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Estrogen blocks the protective action of melatonin in a behavioral model of ethanol-induced hangover in mice

A.G. Karadayian *, M.A. Mac Laughlin, R.A. Cutrera

Laboratory of Neurobiology and Rhythms, Department of Physiology, School of Medicine, Universidad de Buenos Aires, Buenos Aires, Argentina

HIGHLIGHTS

- Exposure to melatonin improved the motor performance in male mice during hangover.
- Melatonin did not enhance motor performance in females during the hangover.
- Estrogens block the neuroprotective action of melatonin during ethanol hangover.

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ABSTRACT

Melatonin has antioxidant and neuroprotective properties in human beings and experimental models, as well as 'anti-estrogenic' effects. Ethanol (EtOH) affects various behavioral parameters during a period known as ethanol-induced hangover. Our study evaluated the neuroprotective effect of melatonin on motor performance during ethanol hangover in male and female Swiss mice. The females were subjected to specific hormonal states: ovariectomized (OVX) and OVX estrogenized (OVX-E_2). Mice received melatonin (25 μg/ml) or vehicle in their drinking water for seven days and were given intraperitoneal (i.p.) injections of EtOH (3.8 g/kg) or saline on the morning of the eighth day. Motor performance was evaluated by the tightrope test 6 h after EtOH exposure (hangover onset). During ethanol hangover, males exhibited lower motor performance than controls ($p<0.01$) but pretreatment with melatonin significantly improved performance during hangover ($p<0.05$). In females, melatonin treatment before ethanol-induced hangover led to a better motor performance in OVX compared with intact females ($p<0.01$) and a lower performance in OVX-E_2 compared with not-estrogenized OVX ($p<0.05$). Consequently, estrogen reversed the motor performance enhancement afforded by melatonin. We conclude that estrogen interferes with the protective action of melatonin on motor performance during ethanol hangover.

1. Introduction

Melatonin is a hormone secreted by the pineal gland at night in all mammals [1]. *In vivo* and *in vitro* studies have shown that it has neuroprotective and antioxidant properties [2]. In this regard, it has been demonstrated that melatonin can provide protection against kainate-induced excitotoxicity [3,4]. Moreover, melatonin has been proven to reduce lipid peroxidation induced by ethanol thus promoting neuroprotection in the central nervous system [5]. Numerous studies have demonstrated the neuroprotective potential of melatonin under various experimental conditions [6]. This neuroprotective action is mainly due to its antioxidant properties, which include direct free radical scavenging and stimulation of antioxidative enzymes [7,8]. A recent review strongly supports the use of melatonin as a possible treatment for several psychiatric disorders due to its anxiolytic, sedative, anticonvulsant and anti-hypertensive properties [9]. In this respect, the pineal hormone could mitigate neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's diseases [10]. Melatonin also has 'anti-estrogenic' effects [11]. It has been shown to decrease gonadal steroidal synthesis, to interfere with activation of the estrogen receptor by estradiol destabilizing the binding of the estradiol–estrogen receptor complex to the estrogen responsive element [12], and to decrease estrogen bioavailability by down-regulating the activity of enzymes involved in its synthesis [13]. Moreover, the effects of melatonin on rodent behavior have also been demonstrated. It induces sedation, elevates the pain threshold, exhibits anxiolytic effects and directly resets circadian rhythms [14].

Ethanol hangover is described as the unpleasant next-day effect following an evening of excessive alcohol consumption that is characterized by physical and psychological symptoms which include headaches, nausea, diarrhea, fatigue and tremors combined with decreased occupational, cognitive and/or visuospatial skills [15,16]. During ethanol hangover, cognitive functions and subjective capacities are affected

* Corresponding author at: Laboratory of Neurobiology and Rhythms, Department of Physiology, School of Medicine, Universidad de Buenos Aires, Paraguay 2155, C1121ABG, Buenos Aires, Argentina. Tel.: +54 11 4950 9500.
E-mail address: analiakaradayian@conicet.gov.ar (A.G. Karadayian).

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along with inefficiency and reduced productivity in the workplace, driving impairments and reductions in motor coordination [17]. Moreover, in experimental animals, body temperature, wheel running activity and pain perception are impaired in this state [18–20]. In direct relation to this, we have recently demonstrated that motor performance in male mice is reduced during hangover [21].

