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Article *in* Addiction Biology · August 2018

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Cognitive and neurobehavioral benefits of an enriched environment on young adult mice after chronic ethanol consumption during adolescence

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ABSTRACT

Binge drinking (BD) is a common pattern of ethanol (EtOH) consumption by adolescents. The brain effects of the acute EtOH exposure are well-studied; however, the long-lasting cognitive and neurobehavioral consequences of BD during adolescence are only beginning to be elucidated. Environmental enrichment (EE) has long been known for its benefits on the brain and may serve as a potential supportive therapy following EtOH exposure. In this study, we hypothesized that EE may have potential benefits on the cognitive deficits associated with BD EtOH consumption. Four-week-old C57BL/6J male mice were exposed to EtOH following an intermittent 4-day drinking-in-the-dark procedure for 4 weeks. Then they were exposed to EE during EtOH withdrawal for 2 weeks followed by a behavioral battery of tests including novel object recognition, novel location, object-in-place, rotarod, beam walking balance, tail suspension, light–dark box and open field that were run during early adulthood. Young adult mice exposed to EE significantly recovered recognition, spatial and associative memory as well as motor coordination skills and balance that were significantly impaired after adolescent EtOH drinking with respect to controls. No significant permanent anxiety or depressive-like behaviors were observed. Taken together, an EE exerts positive effects on the long-term negative cognitive deficits as a result of EtOH consumption during adolescence.

Keywords alcohol, behavior, binge drinking, cognition, environmental enrichment, therapy.

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INTRODUCTION

Ethanol (EtOH) is the most commonly consumed drug in the world and has become the most widely used toxic substance during adolescence (Pautassi, Nizhnikov, & Spear 2009). Binge drinking (BD) is defined as a heavy, episodic and rapid drinking pattern and is characterized by intermittent consumption of large amounts of EtOH in short periods of time followed by a period of abstinence. This pattern of EtOH consumption causes large, rapid spikes in blood EtOH concentrations (BECs) and is particularly common amongst adolescents and young adults and may increase vulnerability to EtOH dependence in adulthood (Amodeo *et al.* 2017).

The neocortex, limbic system and cerebellum are particularly sensitive to the neurotoxic effects of EtOH during early life (Squeglia, Jacobus, & Tapert 2009). Moreover, BD effects are correlated with cognitive damage in young people as abusive EtOH consumption can have deleterious effects on the adolescent brain (Lacaille *et al.* 2015). It causes a significant loss of hippocampal neurons, astrocytes and microglia (Oliveira *et al.* 2015), hippocampal

shrinkage (De Bellis *et al.* 2000) and mitochondrial dysfunction that leads to brain inflammation, synaptic dysfunction and memory loss (Crews *et al.* 2000). Furthermore, EtOH damages the entorhinal cortex (Crews *et al.* 2000) and causes mood alteration (Karanikas, Lu, & Richardson 2013) in adolescent brains. Given the incidence of BD in adolescents and young adults and the damaging effects of EtOH on the central nervous system, it is critical to understand both the long-term consequences of this exposure and methods by which this damage can be overcome by therapeutic interventions.

Numerous studies have reported the significant beneficial effect of environmental enrichment (EE) on several conditions, such as schizophrenia (Pang & Hannan 2013), brain stroke (Chen *et al.* 2017), neurodegenerative (Pang & Hannan 2013) or genetic diseases (Kondo *et al.* 2008), as well as a neuroprotective strategy in age-related cognitive decline and chronic stress (Pang & Hannan 2013). The EE paradigm consists of complex housing conditions characterized by the combination of social interaction, physical exercise and inanimate novel objects performed in a large two-floor cage, equipped with running wheels, toys, ladders and tunnels that promote sensory, cognitive and motor abilities having a deep impact on both brain structure and function (Van Praag, Kempermann, & Gage 2000; Yau *et al.* 2014). EE strengthens plastic changes in the brain and ameliorates spatial memory and hippocampal synaptic plasticity after prenatal ethanol exposure (Hanningan, Berman, & Zajac 1993). The benefits of EE associate with an increase in brain weight and size, cortical thickness, neurotrophic factors, dendritic arborization, synaptogenesis and adult hippocampal neurogenesis (Mora, Segovia, & del Arco 2007; Yau *et al.* 2014). Besides, an increase observed in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and N-methyl-D-aspartic acid receptors of enriched rats is directly related to an improvement observed in cognitive processes (Simpson & Kelly 2011). All these benefits that EE reports have become this paradigm one of the most applied strategies in a wide range of neuroscience research areas (Simpson & Kelly 2011).

The long-lasting effects that binge EtOH consumption during adolescence has on both cognition and neurobehavior at adulthood have been extensively studied (Pascual *et al.* 2007; Rodríguez-Arias *et al.* 2011); however, the potential cognitive and neurobehavioral effects of EE applied in early adulthood after chronic EtOH intake during adolescence remain to be explored. The results of the present investigation on this issue demonstrate that the cognitive impairment, motor incoordination and imbalance observed in young adult mice after adolescent exposure to EtOH can be reversed by EE.

MATERIALS AND METHODS

Experimental animals

A total of 308 C57BL/6J male mice (Janvier Labs) were used (n = 11 for each experimental group and each behavioral test). All procedures were approved by the Committee of Ethics for Animal Welfare at the University of the Basque Country (CEEA/M20/2016/073; CEIAB/2016/074) and were in accordance to the European Communities Council Directive of September 22, 2010 (2010/63/EU) and Spanish regulations (Real Decreto 53/2013). Experiments were carried out in a temperature-controlled room (22°C), and cages were kept on a 12-hour on : 12-hour off lighting schedule (red light on at 9:00 hours). *Ad libitum* food and water were available for the animals, except during EtOH access as noted later.

