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RESEARCH ARTICLE

Role of mTOR-regulated autophagy in spine pruning defects and memory impairments induced by binge-like ethanol treatment in adolescent mice

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Keywords
adolescence, autophagy, binge ethanol treatment, cognitive function, dendritic spines, mTOR, synaptic pruning.

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Abstract

Adolescence is a brain maturation developmental period during which remodeling and changes in synaptic plasticity and neural connectivity take place in some brain regions. Different mechanism participates in adolescent brain maturation, including autophagy that plays a role in synaptic development and plasticity. Alcohol is a neurotoxic compound and its abuse in adolescence induces neuroinflammation, synaptic and myelin alterations, neural damage and behavioral impairments. Changes in synaptic plasticity and its regulation by mTOR have also been suggested to play a role in the behavioral dysfunction of binge ethanol drinking in adolescence. Therefore, by considering the critical role of mTOR in both autophagy and synaptic plasticity in the developing brain, the present study aims to evaluate whether binge ethanol treatment in adolescence would induce dysfunctions in synaptic plasticity and cognitive functions and if mTOR inhibition with rapamycin is capable of restoring both effects. Using C57BL/6 adolescent female and male mice (PND30) treated with ethanol (3 g/kg) on two consecutive days at 48-hour intervals over 2 weeks, we show that binge ethanol treatment alters the density and morphology of dendritic spines, effects that are associated with learning and memory impairments and changes in the levels of both transcription factor CREB phosphorylation and miRNAs. Rapamycin administration (3 mg/kg) prior to ethanol administration restores ethanol-induced changes in both plasticity and behavior dysfunctions in adolescent mice. These results support the critical role of mTOR/autophagy dysfunctions in the dendritic spines alterations and cognitive alterations induced by binge alcohol in adolescence.

INTRODUCTION

Adolescence is an important brain maturation period during which plastic and dynamic processes take place. Structural remodeling occurs in a variety of brain regions, including the hippocampus, where neural plasticity is a characteristic of adolescence, and even in adulthood (51,57). Synaptic pruning, a process in which the selective removal of synapses occurs during brain development, is also a crucial event for neurocircuitry refinement in adolescence (87). Indeed, during the pubertal period, the density of dendritic spines decreases by half in widespread areas of the central nervous system (CNS) (52,79,101), including the hippocampus (58). The correct maturation of this brain area is critical for the accurate development of learning and memory processes (12).

Increasing evidence demonstrates the role of autophagy machinery in synaptic pruning (60). Autophagy is a conserved regulated mechanism that allows cells to remove unnecessary or dysfunctional components, and to deliver them to the lysosome. The main autophagy pathway is regulated by serine/threonine kinase mTOR (mammalian target of rapamycin), which is necessary for phagophore and autophagosome formation. This kinase is essential for cell growth, protein synthesis and metabolism regulation among many other cellular pathways (37). In the CNS, autophagy and its regulation by mTOR (22) and are critical for maintaining neuronal functions (54), such as synaptic remodeling and plasticity associated with long-term memory formation (88). Deficits in mTOR are

associated with mental retardation (18,47,94). Autophagy dysfunctions are also involved in several inflammatory and neurodegenerative diseases (6,64). In fact, modulators of mTOR, such as rapamycin, reduce inflammation and ameliorate its progression (53).

We recently demonstrated that binge ethanol treatment in adolescent mice (PND30 to PND43) alters autophagy machinery by impairing the levels of LC3-II and p62, which leads to dysfunctions in synaptic proteins PSD-95 and SHANK3. Notably, these effects have been associated with the activation of the immune receptor Toll-like receptor 4 (TLR4) response (66), which plays a crucial role in ethanol-induced brain damage in adolescence. Indeed, human and experimental studies demonstrate that binge drinking in adolescence causes myelin alterations [e.g., humans, (90); animals, (67)], hippocampus dysfunctions [e.g., humans, (25,70), animals, (83,92)] and cognitive and behavioral effects [e.g., (24,67,68)]. Studies in mice further demonstrate that ethanol-induced brain injury and behavioral effects are associated with the activation of the TLR4 response, which triggers neuroinflammation as the elimination of TLR4 (using TLR4-KO mice) abolishes the production of the inflammatory cytokines associated with brain damage and behavioral dysfunction in mice adolescents involved in binge ethanol drinking (67,76).

The hippocampus is one of the most important brain areas to develop in adolescence, and is extremely vulnerable to the effects of binge ethanol drinking in both humans (70,80) and rodent adolescents [e.g., (63,65)]. This brain region is one of the most critical brain structures for memory formation and storage (21,55) and many studies in human [e.g., (29,80)] and rodent adolescents [e.g., (67)] show that alcohol binge causes alterations to memory processes. Several behavioral tests have been used to assess cognitive and memory functions in rodents, such as novel object recognition, the Hebb–William maze and the passive avoidance test. Indeed, the Hebb–Williams maze paradigm has been used for assessing memory processes in rodents with hippocampal lesions [e.g., (17,32,48)] and has been a tool used in translational research to measure comparable cognitive functions in human and animal models with different pathologies (72). In addition, the passive avoidance paradigm and novel object recognition performance are also associated with hippocampal functions (26,42,72).

