phase shift. Control mice did not show any significant differences at RD 5. Alcohol-treated mice displayed a significant decrease in %FEO and %TSO from RD 1 to RD 4. These significant differences were found when compared with control mice ($p < 0.001$) and when compared with BD 1 to DB 4 respectively ($p < 0.01$). The number of total entries was significantly reduced only at RD 1 and RD 2 ($p < 0.05$, compared with controls; $p < 0.001$, compared with BD 1 and BD 2 respectively). In addition, hangover mice partially recovered the %FEO, %TSO and TE during the resynchronization period ($p < 0.05$, paired Student *t*-test). At the end of the experiment, hangover animals still exhibited a significant reduction in %TSO while %FEO and TE came back to basal and control levels.

## 4. Discussion

It was widely established that alcohol hangover impairs physical and psychological well-being generating disadvantages that range from occupational, social and individual skills. We previously demonstrated that motor and affective behavior together with an increasing oxidative stress due to mitochondrial dysfunction in brain cortex take place when hangover begins, 6 h after treatment with alcohol [12]. Even when scientific evidence suggests that ethanol modifies physiological and behavioral activities depending on circadian clock, no previous studies have reported the possible effects of photoperiod changes on the different type and length of impairments due to the hangover episode.

The key finding to emerge from this study is that behavioral impairment due to AH could be modulated by particular conditions such as constant darkness and also by circadian resynchronization.

We explored motor performances and anxiety-like behavior under normal light–dark condition. We found that motor function was recovered at 20 h after the beginning of alcohol hangover. In addition, anxiety-like phenotype was observed during the following 4–16 h after AH onset. During the dark period, animals showed an increased general activity in the elevated-plus maze as expected in nocturnal animals. These results were in accordance with the previous obtained by our laboratory when similar behavioral parameters were evaluated across a complete hangover episode [11] and with other researches which verified a 12 h for acute alcohol withdrawal symptoms [30,31]. These results were observed under normal light–dark conditions. We also explored the effect of constant darkness on the recovery of motor impairment and anxiety-like signs during AH. Our results indicate that constant darkness significantly reduced the time for recovery of both motor and affective disruptions. Indeed, motor performance was totally recovered 8 h after AH onset, 16 h before under normal photoperiod. Moreover, it is important to highlight that under constant darkness, neuromuscular coordination was 25% decreased at the beginning of AH while a 95% decrement was found when testing in LD. Along with this, same pattern was obtained for anxiety-like behavior test under constant darkness. In this sense, animals returned to basal levels between 2 and 4 h after AH onset being half of the recovery time compared with same test carried out in standard photoperiod. These results significantly support the hypothesis that darkness plays an important role in the recovery of AH symptoms. Recent scientific evidence indicates that there is a wide range of treatments ranging from pharmacological to behavioral approaches with the aim of mitigate hangover symptoms. Nevertheless, most remedies affect one of hangover symptoms