Taken together, it could be inferred that a neuroprotective agent like melatonin could improve behavior during hangover. At present there is no established casual link between ethanol hangover and melatonin action and it could be hypothesized that melatonin might prevent motor performance impairments induced by the hangover state. Furthermore, due to its antiestrogenic effects, we could also hypothesize that the neuroprotective action of melatonin might be influenced by estrogens. In order to prove this hypothesis, the aim of this study was to research the effect of melatonin on motor performance during ethanol-induced hangover in different conditions: intact, OVX and OVX-E_2 female mice and male mice.

Our findings indicate that melatonin is only significantly neuroprotective in the hangover state in the absence of estrogen.

2. Materials and methods

2.1. Animals

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-hour 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). All efforts were made to minimize suffering and reduce the number of animals used.

The total number of animals used in all experiments was as follows:

49 male mice (9 for experiment I and 40 for experiment II).
59 intact female mice (9 for experiment I, 40 for experiment II and 10 for E_2 serum levels).
50 sham-OVX mice (40 for experiment III and 10 for E_2 serum levels).
70 OVX mice (60 for experiment III and 10 for E_2 serum levels).
30 OVX-E_2 mice (20 for experiment III in 2 groups and 10 for E_2 serum levels).

Mice body weight (BW) was monitored weekly.

2.2. Solution preparation

EtOH (15% w/v) was prepared by diluting 95% EtOH with saline solution. Animals received intraperitoneal (i.p.) injections of 15% EtOH at a dose of 3.8 g/kg. This dose is applied in ethanol-induced hangover animal models [22,23]. Melatonin (Mel) (Sigma-Aldrich, USA) was solubilized in 1 ml of 10% ethanol and mixed with tap water to 100 ml. Accordingly, final concentration of melatonin in drinking water was 25 μg/ml. This concentration has been demonstrated to act as antioxidant and neuroprotective agent [24–26]. EtOH concentration in the melatonin solution was only 0.1%.

2.3. Tightrope test

Motor coordination was evaluated with a modified tightrope test [27]. In brief, the procedure consisted of placing an animal on the middle of a 60-cm long horizontal rope suspended 30 cm above the floor and recording the time until the animal either reached the end of the rope or fell off. A score was assigned as follows: animals that reached the end of the rope in ≤6 s were given 1 point and one point was added for each additional fraction of 6 s needed to complete the test. Animals that stayed on the rope for 60 s without reaching the end were given 11 points. In addition to the 11 points, mice were given 1 extra point for every fraction of 6 s it took them to fall off before the 60 s were up. Therefore, animals that scored the lowest performed the best. It should be noted that the test assesses motor performance as a mean of intrinsic neuromuscular coordination. Mice not only had to reach the end of the rope in a predefined time but they also had to do so on all four extremities, as well as the tail, in a coordinated way. Therefore, animals that did not meet these criteria were excluded (*e.g.* animals that reached the end of the rope in less than 60 s but using only both forelimbs) [28].

2.4. Procedure

2.4.1. Experiment I: determination of onset of ethanol-induced hangover

Male (n = 9) and female mice (n = 9) received an i.p. injection of EtOH (15% p/v, 3.8 g/kg); this dose has been used previously in other studies [19,23,29,30]. Three mice from each group were decapitated 60, 180 or 360 min after the injection. Blood was collected from the trunk and blood alcohol concentration (BAC) was measured by gas chromatography (Hospital Británico, Buenos Aires, Argentina) to determine the animals' response to ethanol and the onset of hangover. Experiments were conducted in the morning (9:00 a.m.). The criteria used to establish onset of ethanol-induced hangover was when BAC was less than or equal to 10% of the maximum value reached. Behavioral tests were performed after onset of hangover.

2.4.2. Experiment II: effect of melatonin on motor performance during ethanol-induced hangover in Swiss mice

Mice received melatonin or vehicle in their drinking water for one week. At 9:00 a.m. on the eighth day animals received an i.p. injection of EtOH (15% p/v, 3.8 g/kg) or saline. Motor performance was evaluated in the early afternoon (3:00 pm), at onset of hangover, with a modified tightrope test [27]. Each of the four groups contained 10 animals.