Therefore, by considering that autophagy pathways participate in synaptic pruning (46,94) and the physiological role of mTOR in the autophagy in memory processes (88), the present study evaluates whether the modulation of the autophagic process, by inhibiting mTOR with rapamycin, is capable of restoring alterations in synaptic plasticity and the cognitive functions induced by intermittent ethanol treatment in adolescence. Herein, we demonstrate for the first time that intermittent ethanol treatment alters the density and morphology of dendritic spines, effects associated with not only memory and learning impairment, but also with alterations in CREB (cAMP-responsive element-binding protein) phosphorylation levels in adolescent mice. We further provide evidence that rapamycin administration restores both the synaptic spine dysfunctions and cognitive alterations induced by ethanol in the adolescent brain. These findings support the critical role of mTOR in regulating the neurochemical autophagy involved in neuronal plasticity and cognitive/behavioral alterations induced by binge-like ethanol treatment in adolescence.

MATERIALS AND METHODS

Animals and treatments

Seventy-two female and male C57BL/6 WT (wild-type) mice (Harlan Ibérica, Barcelona, Spain) were used, which were derived from 17 litters. Mice were housed (3–4 animals/cage) and maintained on a water and solid diet *ad libitum*. Environmental conditions, such as light and dark (12/12 h), temperature (23°C) and humidity (60%), were controlled for all the animals. All the animal experimental procedures were approved by the Ethical Committee of Animal Experimentation of the Principe Felipe Research Center (Valencia, Spain), following the guidelines approved by European Communities Council Directive (86/609/ECC) and Spanish Royal Decree 1201/2005.

The intermittent ethanol treatment was initiated early in adolescence or during the prepubescent period on postnatal day (PND) 30 (1,19). Morning doses of either saline or 25% (v/v) ethanol (3 g/kg) in isotonic saline were administered intraperitoneally to 30-day-old mice on two consecutive days with 2-day gaps without injections for 2 weeks (PND30 to PND43), as previously described (68,74). No signs of irritation of the peritoneal cavity, pain or distress or peripheral inflammation induced by intraperitoneal ethanol concentration was noted, which agrees with other studies that have used intraperitoneal ethanol administration (45). Both female and male mice showed similar or higher blood alcohol levels than human ethanol-intoxicated adolescents (76). To inhibit the mTOR pathway, some animals were intraperitoneally administered rapamycin (3 mg/kg; LC Laboratories, Woburn, Canada), dissolved in 100% DMSO diluted at 60% (v/v) with physiological saline, 1 h prior to each ethanol or saline injection. Animals were randomly assigned to four groups according to their treatments: (i) physiological saline or control (18 females, 18 males); (ii) rapamycin plus physiological saline (18 females, 18 males); (iii) ethanol (18 females, 18 males); (iv) rapamycin plus ethanol (18 females, 18 males). No changes were observed in the animals' body weight or in brain weight during the intermittent treatment (Figure S1). Mice were sacrificed by cervical dislocation 24 h after the last (8th) ethanol or saline administration (PND44). No ethanol levels in serum were detected 24 h after the last ethanol injection. Some animals were used for molecular and Dil staining analyses (24 females, 24 males). One hemisphere of each animal was removed and freshly cut for Dil staining. The hippocampus of the other hemisphere was dissected and immediately snap-frozen in liquid nitrogen until analyzed. The other animals (48 females, 48 males) were used to perform behavioral studies

after ethanol treatment at PND46 in this test daily order: novel object recognition, passive avoidance, Hebb–Williams maze.

Western blot analysis

The Western blot technique was performed in hippocampus tissue lysates (n = 6 samples/group of females or males), as described elsewhere [Montesinos *et al* (68)]. The employed primary antibodies were: p-mTOR, p-ERK1/2 (Santa Cruz Biotechnology, Heidelberg, Germany), p-CREB, p-Akt (Cell Signaling Technology, Leiden, The Netherlands). Membranes were washed, incubated with the corresponding HRP-conjugated secondary antibodies and developed by the ECL system (ECL Plus; Thermo Fisher Scientific, Illinois, USA). All the membranes were stripped and incubated with Coomassie blue brilliant as the total protein loading control (36). Band intensity was quantified with the ImageJ 1.44p analysis software (National Institutes of Health, Bethesda, MD, USA). The densitometry analysis is shown in arbitrary units normalized to the respective Coomassie blue brilliant staining (Figure S2).

Tissue preparation, DiI staining, confocal imaging and spine analysis

Brains (n = 6 brains/group of females or males) were rapidly dissected into 200 μm-thick coronal slices in 4°C PB 0.1 M. They were kept in 1.5% PFA in PB 0.1 M solution for at least 30 minutes. Slices were stored in PB 0.1 M with 0.05 M sodium azide at 4°C until used. DiI stain (1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindocarbocyanine Perchlorate ("DiI"; $DiIC_{18}(3)$), Molecular Probes) was diluted in N,N-dimethylformamide (Sigma) at 5 mg/mL. Slices were incubated in PBS containing DiI at 1:500–1:2000 of the stock solution for 10 minutes. Then, slices were incubated in PB 0.1 M with 0.05 M sodium azide overnight (o/n) at room temperature to allow DiI to fully diffuse along neuronal membranes. Slices were mounted using FluorSafe (Calbiochem, US and Canada) and visualized as soon as the medium was dry. Fluorescence images were taken under a Leica confocal microscope (model TCS-SP8-AOBS, Mannheim, Germany).

As the dentate gyrus shows physiological and anatomical differences, depending on the coronal study level, and by comparing the suprapyramidal and infrapyramidal blades of the granule cell layer (4), slices were selected between Bregma −2.60 mm and Bregma −2.06 mm. Images were always taken on the supra-pyramidal blade. Based on their migration patterns (3), the granule cells located in the medial region of the dentate gyrus were selected to avoid the different maturation stage (oldest and youngest granule cells) of the outer and inner dentate gyrus regions. Spine images were taken around 80–170 μm from soma. Five to twelve dendritic branches from different dentate granule cells were analyzed for each animal. The spine analysis was performed in 3D from the z-stacks with the semiautomatic software NeuronStudio (http://research.mssm.edu/cnic/tools-ns.html), which measures dendritic length and classifies spines into three major morphological types. This method agrees with well-accepted spine type classification methods (16,39) and considers mushroom spines to be the stablest and mature spines and the stubby and thin spines immature and unstable spines (11). To minimize any bias, all the analyses were done blind to the experimental condition. Density was calculated by dividing the total number of spines present by the dendritic length of the segment.