Drinking in the dark procedure and timeline

Series of 44 adolescent male mice [postnatal day (PND) 32] were randomly assigned in pairs of control (n = 22) and EtOH (n = 22). The EtOH groups were subjected to a drinking in the dark (DID) procedure, a model in which BD was simulated for each behavioral task as described previously (Bonilla-del Río *et al.* 2017). They were exposed to 4-day DID over 4 weeks (from PND 32 to PND 56) (Rhodes *et al.* 2007). Each week on days 1, 2, 3 and 4, the animals were weighed at 8:00 hours (1 hour before the light turned red) and were returned with their partners housed in a Plexiglas cage (17 cm × 14.3 cm × 36.3 cm). At 12:00 hours, 3 hours into red lights being turned on, each animal was individually housed and exposed to either a single bottle of 10-ml tap water (control group) or a single bottle of 10-ml EtOH [20 percent EtOH (v/v) prepared from 96 percent EtOH (Alcoholes Aroca S.L., Madrid, Spain)] (EtOH group). During the first 3 days of DID treatment, the animals had access to water or EtOH for 2 hours. On day 4, they were exposed to 4 hours. In the last 3 days of the week, animals were kept resting in their respective Plexiglas cages with food and water *ad libitum*. EtOH intake during each day [grams of EtOH per kilogram of animal per 2 or 4 hours (g/kg/2 or 4 hours)] (Fig. 1a) and total EtOH intake during DID procedure [grams of EtOH per kilogram of animal per hour (g/kg/hour)] (Fig. 1b) were measured, and blood samples were collected from the tail veins 30 minutes after the last 4-hour session to determine BEC using a commercial EtOH assay kit (Sigma-Aldrich) (Fig. 1c). A direct correlation between BEC (mg/dl) and EtOH intake (g/kg/hour) was detected (not shown) that agrees with previous reports (Holgate *et al.* 2017).

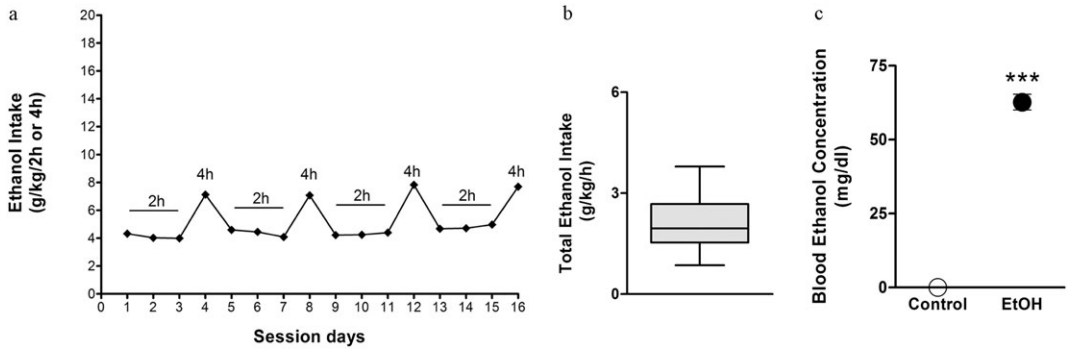

Figure 1 Voluntary oral EtOH consumption and blood EtOH concentration. (a) Daily EtOH intake during drinking in the dark [grams of EtOH per kilogram per 2 or 4 hours (g/kg/2 or 4 hours)], (b) total EtOH intake of C57BL/6J adolescent mice from postnatal day 32 to postnatal day 56. Data are expressed as grams of EtOH or tap water per kilogram of animal per hour (g/kg/hour) and (c) blood EtOH concentration of C57BL6 adolescent mice on the last day, 30 minutes after 4-hour drinking-in-the-dark session treatment. Blood samples were collected from lateral tail veins; data are expressed as milligrams per deciliter (mg/dl). All data are mean ± SEM. *Significance between control and EtOH group (***$P < 0.001$)

Environmental enrichment condition

During withdrawal (PNDs 57–73), 11 EtOH-treated and 11 controls mice were randomly placed into environmental enriched conditions (EE-H_20, EE-OH) within a large two-level cage (50 cm long × 28 cm high × 54 cm wide). The cages for each group were equipped with free access running wheels, toys (objects with different colors, sizes and shape), ladders, tunnels, multiple plastic tubes and a plastic house. In order to promote novel stimuli and increase the animals' exploratory capacity, the EE was provided with new toys twice a week.

Behavioral studies

Given the vulnerability of limbic structures to EtOH, we examined various forms of learning and memory as well as depressive-like and anxious behaviors. The novel object recognition test (NORT) (Redrobe, Bull, & Plath 2010), the novel location test (NOLT) (Moreno-Castilla *et al.* 2017) and the object-in-place test (OPT) (Brown *et al.* 2012) were performed in the last 3 days of the withdrawal period to assess recognition memory, spatial recognition memory and associative recognition memory, respectively (Fig. 2). Mice were placed into a

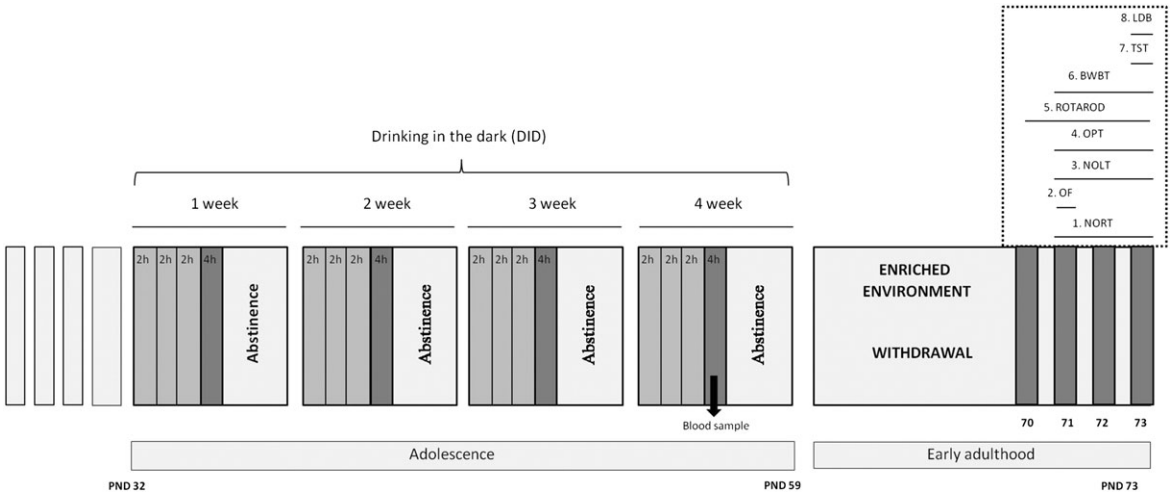

Figure 2 Schematic representation of the experimental timeline. Male adolescent C57BL/6J mice were exposed to a 4-week DID procedure [from PND 32 to PND 56]. Each week, animals were exposed to free ethanol or tap water access for 2-hour sessions over three consecutive days, and a 4-hour session on day 4. In the remaining 3 days of the week, animals were kept resting in their respective cages. Then, animals were submitted to 2-week withdrawal period (from PND 57 to PND 70) and housed under standard laboratory or environmental enrichment conditions. In the last 4 days of the withdrawal period, recognition, spatial and associative memory tasks as well as motor coordination, balance, depressive behavior and anxiety tests were performed. BWBT, beam walking balance test; LDB, light–dark box; NOLT, novel location test; NORT, novel object recognition test; OF, open field; OPT, object-in-place test; TST, tail suspension test