2.4.3. Experiment III: effect of melatonin on motor performance during ethanol hangover in ovariectomized Swiss mice

Ovariectomized mice (OVX) were used and the experiment was divided into two stages. Female mice (n = 40) were first anesthetized (equitesine; 1 mg/35 g BW) and the ovaries removed. For this and subsequent procedures the post-surgical period was 15 days. After recovery, independent groups of mice received the treatments described in Experiment II and were subjected to the tightrope test at the onset of ethanol hangover. Another group of ovariectomized mice (n = 20) was subdivided into two groups of 10 animals each: OVX and ovariectomized estrogenized (OVX-E_2) mice. The OVX group received treatments as described above, and the OVX-E_2 animals were chronically estrogenized for 60 days with implanted Silastic capsules containing 0.1 mg of 17 β-estradiol (E_2) (Sigma Aldrich, USA). This period of time has been proven to be adequate to restore estradiol levels [31,32]. Silastic material allows steady estradiol release (approximately 180 pg/day). Capsules were 10 mm × 2 mm and the fill procedure was a modified technique [33,34]. Capsules were placed in a 3 mm incision in the dorsal part of the neck while the animals were under equitesine anesthesia. All OVX-E_2 mice received melatonin for seven days and then an i.p. injection of EtOH or saline in the morning (9:00 a.m.), with the tightrope test being given in the early afternoon (3:00 p.m.). As a control, a separate group of female mice (n = 40) underwent the same surgical procedure but without removing the ovaries. This sham-OVX group also received Silastic capsule implants that were filled with estradiol diluents and underwent the four different treatments

described in Experiment II before the tightrope test was performed at the onset of ethanol hangover.

2.5. E_2 serum concentration

Ten mice from each group (intact, OVX, OVX-E_2 and sham-OVX) were sacrificed by decapitation to estimate serum levels of E_2 following capsule placement. Trunk blood was collected and centrifuged, and serum samples were stored at −70 °C until they were assayed. Total E_2 measurements were performed in triplicate for each sample using an enzyme-linked immunosorbent assay (ELISA) kit for 17 β-estradiol (EIAgen, Adaltis Italia S.p.A). ELISA results are shown in Table 1.

2.6. Statistical analysis

The results are represented as mean ± standard error of mean (SEM). Prior to each analysis, test variables were checked for normality with the Kolmogorov–Smirnov test to determine whether posterior parametric or nonparametric statistical analysis was appropriate. BAC levels were analyzed using an independent *t*-test. Behavioral results, E_2 serum concentration and BW differences were analyzed using factorial ANOVA and post-hoc Tukey's tests. SPSS statistical software (version 13.0) was used and differences were considered statistically significant when $p<0.05$.

3. Results

3.1. Experiment I: blood alcohol levels and onset of hangover in male and female Swiss mice

To determine the onset of ethanol hangover, BAC was measured 60, 180 and 360 min after an acute injection of ethanol (Fig. 1). As expected, the results showed that BAC dropped significantly between 60 and 180 min in both sexes: 330 ± 10.2 mg/dl *vs.* 237.67 ± 18.52 mg/dl for female ($t=4.98$, df = 2, $p<0.05$) and 318 ± 15.33 mg/dl *vs.* 246 ± 14.64 mg/dl for male mice ($t=3.70$, df = 4, $p<0.05$). A marked decrease was observed 360 min after the injection: 15.33 ± 7.42 mg/dl for female ($t=11.14$, df = 4, $p<0.001$) and 13.67 ± 6.81 mg/dl for male mice ($t=12.77$, df = 4, $p<0.001$) as compared with BAC at 180 min for both sexes. These values were similar to those reported by other laboratories [35] and indicate that 6 h after the injection initial BAC had decreased by 95%. These measurements allowed us to establish the onset of ethanol hangover (see Section 2.4.1).