Behavioral testing

Hebb–Williams maze

This task was used for its advantages over other tests as not only can problem-solving, visuospatial abilities and cognitive performance in rodents (17,82) be assessed, but also easy and difficult learnings and, consequently minor cognitive deficits, can be differentiated (15,32,97). Motivation to perform this maze is not based on reinforcement (i.e., food), but on escaping from a stressful situation (cold water), which can influence learning and memory (40,69).

The maze used in our experiments was made of black plastic and measured 60 cm wide × 60 cm long × 10 cm high. It contained a start box and a goal box (both 14 cm wide × 9 cm long), which were positioned at diagonally opposite corners. The maze contained cold water at a wading depth (15°C, 3.5 cm high), while the goal box was stocked with fresh dry tissue. Several maze designs were produced by fixing different arrangements of barriers to a clear plastic ceiling. This apparatus allows the cognitive process of routed learning and water-escape motivation to be measured. The followed procedure was based on that employed by (33), in which mice must navigate the maze and cross over from the wet start box to the dry goal box to escape cold water. Animals (n = 12 mice/group of females or males) underwent a 5-minute habituation period (dry sand, no barriers) on day 1 and undertook problem A on day 2 and problem D on day 3 (4 trials/day) (practice mazes). Mice were subsequently submitted to mazes 1, 5, 3, 4 and 8 on separate days on which eight trials took place. The time limit for reaching the goal box was 5 min, after which the mouse was guided to the box. The following measurements were recorded: acquisition criterion score, considered to be a task completed in less than 60 s during two consecutive sessions; total latency score (the sum of the latencies in all the problem trials in each maze); latency to reach the goal during the eighth trial; error scores, for which a similar total was used (where "error" was considered the act of entering the error zone as previously described (82). Following (91), mazes were defined as easy (1, 3 and 4) or difficult (5 and 8).

Novel object recognition

Mice (n = 12 mice/group of females or males) performed this test in a black open box (24 cm × 24 cm × 15 cm) using small nontoxic objects: two plastic boxes and a plastic toy. The task procedure is elsewhere described (62,75) and

consists of three phases: habituation, training session (T1) and test session (T2). During the habituation session, mice spent 5 min exploring the open-field arena where T1 and T2 were performed. During the training session, one mouse was placed in the open-field arena containing two identical sample objects placed in the middle of the testing box for 3 min. After a 1-minute retention interval, the animal was returned to the open-field arena with two objects during the test session (3 min): one object was identical to the sample and the other was novel. Object exploration was defined as the orientation of the animal's snout toward the object within a range of ≤2 cm from the object. The recognition index was calculated by measuring the discrimination index [D.I. = $(t_{novel} - t_{familiar})/(t_{novel} + t_{familiar}) \times 100\%$], with "t" taken as the time that each mouse spent exploring an object.

Passive avoidance test

A step-through inhibitory avoidance apparatus for mice (Ugo Basile, Comerio-Varese, Italy) was employed for the passive avoidance test. This cage was made of Perspex sheets and divided into two compartments (15 cm × 9.5 cm × 16.5 cm each). The safe compartment was white and illuminated by a light fixture (10 W) fastened to the cage lid, whereas the "shock" compartment was dark and made of black Perspex panels. The two compartments were divided by an automatically operated sliding door at the floor level. The floor was made of 48 stainless steel bars with a 0.7 mm diameter, placed 8 mm apart. Passive avoidance tests were carried out following the procedure previously described (13). On the training day, each mouse (n = 12 mice/group of females or males) was placed in the illuminated compartment facing away from the dark compartment. After a 60-second habituation period, the door leading to the dark compartment was opened. When the animal had placed all four paws in the dark compartment, a footshock (0.5 mA, 3 s) was delivered and the animal was immediately removed from the apparatus and returned to its home cage. The time taken to enter the dark compartment (step-through latency) was recorded. Retention was tested 24 h later following the same procedure, but with no footshock. The maximum step-through latency lasted 300 s.

RNA isolation, reverse transcription and quantitative RT-PCR

Frozen hippocampus samples (100–200 mg) (n = 6 samples/group of females or males) were used for total RNA extraction. Tissue was disrupted using 1 mL of TRIzol (Sigma-Aldrich) and the total RNA fraction was extracted following the manufacturer's instructions. Next, 500 ng of total RNA were reverse-transcribed in parallel with the TaqMan MicroRNA Reverse Transcription Kit and the TaqMan Advanced miRNA cDNA Synthesis kit (ThermoFisher Scientific, USA) following the manufacturer's protocols. The expression levels of the selected miRNAs (miR-155-5p, miR-96-5p and miR-182-5p) involved in neuroinflammatory response (89,96) were confirmed by quantitative real-time PCR (RT-qPCR) using the TaqMan universal PCR Master miX no AmpErase UNG (ThermoFisher Scientific, USA) for the endogenous control (RNAU6) and the TaqMan Fast Advanced Master Mix for the selected miRNAs assays (ThermoFisher Scientific, USA) following the manufacturer's protocols. Quantitative two-step RT-PCR (real-time reverse transcription) was performed in the Light-Cycler 480 detection system (Roche Diagnostics). Table 1 shows the TaqMan assays employed.