temperature-controlled (22°C) behavioral room and kept under red light 2 hours before each behavioral test. The procedures were carried out with some modifications (Fig. 3). The rotarod test (Aristieta *et al.* 2014) and beam walking balance test (BWBT) (Kossatz, Maldonado, & Robledo 2016) were used to explore motor coordination and balance, respectively. Finally, the tail suspension test (TST) for detecting depression-related behaviors (Imperatore *et al.* 2015) and the light–dark box (Imperatore *et al.* 2015) and open field (OF) tests for anxiety (Byrns *et al.* 2014) were applied.

Novel object recognition test

Recognition memory was assessed in a square-shaped open box (dimensions: 40-cm length × 45-cm height × 40-cm width) under red 10 lux lighting conditions. On the first 2 days of the behavioral test (PNDs 71–72), animals were habituated to the apparatus for 10 minutes each day. On the third day (PND 73), an acquisition session was carried out where two identical familiar objects were placed at an equal distance in two adjacent corners of the arena and a mouse placed in the middle of the square was allowed to explore them for 10 minutes. Mice that did not explore either object for more than 20 seconds were excluded from the study. Following a single acquisition session, the mice were returned to their home cages for 1 hour, at which time the test session was performed. For testing (10 minutes per animal), one of the two familiar objects from acquisition was replaced by a novel object (Fig. 2a). Total exploratory time was measured, and the discrimination index (DI) [(time spent exploring the novel location − time spent

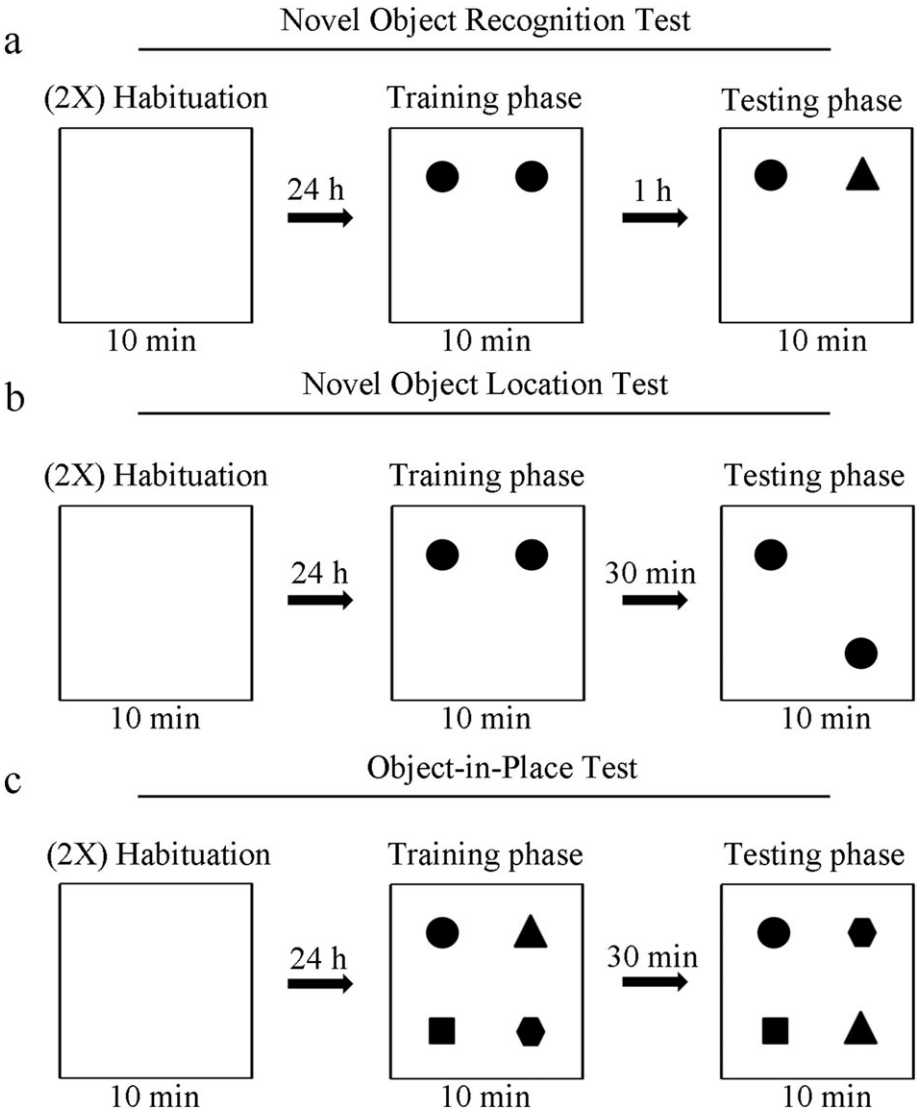

Figure 3 Schematic diagram illustrating the three memory tests assessed. (a) Novel object recognition test, (b) novel object location test and (c) object-in-place test

exploring familiar location/total time exploring objects)] during the test session was calculated.

Novel object location test

This task probes spatial recognition memory using the same apparatus and conditions used for NORT but with additional visual cues fixed on the walls constantly visible from the arena to help the spatial orientation of the mouse. On the first 2 days of the behavioral test (PNDs 71–72), animals were habituated to the apparatus for 10 minutes per day. On the third day (PND 73) during acquisition, each mouse was allowed to explore two identical parallel objects for 10 minutes. Mice that did not explore the objects for more than 20 seconds were excluded. Following acquisition, the mice were returned to their home cages for 30 minutes. Subsequently, they were subjected to the testing session in which one of the two identical objects was set to a new location while the other object remained in the same position. The mice were allowed for 10 minutes to freely investigate and explore the apparatus with one of the objects in a novel location (Fig. 2b). Total exploratory time was measured, and DI was calculated as in the NORT.