3.2. Experiment II: effect of melatonin pretreatment on motor performance during ethanol-induced hangover in male and female intact mice

As explained, a lower score reflects better motor performance in the tightrope test. Results for male control animals did not differ from those for male mice that had been pretreated with melatonin (vehicle-control *vs.* melatonin-control). During ethanol-induced hangover, the average score was almost three times higher than control scores (vehicle-control *vs.* vehicle-EtOH-hangover; $F[1,15]=14.79$, $p<0.01$, Fig. 2). Pretreatment with melatonin significantly reduced scores during ethanol hangover ($F[1,17]=14.49$, $p<0.05$ compared with scores without pretreatment, Fig. 2). Furthermore, there was an interaction between melatonin pretreatment and ethanol hangover (melatonin * EtOH-induced hangover, $F[1,17]=19.9$, $p<0.001$). Similarly, scores for females pretreated with melatonin were not significantly different from those for females that had been given saline. During hangover, females obtained significantly higher scores, whether receiving melatonin or not, compared with controls ($F[1,22]=73.82$, $p<0.001$; $F[1,27]=38.91$, $p<0.001$ respectively, Fig. 3A). However, melatonin pretreatment increased scores during ethanol hangover ($F[1,21]=5.77$, $p<0.01$ compared with scores without pretreatment, Fig. 3A). Statistically, scores for females pretreated with melatonin were higher than scores for males under the same conditions ($F[1,19]=49.09$, $p<0.001$, statistical comparisons are not shown). Moreover, as in male mice, a statistical interaction between melatonin pretreatment and ethanol hangover was found in intact females (melatonin * EtOH-induced hangover, $F[1,49]=11.59$, $p<0.001$).

Table 1
Estradiol serum concentration and body weight at the end of the second month of estrogen replacement for control, sham-OVX, and OVX-E_2 mice.

	Estradiol (pg/ml)	BW (g)
Intact	35.49 ± 9.65***	32.52 ± 0.64
OVX	2.66 ± 1.98	33.07 ± 0.72
Sham-OVX	45.96 ± 10.82***	33.21 ± 0.68
OVX-E_2	44.46 ± 10.18***	33.25 ± 0.68

Values are expressed as mean ± SEM (N = 10 each group).
*** $P<0.001$ in intact, sham-OVX and OVX-E_2 *vs.* OVX group. ANOVA, Tukey's test.

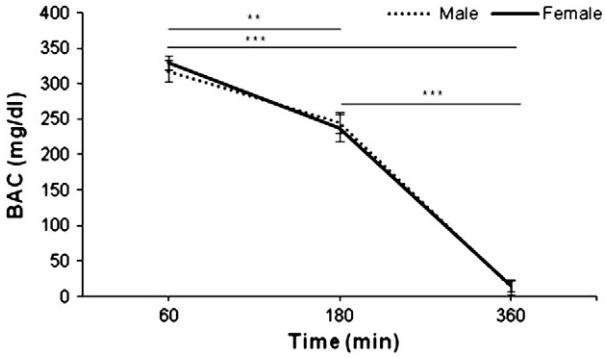

Fig. 1. Blood alcohol concentration during and after EtOH treatment. Blood alcohol concentration in male (solid line) and female (dotted line) Swiss mice was measured 60, 180 and 360 min after acute ethanol injection. Values are expressed as mean ± SEM (n = 9 each group). **p<0.01, ***p<0.001. Independent samples *t*-test.

3.3. Experiment III: effect of estrogen replacement on motor performance during ethanol-induced hangover

In view of the significant differences between male and female mice, an OVX mice group was included to clarify the role of estrogens in our experimental design. OVX females showed no differences with controls (vehicle-control *vs.* melatonin-control, Fig. 3A). OVX mice obtained higher scores in the tightrope test during hangover ($F[3,22]=12.13$,

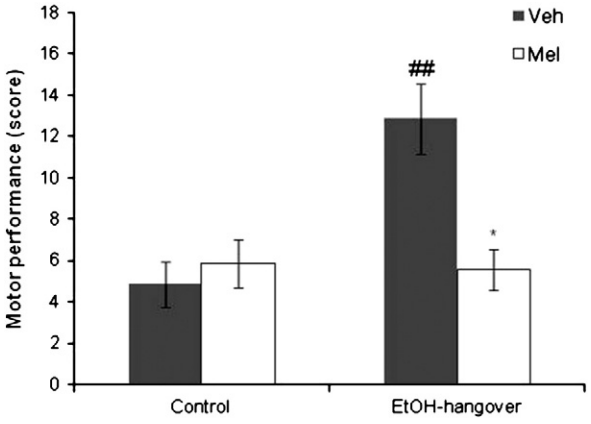

Fig. 2. Male tightrope performance during ethanol hangover. Values are expressed as mean ± SEM (n = 10 each group). *p<0.05, melatonin–EtOH hangover *vs.* vehicle–EtOH hangover; ##p<0.01, vehicle–EtOH hangover *vs.* vehicle control. ANOVA and Tukey's test. Lower scores represent better performance. Bar shading indicate pretreatment: gray, vehicle; white, melatonin.