Statistical analysis

The results are reported as the mean ± SEM. All the employed statistical parameters were calculated with SPSS v24. The Western blot data were performed by a three-way (Sex, Binge Ethanol Treatment and Rapamycin Treatment) ANOVA, followed by the Bonferroni post hoc test. If the latter failed, Kruskal–Wallis nonparametric tests followed by Dunn post hoc tests were conducted. Confocal imaging and quantitative RT-PCR were analyzed with a two-way (Sex and Treatments) ANOVA, followed by the Bonferroni post hoc test. The data from the Hebb–Williams maze were analyzed by a mixed three-way ANOVA with the aforementioned three between-subject variables and one within subject variable "Maze Difficulty" at two levels (easy and difficult). Passive avoidance was analyzed by a repeated measures ANOVA with three "between" subject variables: Gender, with two levels (male and female); Binge-like Ethanol Treatment (saline and ethanol); Rapamycin Treatment, with

Table 1. TaqMan assays description.

TaqMan assay	Mature sequence	Chromosome location
U6 snRNA	GTGCTCGCTTCGGCAGCACATATACTAAAATTGGAACGATACAGAGAAGATTAGCA TGGCCCCTGCGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTT	Chr.10 LOC115487555
mmu-miR-96-5p	UUUGGCACUAGCACAUUUUUGCU	Chr.7: 129774692 - 129774769 [-] on Build GRCm38
mmu-miR-155-5p	UUAAUGCUAAUUGUGAUAGGGGU	Chr.16: 84714140 - 84714204 [+] on Build GRCm38
mmu-miR-182-5p	UUUGGCAAUGGUAGAACUCACACCG	Chr.6: 30165918 - 30165992 [-] on Build GRCm38

two levels (with and without rapamycin); Days (Training and 24-hour test) as a within subject variable. The novel object recognition test data were analyzed by a mixed ANOVA with the above-cited three between variables. Bonferroni adjustment was employed for the post hoc comparisons.

RESULTS

The mTOR-autophagy pathway is involved in the synaptic pruning alterations induced by binge-like ethanol treatment in adolescence

We previously demonstrated that binge-like ethanol exposure in adolescence impairs autophagy machinery by lowering LC3-II levels and accumulating p62 in prefrontal cortices, effects that are associated with alterations in the levels of excitatory scaffolding synaptic proteins PSD-95 and SHANK3 (66). Considering that kinase mTOR is a critical regulator of the autophagic process (14,47), n, modulates synaptic pruning (94), we first evaluated the effects of ethanol on mTOR phosphorylation in the presence or absence of its inhibitor rapamycin. Figure 1 illustrates that ethanol treatment upregulated mTOR phosphorylation in the hippocampus of both the female [$F(2,14) = 12.502$, $P = 0.001$] and male [$F(1,34) = 8.543$, $P < 0.01$] mice and these effects were abolished in the animals treated with rapamycin plus ethanol (females: [$F(2,14) = 27.192$, $P < 0.0001$] and males: [$F(1,34) = 20.288$, $P < 0.0001$]). The three-way ANOVA revealed a significant main effect for Binge-like Ethanol Treatment [$F(1,34) = 5.714$, $P < 0.05$], Rapamycin Treatment [$F(1,34) = 28.934$, $P < 0.0001$] and Binge-like Ethanol Treatment and Rapamycin Treatment interaction [$F(1,34) = 20.857$, $P < 0.01$].

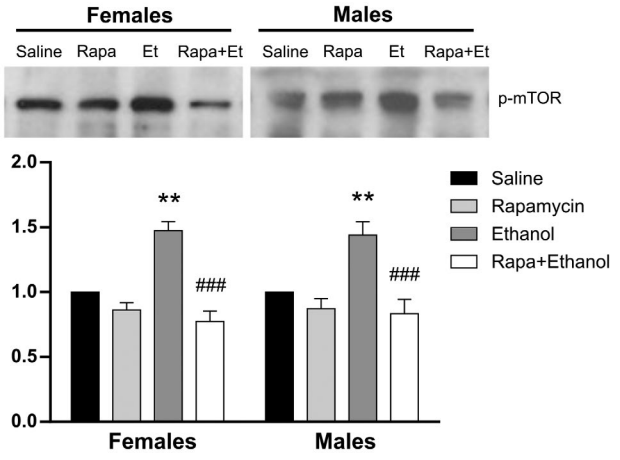

Figure 1. *Rapamycin restores the ethanol-induced impairment of mTOR phosphorylation in the hippocampus of adolescent female and male mice.* The immunoblot analysis and the quantification of mTOR phosphorylation (p-mTOR) in the hippocampus of PND44 female and male animals intermittently treated with saline, rapamycin, ethanol or rapamycin plus ethanol. Figure S2 includes the full-length Coomassie blue staining as the loading control. A representative immunoblot is shown. Values represent mean ± SEM, n = 6 mice/group. $^{**}P < 0.01$, compared to their respective saline-treated group; $^{###}P < 0.001$ compared to their respective ethanol-treated group.