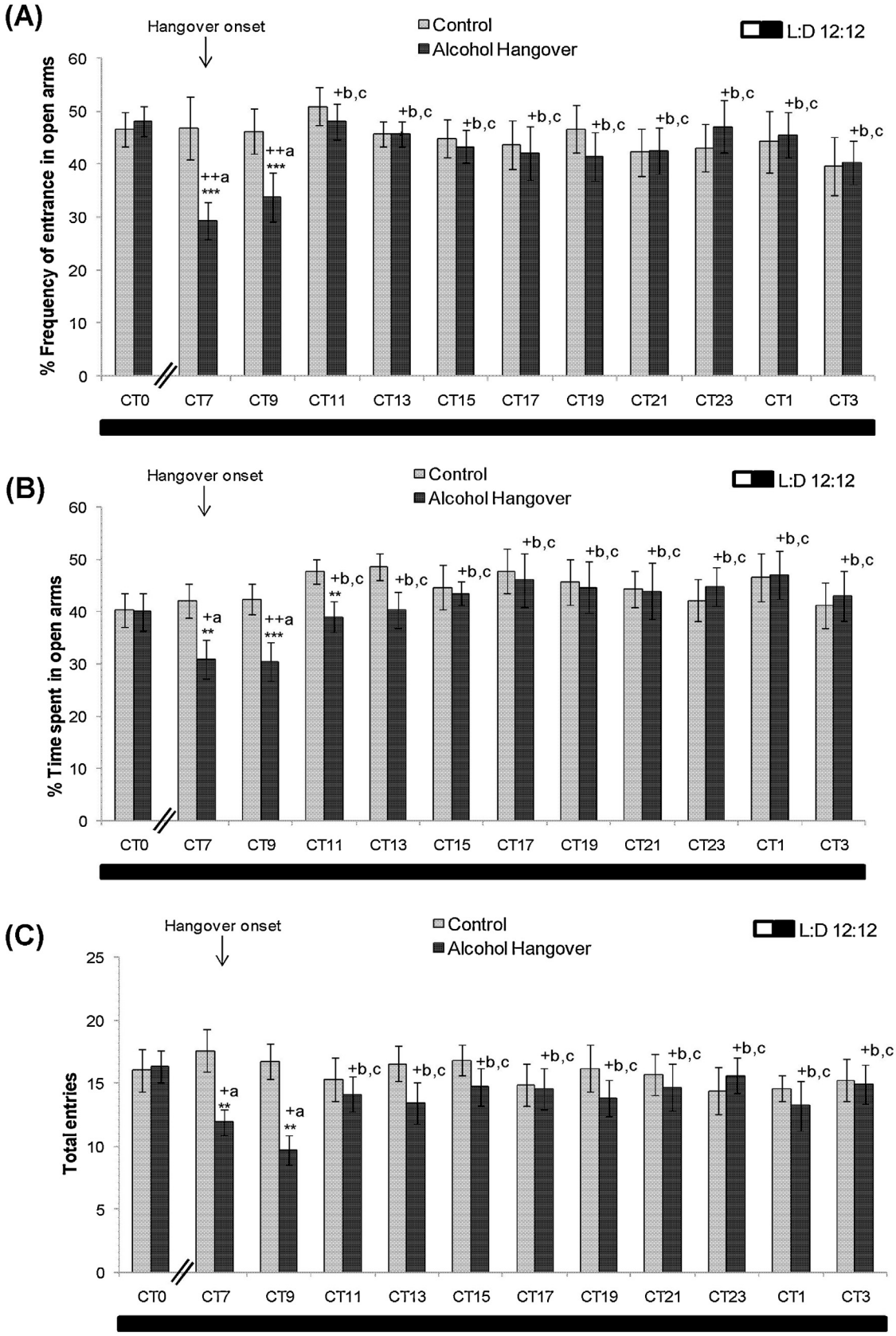


**Fig. 5.** Effect of constant darkness on the time extension of anxiety-like phenotype during alcohol hangover. Values are expressed as mean ± SEM ($n = 10$ each group). CT: circadian time; L:D, light:dark. Unpaired independent samples Student's $t$-test was used ($^{**}p < 0.01$, $^{***}p < 0.001$) for intergroup differences. Repeated-measures two-factor ANOVA was used ($^{+}p < 0.05$, $^{++}p < 0.01$) for in-group difference. (A) Frequency (%) of entrance into open arms, (B) proportion (%) of time spent in open arms and (C) total number of entries. Letters indicate the time point of the comparison as follows: a: ZT0, b: ZT7, c: ZT9, d: ZT11, e: ZT13, f: ZT15, g: ZT17, h: ZT19, i: ZT21, j: ZT23 and k: ZT1. Bars shading indicate group: gray, control; black, alcohol hangover.