Object-in-place test

Associative recognition memory was analyzed in the same apparatus and conditions that were used for NORT and NOLT. The habituation was carried out during the first 2 days of the behavioral test (PNDs 71–72), and each animal was able to explore the empty apparatus for 10 minutes. In the acquisition phase next day (PND 73), each mouse was placed in the center of the arena with one different object in each corner and was allowed to explore them for 10 minutes. Mice with less than 20 seconds of total object exploration time were excluded. In the test phase 30 minutes later, two of the objects were exchanged, while the other two remained in the same location. The mouse was allowed to explore this new environment for 10 minutes (Fig. 2c). Total exploratory time was measured, and DI during test session was calculated.

Open field

The OF test was used in order to assess anxiety-like behavior. Animals were individually taken from the home cage and placed in the same square-shaped open area as for NORT. It was subdivided into a 28-cm-inner and 6-cm-outer perimeter zone. During the first 5 minutes of NORT habituation, each animal was allowed to freely explore the square, and then, it was returned to the home cage. The time spent exploring the outer perimeter zone (6 × 6 cm from the wall) was measured.

Rotarod

The rotarod test serves to evaluate motor coordination. The mice were trained over 3 days before the test phase. In the first 2 days (PNDs 70–71), they were placed on the rotating rod facing away from the direction of rotation and the experimenter. The mice walked at a constant rotation speed (4 rpm at PND 70; 20 rpm at PND 71) for 5 minutes at 30-minute intervals three times per day. They were placed back on the rod each time they fell off until the 5-minute session was completed. On the third day (PND 72), the rotation speed increased steadily from 4 to 40 rpm over 5 minutes. The mice underwent three training sessions with increasing speed separated by 30-minute rest in the home cage. On the fourth day (PND 73), the final test was performed under the third day's accelerating protocol. The latency of each mouse to fall off from the rotarod and falling speed were measured.

Beam walking balance test

This task is useful for detecting subtle deficits in balance. Two circular wood beams of different diameters were used (wide, 2-cm diameter; narrow, 1-cm diameter). On the first day of training phase (PND 71), mice were trained to traverse the wide (2 cm) beam three consecutive times. The mouse was set to restart if a fall in the course of training occurred. On the second training day (PND 72), the mouse was to cross the wide beam for three consecutive times and then the narrow (1 cm) beam for another three consecutive times. This same protocol was applied during the testing session next day (PND 73). Then, the time taken to cross the wide and narrow beams and the number of foot slips were assessed.

Tail suspension test

The TST analyzes depressive-like behavior and learned helplessness in rodents. The mouse was faced downwards and suspended by the tail 60 cm above a padded floor using tape wrapped around the tail (1 cm from the tip) for 6 minutes. The immobility time during the last 4 minutes was measured. Immobility was considered when the animal was in full stillness.

Light–dark box

The light–dark box test evaluates unconditioned anxious behavior, taking advantage for rodents' avoidance of open, brightly lit environments. Each mouse was placed in the dark box for 10 seconds to acclimate. Then the animal was allowed to explore the dark and the light box through an open gate for 10 minutes. The percentage of time that the mouse spent in the light box was measured.

Statistical analysis

SPSS statistical software version 22.0 (IBM, Spain) program was used to perform all statistical analysis. First, data were examined for normal distribution and homogeneity of variances using the Kolmogorov–Smirnov test and Levene's test, respectively. Two-way analysis of variance (ANOVA) was carried out to evaluate the long-term DID effect, EE condition and the interaction between DID and EE in each behavioral test. Then, to explore further the effect of DID in different experimental conditions, a one-way ANOVA with *post hoc* analysis (Bonferroni correction for equal variances or Tamhane's T2 correction for unequal variances) was used. BEC differences between control and EtOH groups were analyzed with Student's *t*-test. $P < 0.05$ was considered statistically significant. Data were shown as mean ± SEM.

RESULTS

Environmental enrichment in early adulthood recovers cognitive impairment after ethanol exposure during adolescence

One-way ANOVA showed statistical differences between standard rearing control and DID EtOH groups. In particular, a significant decrease in the DI was observed in NORT (control: 0.44 ± 0.05; EtOH: 0.034 ± 0.03) ($P < 0.001$; Fig. 4b), NOLT (control: 0.18 ± 0.03; EtOH: −0.01 ± 0.03) ($P < 0.05$; Fig. 4d) and OPT (control:

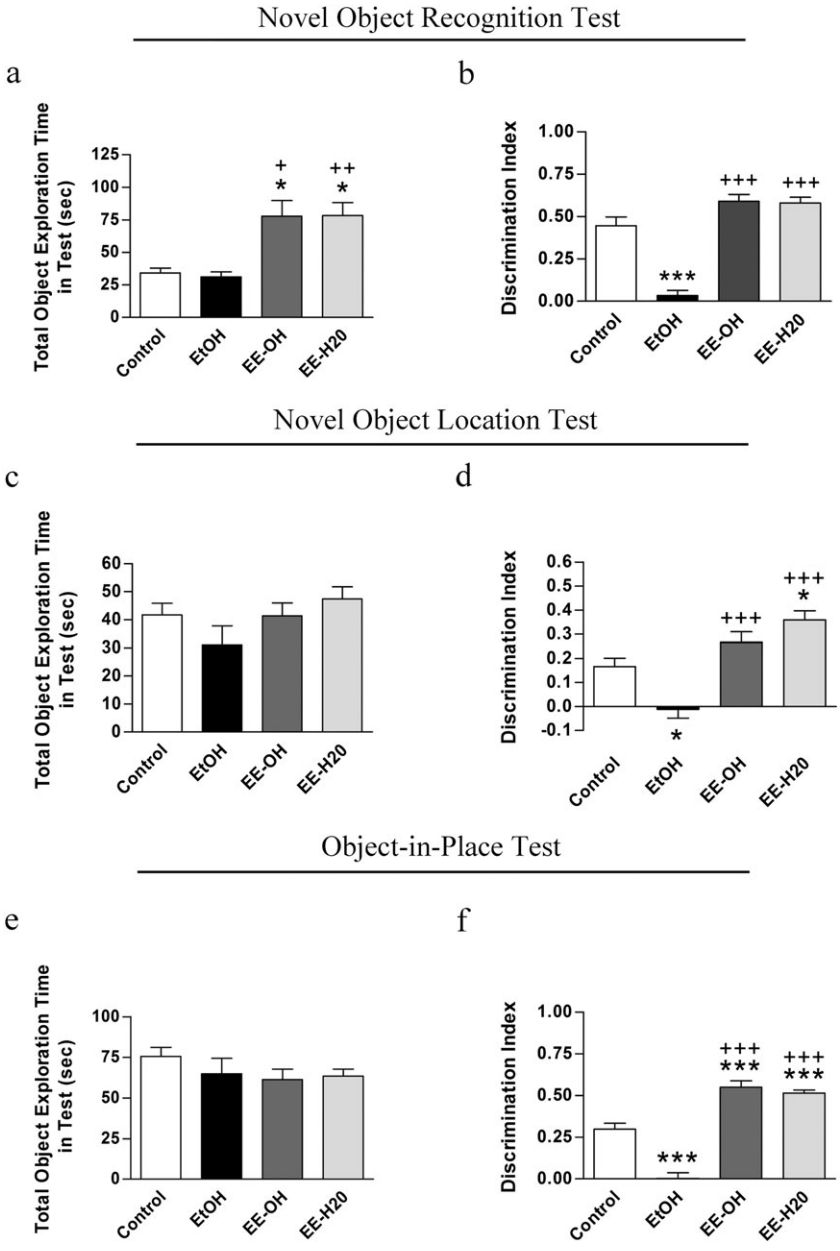

Figure 4 Environmental enrichment (EE) in early adulthood (postnatal days 57 to 70) recovers cognitive impairment after drinking in the dark during adolescence. (a) Total object exploration time (seconds) and (b) discrimination index of control, EtOH, EE-OH and EE-H_20 experimental groups ($n = 11$ for each group) during novel object recognition test phase. (c) Total object exploration time (seconds) and (d) discrimination index of control, EtOH, EE-OH and EE-H_20 experimental groups ($n = 11$ for each group) during novel location test phase and (e) total object exploration time (seconds) and (f) discrimination index of control, EtOH, EE-OH and EE-H_20 experimental groups ($n = 11$ for each group) during object-in-place test phase. All data are expressed as mean ± SEM. *Significance between control and EtOH, EE-OH and EE-H_20 groups ($^{*}P < 0.05$; $^{***}P < 0.001$). $^{+}$Significance between EtOH and EE-OH and EE-H_20 groups ($^{+}P < 0.05$; $^{++}P < 0.01$; $^{+++}P < 0.001$)

0.29 ± 0.03; EtOH: 0.0029 ± 0.03) ($P < 0.001$; Fig. 4f) of EtOH-treated compared with young adult control mice. However, their total exploration time between the familiar and the new object was similar in the three tests (control: 34.09 ± 3.77 seconds; EtOH: 31.14 ± 3.86 seconds in NORT) ($P > 0.05$); (control: 41.73 ± 4.16 seconds; EtOH: 31.10 ± 6.77 seconds in NOLT) ($P > 0.05$); and (control: 75.71 ± 5.50 seconds; EtOH: 64.90 ± 9.45 seconds in OPT) ($P > 0.05$) (Fig. 4a,c,e). These results suggest that chronic binge EtOH consumption during adolescence leads to long-term cognitive impairments. Interestingly, the EE applied in young adult mice (EE-OH group) increased the DI in NORT (EE-OH: 0.58 ± 0.04) ($P < 0.001$; Fig. 4b), NOLT (EE-OH: 0.26 ± 0.04) ($P < 0.001$; Fig. 4d) and, particularly, OPT (EE-OH: 0.54 ± 0.03) ($P < 0,001$; Fig. 4f) with respect to the EtOH group without EE. EE led also to an improvement in NOLT (EE-$H_2$0: 0.35 ± 0.03) ($P < 0.05$; Fig. 4d) and OPT (EE-$H_2$0: 0.51 ± 0.018) ($P < 0.001$; Fig. 4f) DI parameter in controls. Furthermore, an increase in NOLT DI and in OPT DI was observed compared with controls, indicating that EE *per se* has a potential cognitive benefit. Altogether, the EE applied in early adulthood recovers the persistent memory impairment observed after BD during adolescence.

No significant interaction between EtOH and EE was observed in total exploration time in NORT [$F_{(1.47)} = 0.89$, $P > 0.05$], NOLT [$F_{(1.39)} = 0.205$, $P > 0.05$] and OPT [$F_{(1.41)} = 0.446$, $P > 0.05$]. However, two-way ANOVA revealed a statistically significant effect in the NORT DI of DID [$F_{(1.47)} = 24.55$, $P < 0.001$], EE [$F_{(1.47)} = 72.42$, $P < 0.001$] and the interaction between them [$F_{(1.47)} = 27.15$, $P < 0.001$] (Fig. 4b). Likewise, a significant effect of the DID [$F_{(1.41)} = 15.45$, $P < 0.001$], EE [$F_{(1.41)} = 131.75$, $P < 0.001$] and their interaction [$F_{(1.41)} = 24.96$, $P < 0.001$] was observed in the OPT DI (Fig. 4f). Furthermore, two-way ANOVA revealed a significant effect of DID [$F_{(1.38)} = 13.56$, $P < 0.001$] and EE [$F_{(1.38)} = 32.32$, $P < 0.001$], but no significant effects were found in the interaction between EtOH intake and EE [$F_{(1.38)} = 1.76$, $P > 0.05$] in NOLT DI (Fig. 4d).

Environmental enrichment in early adulthood reverses persistent motor incoordination after chronic ethanol consumption during adolescence

In the rotarod test, DID EtOH mice relative to the control group exhibited a significant lower latency to fall off (EtOH: 80.60 ± 11.79 seconds; control: 134.6 ± 12.13 seconds) ($P < 0.05$; Fig. 5a) and a lower rotating speed at falling (EtOH: 13.45 ± 1.44 rpm; control: 20.15 ± 1.49 rpm) ($P < 0.05$) (Fig. 5b). These results suggest a direct negative effect of adolescent BD on young adult motor coordination. However, the parameters were improved when young adult animals were exposed to EE after adolescent chronic EtOH exposure (EE-OH: 139.00 ± 14.52 seconds; EE-$H_2$0: 154.00 ± 13.17 seconds) (EE-OH: 20.95 ± 1.46 rpm; EE-$H_2$0: 22.50 ± 1.58 rpm) ($P < 0.05$; $P < 0.01$; Fig. 5a,b).