We next analyzed synaptic pruning in the control and ethanol-treated mice using the density and morphology of the dendritic spines obtained from the 3D hippocampal images of the dentate gyrus of granule cells. Spines are classified as mushroom, the stablest and mature spines and stubby and thin as immature and unstable spines (11) (Figure 2). As shown in Figure 3, the two-way ANOVA with the Bonferroni post hoc test revealed a significant increase in the overall spine density after ethanol treatment in female [$F(3,17) = 8.077$, $P < 0.05$] and male [$F(3,13 = 4.957$, $P < 0.05$] mice compared to their control counterparts group. The spine density classification based on morphology revealed that ethanol increased the thin spines in females [$F(3,17) = 24.62$, $P < 0.0001$] and the stubby spines in males [$F(3,14) = 5.197$, $P < 0.05$]. No changes were observed in the mushroom spine density in the ethanol-treated female and male mice. Rapamycin administration before ethanol treatment was able to restore the overall spine density in females [$F(3,17) = 8.077$, $P < 0.0018$) and males [$F(3,13) = 4.957$, $P < 0.05$]), as demonstrated by both the thin [$F(3.17) = 24.62$, $P < 0.0001$] and stubby [$F(3,14) = 5.197$, $P < 0.05$] spine densities in the female group and the male group, respectively, similarly to their control counterparts groups. No differences were found between the animals treated with saline and rapamycin (data not shown).

Inhibition of mTOR is able to restore the cognitive dysfunctions induced by adolescent ethanol intermittent treatment

We next assessed whether the dendritic pruning alterations induced by ethanol through the autophagy pathway in the hippocampus of the adolescent mice were associated with cognitive impairments. For this purpose, we performed several spatial learning and memory paradigms, including Hebb–Williams maze, novel object recognition and passive avoidance tasks, which have been related to hippocampal cognitive function (5,34,71).

Hebb–Williams maze

The ANOVA on time to reach the goal (Figure 4A) revealed a significant effect for Difficulty [$F(1,97) = 81.172$, $P < 0.001$] and a significant Binge-like Ethanol Treatment × Rapamycin Treatment interaction [$F(1,97) = 8.352$, $P < 0.01$]. Mice spent more time reaching goals in the difficult mazes than in the easy ones ($P < 0.001$). The ethanol-treated mice needed more time to complete the mazes compared to both the group of animals treated with saline ($P < 0.01$) and the group of animals exposed to rapamycin ($P = 0.01$).

Novel object recognition

The three-way ANOVA analysis in the object recognition test (Figure 4B) provided a significant effect of the main