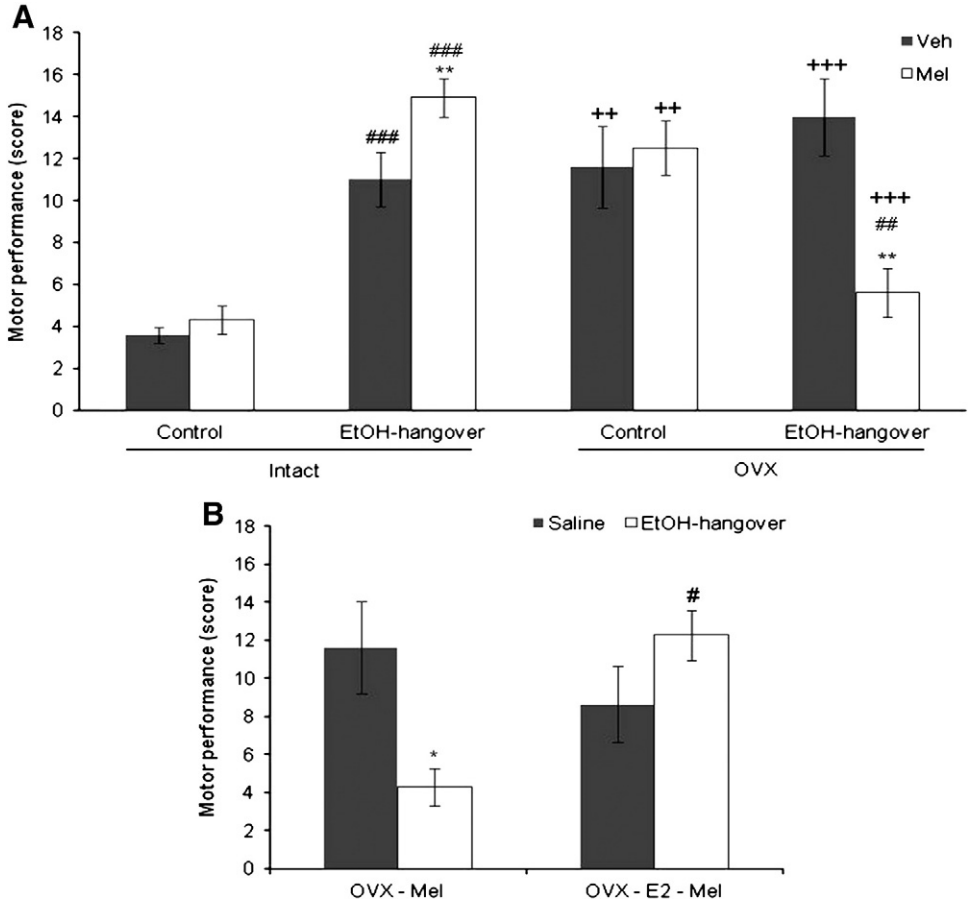

Fig. 3. Female tightrope performance during ethanol hangover. Effect of ovariectomy and estrogen replacement. (A) Comparisons between intact and OVX female mice during ethanol hangover. Values are expressed as mean ± SEM (n = 10 each group). **p<0.01, melatonin–EtOH hangover *vs.* vehicle–EtOH hangover in intact and OVX. ##p<0.01, melatonin–EtOH hangover *vs.* melatonin control in OVX; ###p<0.001, melatonin–EtOH hangover *vs.* melatonin control and vehicle–EtOH hangover *vs.* vehicle control in intact females; ++p<0.01, vehicle control and melatonin control OVX *vs.* intact; +++p<0.001, vehicle–EtOH hangover and melatonin–EtOH hangover OVX *vs.* intact. ANOVA, Tukey's test. Lower scores represent better performance. (B) Comparisons between OVX and OVX-E_2 mice during ethanol hangover. Values are expressed as mean ± SEM (n = 10 each group). *p<0.05, EtOH hangover *vs.* control in OVX-mel; #p<0.05, EtOH hangover OVX-E_2-mel *vs.* EtOH hangover OVX-mel. ANOVA, Tukey's test. Lower scores represent better performance.