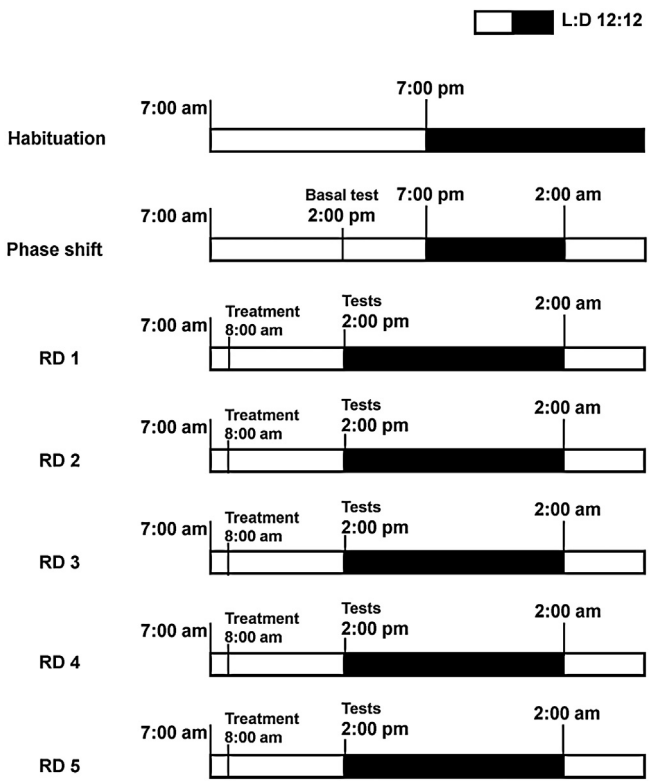


**Fig. 6.** Timeline for the experiment of 5-h phase shift. Male Swiss mice ($n = 70$) were habituated to 12:12 h light/dark cycle (ZT12: 7:00 pm.). A basal test was done before a 5-hours phase advance (ZT12: 2:00 p.m.). Animals were randomly divided in five groups ($n = 14$) and for each resynchronization day every group ($n = 7$, controls; $n = 7$, treated with alcohol) were evaluated at 2:00 p.m. in two behavioral test. Mice received intraperitoneal treatment with saline or ethanol at a dose of 3.8 mg/kg or an equivalent of normal saline at 8:00 a.m. Behavioral tests were performed six hours after treatment when alcohol hangover began. L:D, light:dark; RD: resynchronization day.

rather than overall hangover severity [32,33]. The results here presented lead us to propose a possible tool that considers the darkness to relieve symptoms of photophobia, among others, due to hangover.

Previous researches have studied the effect of withdrawal and hangover on circadian clock functions due to chronic or acute ethanol exposure. For instance, Rosenwasser [16] have established that abstinent alcoholics during acute and/or longer term alcohol withdrawal exhibited abnormalities in the amplitude, timing, and/or patterning of circadian rhythms. Also, it was likened an alcohol hangover to a jet-lag-like circadian disruption (e.g. phase shift) of the body's normal rhythm [34]. Both evidences are related to ethanol' post effects on circadian clock and join to several scientific papers which explored the complex interaction between alcohol and the biological pacemaker [35]. Here, we studied the inverse relationship: the effect of a phase shift as a chronodisruption in order to evaluate the effect of the resynchronization process on the motor and affective impairment seen at the beginning of AH. Mice which underwent a phase shift and suffer from hangover symptoms showed a recovery of about 65% in their neuromuscular coordination which was also similar than the performance reached at the onset of AH under constant darkness. At the end of the resynchronization process, no recovery was observed for hangover groups; however, during the course of the resynchronization animals partially reversed motor injuries. Together with this, signs of anxiety in hangover animals were in part reverted during the resynchronization period; nevertheless, animals still exhibited a significant pattern of general anxiety at the end of the experiment. As expected, our results showed that control mice displayed changes in their reaction into the EPM due to the chronodisruption by the phase shift. These are in accordance with previous evidence of discomfort, insomnia, anxiety and motor impairment due to a phase shift or jet-lag described in humans [36,37].