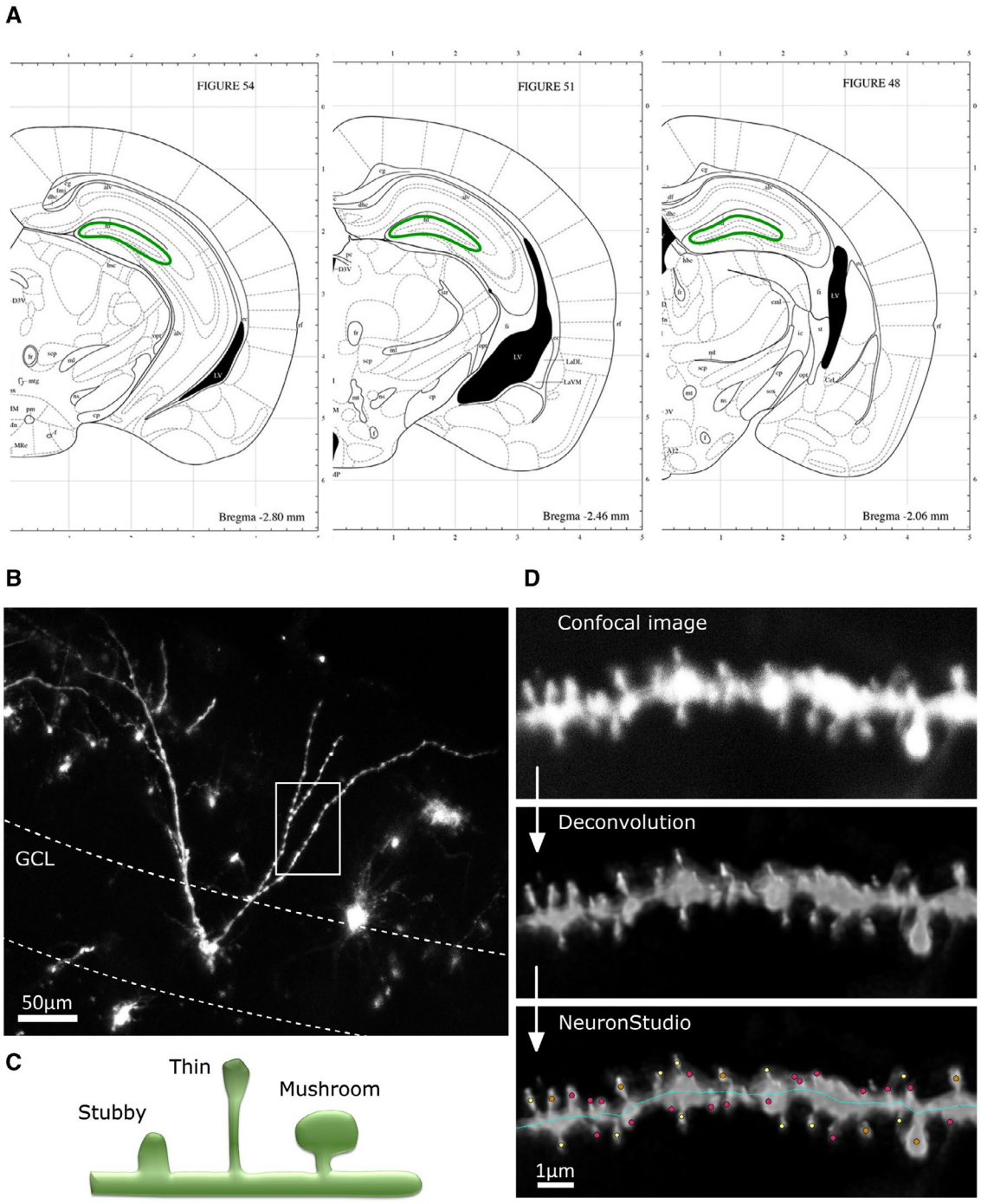

Figure 2. *Illustration of the brain area and spine classification, used to study DiI staining.* **(A)** In the top panel, a schematic of brain slices is shown at distances of 2.80, 2.46 and 2.06 mm from the bregma. The green line denotes the granule cells layer (GCL) of the dentate gyrus of the hippocampus, where images were taken. **(B)** A representative image is provided from a granule cell in the dentate gyrus of the hippocampus. **(C)** Illustration represents a common morphological classification of dendritic spines. The spines with a head/neck diameter ratio above 1.1 µm are considered thin or mushroom. The spines that do not meet the neck ratio value and have a length to spine to head diameter above 2.5 µm are classified as thin, otherwise as stubby. The spines that meet the neck ratio value and have a head diameter that equals or exceeds 0.35 µm are labeled as mushroom, otherwise as stubby. **(D)** The NeuronStudio process used previously to analyze the spine morphology. [Colour figure can be viewed at wileyonlinelibrary.com]

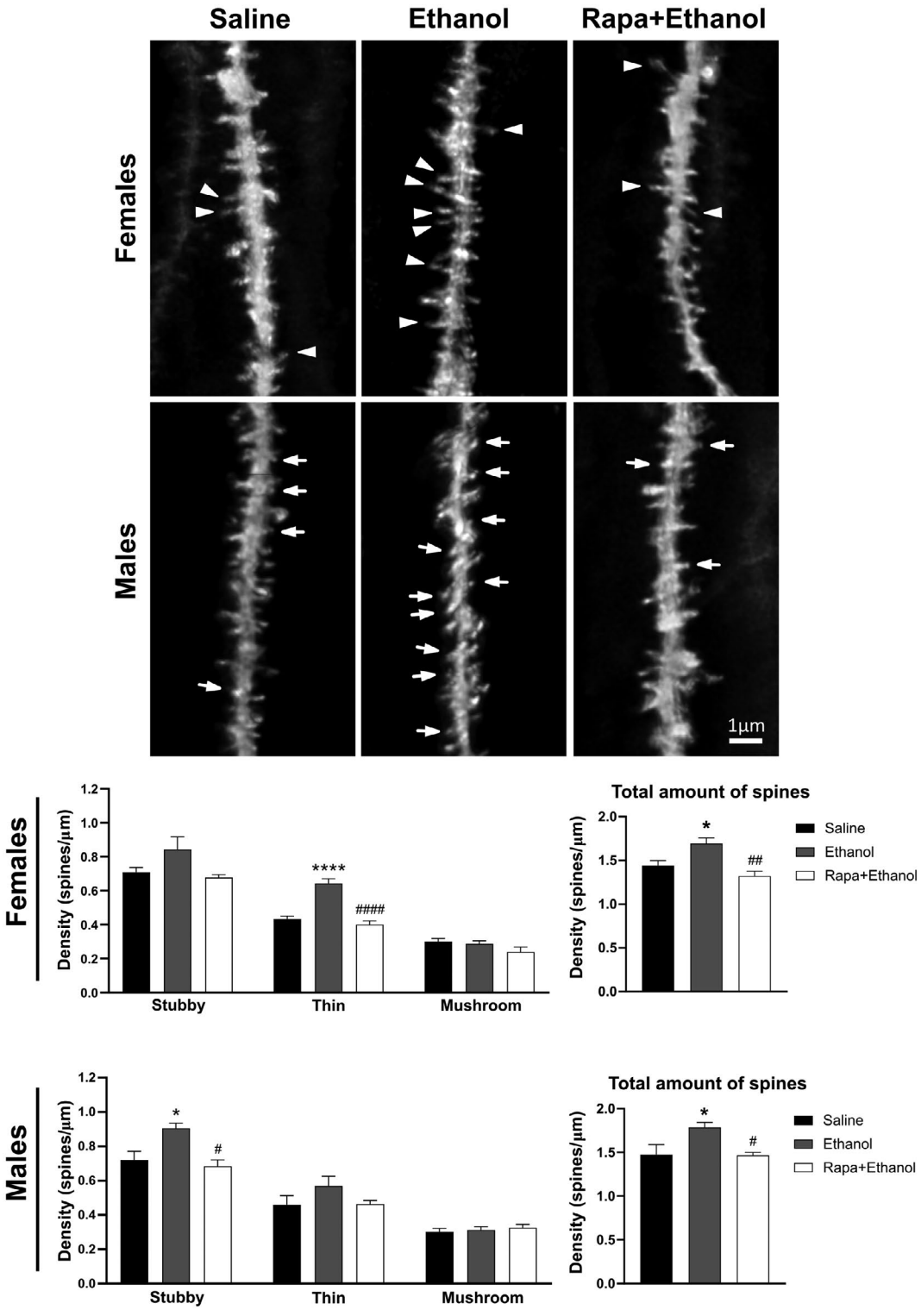

Figure 3. *Rapamycin restores ethanol-induced alterations in the spine morphology of the dentate gyrus of granule cells in the hippocampus of adolescent female and male mice treated with ethanol.* Representative images of the DiI stain on the medial granular dendrites of each group are shown. Arrowheads indicate thin spines in female mice and arrows indicate stubby spines in male mice. Graph bars denote the quantification of spine density in each spine type and in the total amount of spines (spines/µm). Data represent mean ± SEM, n = 6 mice/group. $^{*}P < 0.05$, $^{****}P < 0.0001$ compared to their respective saline-treated group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.0001$ compared to their respective ethanol-treated group. Scale bar = 1 µm.