p<0.001 compared with intact controls, Fig. 3A). However, melatonin pretreatment resulted in lower scores during ethanol hangover ($F[3,19] = 12.12$, p<0.001 compared with intact female mice pretreated with melatonin and suffering from hangover, Fig. 3A). These data (OVX, Mel, and EtOH hangover) were compared with those obtained for males under the same treatment conditions (Mel and EtOH hangover), but no significant differences were found (statistical comparisons are not shown). Another group of OVX mice was divided into two groups to verify the possible effect of estrogen on melatonin action. All the animals were pretreated with melatonin and half of them were chronically estrogenized (OVX-E_2) (see Section 2.4.3). In OVX-E_2 mice, there were no significant differences between the control (saline) and the ethanol hangover group; however, OVX-E_2 mice obtained significantly higher scores compared with non-estrogenized OVX mice receiving EtOH ($F[1,13] = 22.48$, p<0.05, Fig. 3B). Moreover, scores for OVX-E_2 mice were not significantly different from those of intact females that had received identical treatments (see Fig. 3A). OVX mice obtained significantly lower scores during ethanol hangover compared with controls ($F[1,13] = 7.16$, p<0.05, Fig. 3B). The sham-OVX group behaved similarly to intact females (data not shown). Serum 17-β estradiol levels were determined (Table 1) and no significant differences were found between the intact, sham-OVX and E_2 groups. Nevertheless, OVX serum estradiol levels were significantly lower compared with intact, sham OVX and OVX-E_2 (p<0.001). Estrogen replacement did not affect body weight in the second month of treatment (Table 1).

4. Discussion

The key finding to emerge from this study is that estrogen blocks the protective action afforded by melatonin on motor performance in an experimental model of ethanol hangover.

In the hangover state, hypo-activity [22], anxiety-like behavior [36], alterations in body temperature, wheel running activity and pain perception [18–20] have been described to occur. In the present study we found that animals displayed reduced motor coordination during EtOH hangover.

Melatonin has different effects in male humans. For example, in fit subjects like elite soccer players, melatonin is able to synchronize sleep–wake cycles making them perform better [37]. However, the pineal hormone does not exhibit any meaningful effect on physical performance during hangover [38]. Other studies have demonstrated that a single dose of melatonin shortens the duration of ethanol-induced sleep and decreases ethanol-induced hyperactivity in male mice; the same authors suggest that central interaction of ethanol and melatonin may be antagonist or synergistic in nature [39]. Our study shows a possible interaction of melatonin and ethanol in male mice (Fig. 2), although we could not verify whether this interaction is a synergistic or antagonist one. Furthermore, another study has demonstrated that acute melatonin exposure improves water maze performance and decreases ethanol-induced oxidative stress in male rat hippocampus when ethanol remains in the blood [5]. Chronic alcohol consumption produces

alterations in the daily melatonin synthesis profile of alcoholic rats [40]. We have now demonstrated that melatonin supplied in drinking water for a week improves motor performance during ethanol hangover in males (Fig. 2). We hypothesize that melatonin could act as a neuroprotective agent in the same way as in the central nervous system [8,41]. When female mice were studied, contrary to the results obtained in male mice, melatonin treatment not only failed to improve motor performance but made it worse during hangover (Fig. 3A). Although we do not have a conclusive explanation why melatonin could impair motor performance in intact females in the hangover state, other researchers have studied possible gender differences in response to melatonin in basal conditions [42,43]. Paterson and Vickers (1984) have demonstrated that acute melatonin exposure has different effects on male and female locomotion and social activity, and while males exhibit more social activity, females do not respond to hormone treatment [42]. Likewise, Brotto et al. (2000) have established that the effects of chronic melatonin administration on swimming, struggling and immobility in the forced swim test are sex-dependent in rats [43]. Melatonin significantly increases struggling in males but fails to do so in females, and it decreases swimming and increases open-field ambulatory behavior in both sexes [43]. Although these previous studies were conducted under basal conditions, they imply that the presence of estrogens might explain impairment of melatonin's neuroprotective effects in females. However, little is known about the possible interaction between melatonin and estrogens in hangover. Our experiments with OVX mice allowed us to test the role of estrogen in the neuroprotective effects of melatonin during hangover (Fig. 3A). We found that OVX mice showed better motor performance when melatonin had been administered. We believe that our results are in agreement with the motor coordination evidence provided by Brotto et al. (2000) [43].