Two-way ANOVA analysis revealed a significant effect of DID [$F_{(1.38)} = 8.86$, $P < 0.01$] and EE [$F_{(1.38)} = 6.95$, $P < 0.05$] in the latency to fall. However, no significant differences were found between DID and EE interaction [$F_{(1.38)} = 2.21$, $P > 0.05$] (Fig. 5a). Also, although significant differences were detected for DID [$F_{(1.38)} = 10.74$, $P < 0.05$] and EE [$F_{(1.38)} = 7.52$, $P < 0.01$], the interaction in the velocity at falling was not significant between them [$F_{(1.38)} = 2.94$, $P > 0.05$] (Fig. 5b).

Environmental enrichment in early adulthood ameliorates the long-term balance disturbance elicited by binge drinking during adolescence

Young adult DID EtOH mice needed more time to cross the narrow beam (EtOH: 46.25 ± 4.92 seconds; one-way ANOVA) ($P < 0.05$; Fig. 5c) and exhibited a higher number of slips (EtOH: 8.60 ± 0.62 slips) ($P < 0.001$) than controls (22.77 ± 4.93 slips; 5.91 ± 0.32 slips; Fig. 5d). Unlike the EtOH group, the EE-OH and the EE-H_2O mice needed significantly less time to cross the beam (EE-OH: 11.30 ± 1.15 seconds; EE-$H_2$0: 11.72 ± 1.20 seconds) ($P < 0.001$; Fig. 5c) and performed with a lower number of slips (EE-OH: 2.95 ± 0.25 slips; EE-$H_2$0: 2.62 ± 0.24 slips) ($P < 0.001$; Fig. 5d).

Two-way ANOVA analysis revealed a significant effect of DID [$F_{(1.40)} = 12.27$, $P < 0.001$], EE [$F_{(1.38)} = 48.82$, $P < 0.001$] and the interaction between them [$F_{(1.38)} = 13.17$, $P < 0.001$] in the latency to cross the narrow beam (Fig. 5c). Alike results were observed when the number of slips was analyzed. Finally, a significant effect of DID [$F_{(1.36)} = 20.42$, $P < 0.001$], EE [$F_{(1.36)} = 177.44$, $P < 0.001$] and the interaction between them [$F_{(1.36)} = 12.43$, $P < 0.001$] was found in the number of slips in BWBT (Fig. 5d).

Environmental enrichment in early adulthood promotes anxiolysis

No significant changes in long-term depressive-like behaviors were observed after DID EtOH exposure during adolescence (EtOH: 119.7 ± 11.73 seconds; control: 95.18 ± 12.90 seconds) ($P > 0.05$; Fig. 6a). Similarly, EE-OH and EE-$H_2$0 showed similar immobility times (EE-OH: 112.0 ± 7.11 seconds; EE-$H_2$0: 101.8 ± 7.75 seconds) to control ($P > 0.05$; $P > 0.05$; Fig. 6a) and EtOH

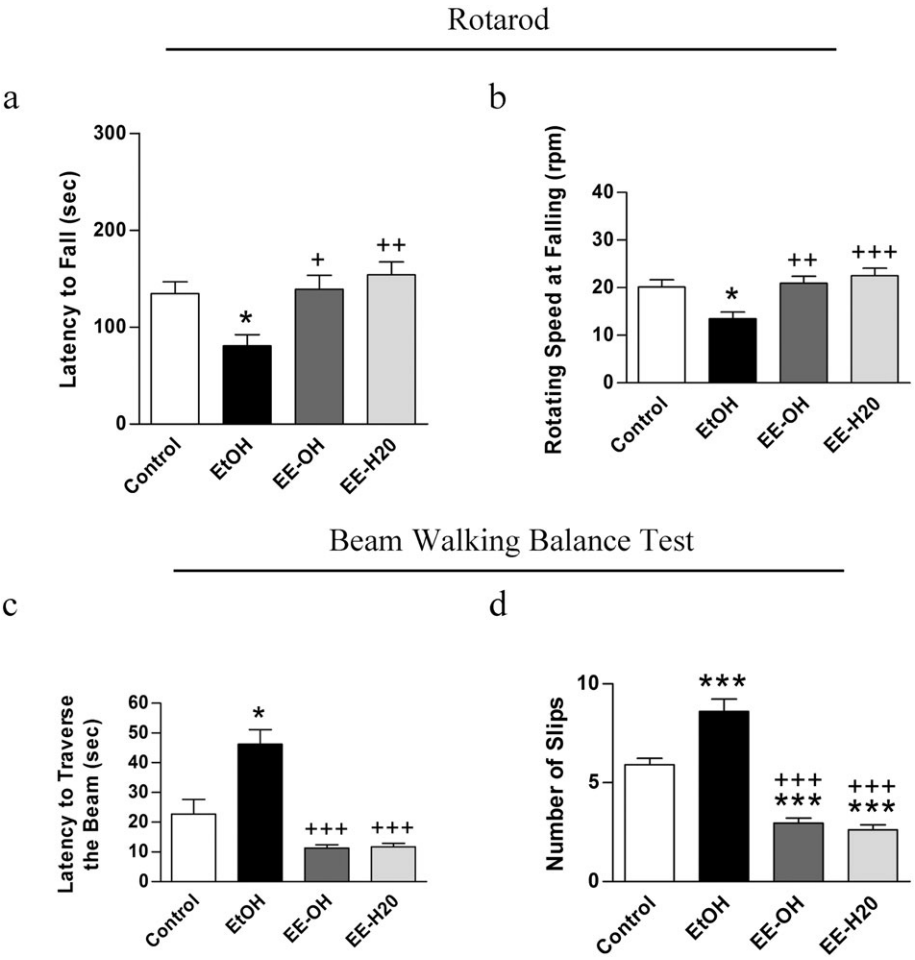

Figure 5 Environmental enrichment (EE) in early adulthood (postnatal days 57 to 70) recovers motor incoordination and balance disturbance after drinking in the dark during adolescence. (a) Latency to fall (seconds) and (b) rotating speed at falling (rpm) in control, EtOH, EE-OH and EE-H_20 experimental groups (n = 11 for each group) during rotarod test phase. (c) Latency to traverse the beam (seconds) and (d) number of slips in control, EtOH, EE-OH and EE-H_20 experimental groups (n = 11 for each group) during beam walking balance test phase. All data are expressed as mean ± SEM. *Significance between control and EtOH, EE-OH and EE-H_20 groups ($^{*}P < 0.05$; $^{***}P < 0.001$). $^{+}$Significance between EtOH and EE-OH and EE-H_20 groups ($^{+}P < 0.05$; $^{++}P < 0.01$; $^{+++}P < 0.001$)

($P > 0.05$; $P > 0.05$; Fig. 6a), respectively. Furthermore, two-way ANOVA revealed no significant changes in the effect of DID [$F_{(1.38)}$ = 2.76, $P > 0.05$], EE [$F_{(1.38)}$ = 0.02, $P > 0.05$] and their interaction [$F_{(1.38)}$ = 0.47, $P > 0.05$] in the immobility time in the TST (Fig. 6a).