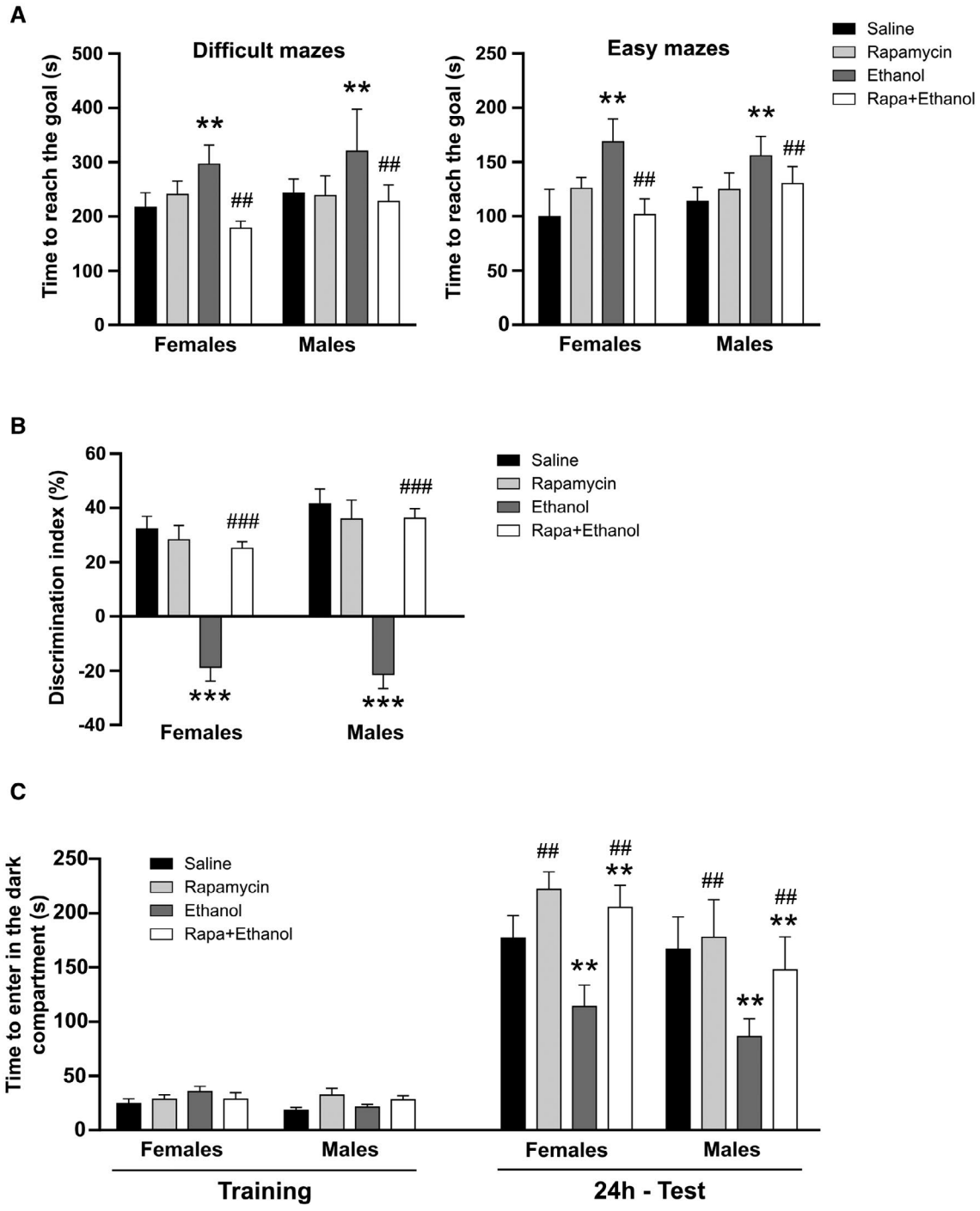

Figure 4. *Rapamycin restores ethanol-induced cognitive dysfunction in adolescent female and male mice.* **(A)** Bars represent the mean (± SEM, n = 12 mice/group) of the time to reach the goal in the difficult and easy mazes in the Hebb–Williams mazes. $^{**}P < 0.01$ compared to their respective saline-treated group. $^{\#\#}P < 0.01$ compared to their respective ethanol-treated group. **(B)** Bars represent the mean (± SEM, n = 12 mice/group) of the discrimination index during the novel object recognition task. $^{***}P < 0.001$ compared to their respective saline-treated group. $^{\#\#\#}P < 0.01$ compared to their respective ethanol-treated group. **(C)** Bars represent the time taken to enter the dark compartment of the passive avoidance test during the training and test sessions (24 h and 72 h after training). Data are presented as mean (± SEM), n = 12 mice/group. $^{**}P < 0.01$ compared to ethanol-treated groups and their control counterparts. $^{\#\#}P < 0.01$ compared to rapamycin-treated groups and their control counterparts.

variables Binge-like Ethanol Treatment [$F(1,96) = 63.729$, $P < 0.001$] and Rapamycin Treatment [$F(1,96) = 39.366$, $P < 0.001$] and of the Binge-like Ethanol Treatment × Rapamycin Treatment interaction [$F(1,96) = 58.384$, $P < 0.01$]. Moreover, the ethanol-treated mice failed to recognize the novel object and its discrimination index was significantly lower than for the same group treated with rapamycin and the mice exposed to saline ($P < 0.001$, in both cases). Conversely, the animals treated with rapamycin plus ethanol were able to distinguish the new object.