The results presented in this study, strongly demonstrated that light–dark conditions and circadian clock functioning are two of the essential parameters necessary to analyze the different effects of alcohol withdrawal and hangover. This also joins to other

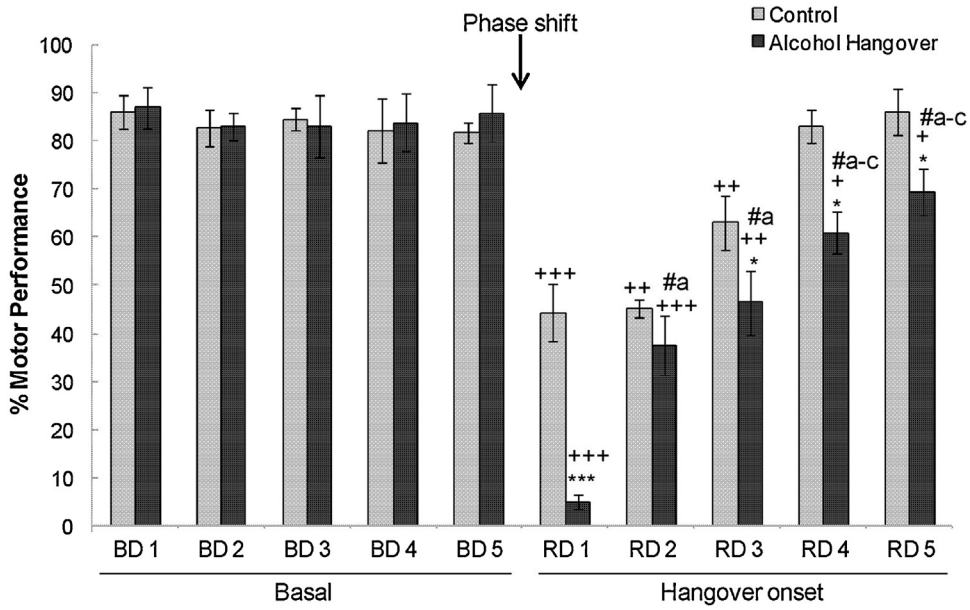


**Fig. 7.** Effect of phase shift and resynchronization on the motor impairment at the onset of alcohol hangover. Values are expressed as mean ± SEM ($n = 14$ each group of resynchronization day: $n = 7$, control; $n = 7$, treated with alcohol). Unpaired independent samples Student's $t$-test was used for intergroup differences (control vs. hangover: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) and for differences between same groups of treatment across the resynchronization process ($\#p < 0.05$). Paired sample Student's $t$-test was used to analyze differences due to the phase shift, e.g. hangover group BD 1 vs. hangover group RD 1 ($^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$). Letters indicate the time point of the comparison as follows: a: RD 1, b: RD 2, c: RD 3 and d: RD 4. Bars shading indicate group: gray, control; black, alcohol hangover. BD: basal day; RD: resynchronization day.