In opposition to our results, a neuroprotective effect of estrogens related to ethanol has been described in a recent review by Jung and Metzger (2010). They explain that ethanol withdrawal induces hyperexcitatory neurotransmission and intense generation of reactive oxygen species, but 17 β-estradiol protects neurons and mitochondria, as well as against impairments of motor behavior and motor learning [44]. Conversely, in a model of chronic ethanol consumption, the beneficial effects of melatonin seem to be independent of estrogens. Thus, in OVX rats that receive estrogen, a one-week melatonin treatment prevents DNA damage in uterus induced by drinking ethanol 5% [45]. This apparent discrepancy can be explained if we take into account that our experimental conditions were considerably different from Bershtein et al.'s (2002) [45]. Mice in our study received an acute dose of ethanol and were studied when ethanol was no longer detected in the blood (see Section 2.4.1). We surprisingly found that ovariectomy significantly reduced motor performance in controls and performance was not enhanced by melatonin treatment (Fig. 3A). Studies have shown that estrogen has important behavioral effects. High levels of estradiol impair spatial performance in the Morris water maze and increase 'depressive-like' behaviors in the female meadow vole [46]. Administration of an acute estradiol regimen to aged female C57BL/6 mice produces specific anti-anxiety and anti-depressant effects, independent of the effects on motor behavior [47]. It would seem that estrogens exert different effects on behavior that are both age- and treatment-dependent. Moreover, our results showed that OVX mice behaved differently compared with intact or OVX-E_2 animals. These results and those provided by other referenced authors lead us to suggest that estrogens themselves would have an effect on behavior.

The relationship between melatonin and estrogens has also been researched. Mediavilla et al. (2010) have demonstrated that the oncostatic properties of melatonin on breast cancer could be attributed to its regulation of the estrogen receptor, as well as to modulation of enzymes involved in local estrogen synthesis [48]. It has also been established that melatonin destabilizes the binding of the estradiol–estrogen receptor complex to the estrogen responsive elements in MCF7 breast cancer cells [12]. The beneficial effects of co-treatment with estradiol and melatonin in the post-menopausal period have also been established [49]. In our study, motor performance of OVX mice was similar to that of males during hangover, both under basal conditions and after melatonin pretreatment (Fig. 3A). Furthermore, when a second group of OVX mice was used to test the effect of melatonin during hangover, the results that had been obtained for the first OVX group were replicated. When estrogen replacement and melatonin pretreatment were administered together with acute exposure to EtOH or saline, the same results as in intact females were obtained. This remarkable observation leads us to suggest that estrogen plays a crucial role in the protective action of melatonin on motor performance during hangover. It is interesting to highlight that plasma estrogen levels of estrogenized OVX mice were similar to those of intact female mice (Table 1), showing the effectiveness of estrogen replacement. Unexpectedly, we did not observe any body weight changes throughout treatments (Table 1). Indeed, it has been widely demonstrated that ovariectomy increases food intake and body weight [50]. There is no conclusive explanation for the discrepancy between this and our results. In summary, melatonin pretreatment, in the absence of estrogens, reverses motor performance deficits induced by EtOH hangover. Estrogen replacement allowed us to test this hypothesis. The results of the present study suggest that melatonin might be a future therapy for hangover symptoms. Nevertheless, some authors consider that treating alcohol hangover with melatonin could be expected to promote binge drinking and thus would be hazardous to public health [51].

As a whole, this study shows that melatonin improves motor coordination in male mice during hangover. In addition, our findings suggest that melatonin and estrogens would be inversely related. Additional experiments should be conducted in order to better elucidate the mechanisms by which melatonin improves motor coordination during ethanol hangover.

5. Conclusion

This study demonstrates that a week of melatonin treatment improves motor coordination in male mice at the onset of ethanol-induced hangover. However, melatonin does not enhance female motor performance. We therefore conclude that estrogen blocks the neuroprotective action of melatonin.

List of abbreviations

BAC	Blood alcohol concentration
EtOH	Ethanol
Mel	Melatonin
OVX	Ovariectomized
OVX-E_2	Ovariectomized estrogenized
Veh	vehicle

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