Anxiety-like behavior was measured by the light–dark box and OF tests. One-way ANOVA showed no statistical differences between EtOH and control in both light–dark box (EtOH: 165.5 ± 22.59 seconds; control: 222.9 ± 19.56 seconds) ($P > 0.05$; Fig. 6b) and OF (EtOH: 198.2 ± 11.53 seconds; control: 194.8 ± 6.46 seconds) ($P > 0.05$; Fig. 6c). However, EE in the EtOH and H_20 groups caused an anxiolytic effect in the OF, as the mice spent less time near the walls compared with EtOH (EE-OH: 119.4 ± 4.83 seconds; EE-H_20: 126.6 ± 9.40 seconds) ($P < 0.001$; $P < 0.001$; Fig. 6c) and control ($P < 0.001$; $P < 0.001$; Fig. 6c), respectively.

Although no differences were found in the effect of DID [$F_{(1.44)}$ = 0.49, $P > 0.05$] and in the interaction between EtOH and EE [$F_{(1.44)}$ = 0.392, $P > 0.05$], the two-way ANOVA showed significant differences in the effect of EE in the time spent on the wall in OF [$F_{(1.44)}$ = 75.36, $P < 0.001$] (Fig. 6c). This finding corroborates the idea that EE has a natural anxiolytic effect.

DISCUSSION

In the present study, we delved into the effects of EE on the cognitive and behavioral impairments in young adult mice caused by chronic EtOH consumption during adolescence. The brain undergoes important structural and functional changes along the adolescent period that makes it more vulnerable to the deleterious effects EtOH (Bonilla-del Río *et al.* 2017) that can persist long after EtOH consumption is terminated. Our findings showed

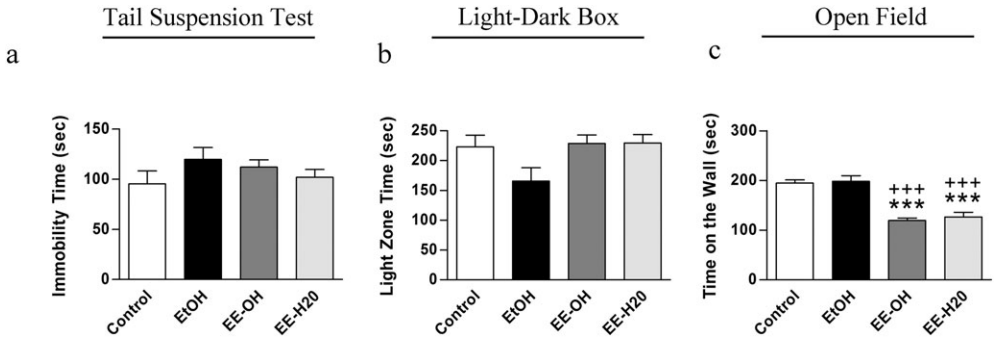

Figure 6 No persistent depressive or anxiety-like behaviors are in young adult mice after drinking in the dark during adolescence. However, environmental enrichment (EE) in adulthood promotes anxiolysis. (a) Immobility time (seconds) of control, EtOH, EE-OH and EE-H_20 experimental groups (n = 11 for each group) during tail suspension test phase. (b) Time in light zone (seconds) of control, EtOH, EE-OH and EE-H_20 experimental groups (n = 11 for each group) during light–dark box test phase and (c) time spent in the wall (seconds) of control, EtOH, EE-OH and EE-H_20 experimental groups (n = 11 for each group) during open field test phase. All data are expressed as mean ± SEM. *Significance between control and EtOH, EE-OH and EE-H_20 groups ($^{***}P < 0.001$). $^{+}$Significance between EtOH and EE-OH and EE-H_20 groups ($^{+++}P < 0.001$)

that the perdurable cognitive and behavioral changes elicited by adolescent EtOH BD can be overcome by an EE in early adulthood.

Long-lasting effects of binge drinking on young adult C57BL/6J mice

The recognition, spatial and associative memory impairment detected in early adulthood by chronic EtOH intake during adolescence is consistent with recent findings showing cognitive and behavioral deficits (Sanchez-Marin *et al.* 2017) and previous observations demonstrating that adolescent BD causes a decrease in hippocampal neurogenesis that persists into adulthood, altering brain plasticity and perturbing cognitive function (Pascual *et al.* 2007; Rodríguez-Arias *et al.* 2011; Vetreno & Crews 2015). Newborn neuronal generation is directly related to hippocampal-dependent cognitive processes (Shors *et al.* 2001) and is highly sensitive to dysregulation by EtOH (Crews *et al.* 2006; Patten *et al.* 2016). In fact, adolescent rats subjected to alcohol intermittent exposure exhibit a reduction in dentate neurogenesis lasting into adulthood (Vetreno & Crews 2015). Also, white matter volume deficits, selective gray matter damage and changes in neuroprogenitor proliferation (by Ki-67 immunopositivity) and caspase-3 expression in the dentate gyrus have been shown to be involved in this cognitive impairment (Crews *et al.* 2016). Together, these alterations can culminate in a reduction in hippocampal volume (De Bellis *et al.* 2000) and BD-induced reduction in brain-derived neurotrophic factor in the adult hippocampus (Sakharkar *et al.* 2016). In line with this, recent studies have demonstrated the importance of the immune system in the neuropathological consequences of adolescent EtOH. BD activates the inflammatory TLR4/NFκB signaling response in glial cells, which leads to the release of cytokines/chemokines and free radicals that correlates with neurophysiological, cognitive and behavioral dysfunction (Pascual, Montesinos, & Guerri 2018). Like the hippocampus, a loss of prefrontal gray matter is correlated with motor, emotional and memory impairments in human alcoholics (West, Maynard, & Leasure 2018). Importantly, prefrontal development persists into adolescence and may be particularly vulnerable to EtOH-induced damage. We have observed that chronic EtOH exposure in adolescence leads to long-term impairment of motor coordination and balance as shown in the rotarod and the BWBT. These results are consistent with previous reports (Forbes *et al.* 2013) showing that early EtOH consumption alters cerebellar function, indicating Purkinje cell vulnerability to EtOH (Jaatinen & Rintala 2008) that leads to loss of these cells (Forbes *et al.* 2013), cerebellar atrophy (Jaatinen & Rintala 2008) and motor deficits (Forbes *et al.* 2013).