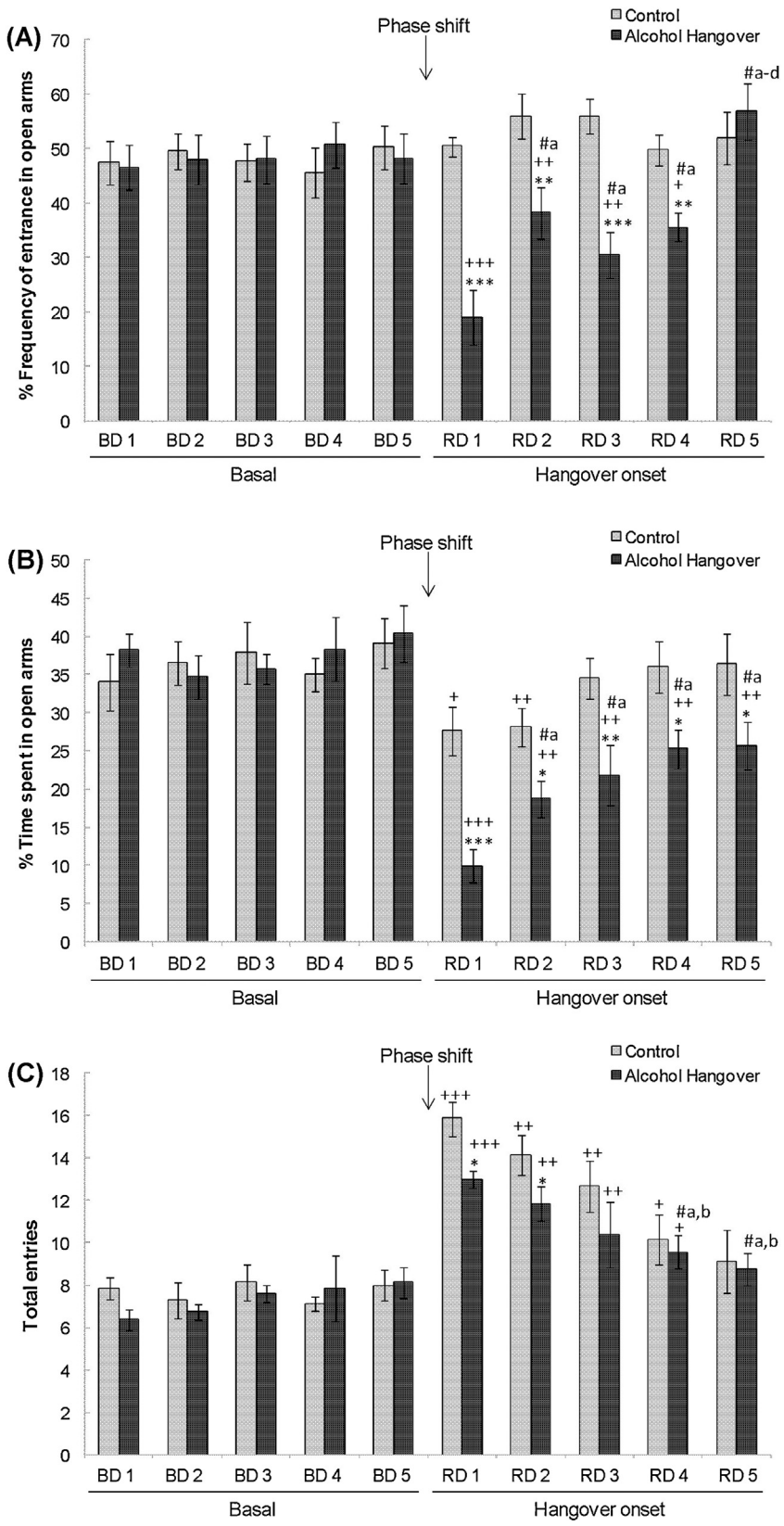


**Fig. 8.** Effect of phase shift and resynchronization on anxiety-like behavior at the onset of alcohol hangover. Values are expressed as mean ± SEM ($n = 14$ each group of resynchronization day: $n = 7$, control; $n = 7$, treated with alcohol). Unpaired independent samples Student's *t*-test was used for intergroup differences (control vs. hangover: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) and for differences between same groups of treatment across the resynchronization process ($^{\#}p < 0.05$). Paired sample Student's *t*-test was used to analyze differences due to the phase shift, e.g. hangover group BD 1 vs. hangover group RD 1 ($^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$). (A) Frequency (%) of entrance into open arms, (B) proportion (%) of time spent in open arms and (C) total number of entries. Letters indicate the time point of the comparison as follows: a: RD 1, b: RD 2, c: RD 3 and d: RD 4. Bars shading indicate group: gray, control; black, alcohol hangover. BD: basal day; RD: resynchronization day.

scientific evidences by [38] by which it was established that assessing the duration for hangover symptoms could provide important descriptive information, such as an indicator of the burden imposed by hangovers in daily life. This could be the key to develop a future treatment for the hangover deleterious effects. In addition, our results contribute to the knowledge of one of the interests of the chronopharmacology of alcohol which studies, among other features, the effect of time-of-day on the action of drugs such as alcohol in body's behavioral response [39]. Furthermore, other studies should be developed in order to precisely define how circadian clock is involved in the recovery of AH such us the possible effect of a phase delay, changes in photoperiods, light pulses during the dark phase and also the evaluation of hangover severity depending on different circadian resynchronizations.

## 5. Conclusion

Alcohol hangover compromises both motor and affective behavior during several hours. A particular photoperiod condition such as constant darkness proved to be effective to significantly reduce the recovery time for the impairments due to hangover. Circadian clock was found to be involved in the recovery of alcohol hangover symptoms. Summing up, the results obtained in the present work significantly advance our understanding of how ethanol and its after-effects interact with the circadian system.

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