Significant differences in long-term anxiety and depressive-like behaviors between control and EtOH groups were not found in this study which could be due to the use of male mice in the experimental sampling. Evidences from human and animal studies suggest that the female brain is more negatively affected by EtOH than the male brain (West *et al.* 2018). Besides, female gender is at greater risk of EtOH-induced brain injury (Prendergast 2004) and exhibited higher rates of anxiety and depression than male gender (Harris *et al.* 2017). Furthermore, longer EtOH withdrawal, like in our study, could lead to adaptations that may reduce the long-term anxiety and depressive-like behaviors, because other investigations reported that EtOH-exposed mice have abnormal plasticity in amygdala and prefrontal cortex (Stephens & Duka 2008; Kroener *et al.* 2012) as well as anxiety at shorter withdrawal periods (Sanchez-Marin *et al.* 2017).

Effects of environmental enrichment in early adulthood

Environmental enrichment promotes multisensory, social, physical and cognitive stimulation that impacts on brain structure and function (Van Praag *et al.* 2000). Our results have shown that EE leads to a significant recovery of recognition, spatial and associative memories. Moreover, a significant interaction between EtOH and EE was detected in NORT and OPT. Interestingly, the cognitive improvement observed in EE-OH group was similar to EE-H_20, suggesting that the positive effect elicited by EE would reach a ceiling in each behavioral test. The recovery observed agrees with the beneficial outcome of EE on cognitive performance that might be linked to a reduction in hippocampal apoptotic cell death, an increase in cortical weight and thickness and hippocampal volume, as well as an increase in neurotransmitters and neurotrophic factors (Nithianantharajah & Hannan 2006; Simpson & Kelly 2011). EE promotes cortical dendritic branching, hippocampal synaptogenesis and neurogenesis (Nithianantharajah & Hannan 2006) that are directly related to cognitive improvement (Triviño-Paredes *et al.* 2016), in particular, memory processes (Sampedro-Piquero *et al.* 2013). Actually, EE during adolescence restores adult neurogenesis in the dentate gyrus and mitigates the effects of alcohol in neonatal rats (Hamilton *et al.* 2014). In agreement, our data show that EE improves certain memories in young adult mice after EtOH intake during adolescence.

Environmental enrichment also stimulates angiogenesis (Chen *et al.* 2017) providing the specific neurovascular substrates for neuronal remodeling (Arai *et al.* 2009). The EE paradigm in this study provided access to toys and other forms of enrichment and also incorporated access to a running wheel. This physical exercise might also be promoting adult hippocampal neurogenesis and cognitive improvement (Yau *et al.* 2014) that could ultimately be related to the beneficial memory effects of EE observed in this study.

Environmental enrichment also recovers young adult motor incoordination and balance disturbance produced after abusive adolescent EtOH consumption. These findings coincide with previous reports showing an improvement and recovery of motor dysfunction after housing animal models of Parkinson's disease and Rett syndrome in EE conditions (Kondo *et al.* 2008) that stimulate brain-derived neurotrophic factor production (Simpson & Kelly 2011) promoting neuroplasticity, motor learning and stroke recovery (Chen *et al.* 2017). Also, EE housing mitigates brain injury leading to a rapid habituation of locomotor activity and an improvement of motor skills indicating that a favorable environment facilitates synapse maturation (Kim *et al.* 2008). Furthermore, we observed a significant decrease in anxiety in both EE-OH and EE-H_20 which is consistent with a decrease in fearfulness, faster habituation of EE-housed animals to the new environment, reduced thigmotaxis activity and increased time in the center of the OF test (Simpson & Kelly 2011; Hüttenrauch, Salinas, & Wirths 2016) as we noticed.

Interestingly, no significant interactions were observed between EtOH and EE in total exploration time, motor coordination behavioral tasks and depressive and anxiety-like behaviors, which are not specifically related to hippocampal functioning. This could suggest that the interaction between EtOH consumption and EE may be restricted to some brain areas. Actually, the behavioral and cognitive alterations observed in adult rats exposed to adolescent intermittent alcohol were associated with changes in gene expression of endocannabinoids and neuroinflammatory molecules in a brain region-specific manner (Sanchez-Marin *et al.* 2017). This is also surprising, specifically for the lack of improvement in the motor tasks given that exercise has been shown to improve motor performance and improve Purkinje cell function. It is possible that involuntary or controlled exercise regimes are necessary in order to achieve these improvements.

In conclusion, the results of the present investigation suggest that EE has potential benefits for the recovery of the cognitive and neurobehavioral impairments observed in young adults after chronic EtOH exposure during adolescence. Thus, this paradigm may serve as a useful tool for lifestyle-based treatment for EtOH-induced brain disorders.

Acknowledgements

This work was supported by Red de Trastornos Adictivos, Instituto de Salud Carlos III (ISC-III) ERDF-EU (grant RD16/0017/0012 to P.G.; grant RD16/0017/001 to F. R.F.); Basque Government (grant BCG IT764-13 to P.G.); MINECO/FEDER, UE (grant SAF2015-65034-R to P.G.); University of the Basque Country (grant UFI11/41 to P.G.); Proyectos de investigación en Salud (grant PI16/01698 to F.R.F.); and Vanier Canada Graduate Scholarship (NSERC to C.J.F.). The authors thank Dr. Cristina Miguelez (Department of Pharmacology of the University of the Basque Country) for borrowing and setting up the Rotarod apparatus and Dr. Laura Cutando for helping with the beam walking balance test.

AUTHORS CONTRIBUTION

IR-B, SP, NP, MEG, IT, LL and IB performed the experiments and contributed to the acquisition of animal data. IE, AR, LR, JM-Z, CJF, IG and FRF assisted with data analysis and interpretation of findings. PG was

responsible for the study concept and design and wrote the manuscript. All authors critically reviewed content and approved final version for publication.

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