Passive avoidance test

The ANOVA for the passive avoidance test (Figure 4C) showed an effect for Days [$F(1,83) = 239.729$; $P < 0.001$], Binge-like Ethanol Treatment [$F(1,83) = 6.263$; $P < 0.05$] and Rapamycin Treatment [$F(1,83) = 9.853$, $P < 0.01$] and revealed significant differences for the Days × Binge-like Ethanol Treatment [$F(1,83) = 5.942$, $P < 0.01$] and Days × Rapamycin Treatment [$F(3,83) = 7.438$, $P < 0.01$] interactions. All the groups of animals (regardless of the treatment received and binge-like ethanol treatment) displayed a longer latency to enter the dark compartment during the 24-hour test ($P < 0.001$) compared to the training day. In addition, the animals exposed to binge-like ethanol treatment exhibited shorter latency to cross to the dark compartment during the 24-hour test vs. the group of saline-treated mice ($P < 0.01$). Furthermore, the mice treated with rapamycin exhibited longer latency to enter the dark compartment during the 24-hour test compared to the control group ($P < 0.01$). This finding suggests that ethanol causes poor retention in the memory task, while rapamycin treatment is able to preserve cognition under harmful ethanol effects.

Role of the CREB pathway in the autophagy impairment induced by adolescent intermittent ethanol treatment

We next determined the possible involvement of transcriptional factor CREB in the dysfunctions of synaptic pruning and cognitive impairments induced by ethanol treatment as this transcription factor plays a fundamental role in long-term synaptic plasticity and memory formation (2,9,85). To this end, we analyzed CREB phosphorylation under different experimental conditions. Figure 5 shows that ethanol treatment decreased CREB phosphorylation in the hippocampus of the female [Kruskal–Wallis test = 9.214, $P < 0.05$] and male [Kruskal–Wallis test = 9.185, $P < 0.05$] mice compared to their saline counterparts. Notably, mTOR inhibition by rapamycin administration prior to ethanol was able to restore the CREB phosphorylation levels in the female [Kruskal–Wallis test = 9.214, $P < 0.05$] and male [Kruskal–Wallis test = 9.185, $P < 0.05$] mice compared to the ethanol-treated mice. No significant difference was found for the variable Sex ($P = 0.319$).

As CREB is a regulatory target for both protein kinase Akt/PKB (27) and MAPK/ERK (61), and contributes to memory consolidation (77), we also evaluated the phosphorylation levels of ERK1/2 and Akt to elucidate if both kinases could be involved in the CREB phosphorylation alterations. Figure 5 shows that binge ethanol treatment lowered the ERK1/2 phosphorylation levels in the hippocampus of both the female [Kruskal–Wallis test = 9.870, $P < 0.05$] and male [Kruskal–Wallis test = 9.242, $P < 0.05$] mice compared to their saline counterparts, whereas no changes were observed in Akt phosphorylation. Notably,

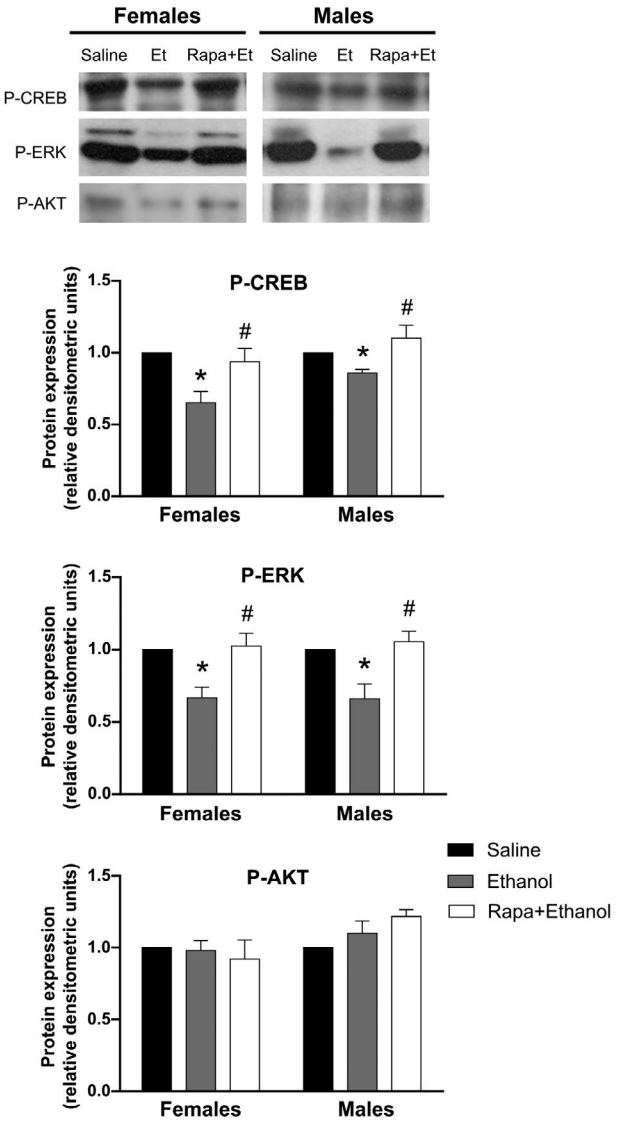

Figure 5. *Rapamycin restores the ethanol-induced impairment of transcription factor CREB in female and male mice.* The immunoblot analysis and quantification of p-CREB, p-ERK and p-Akt in the hippocampus lysates of the PND44 female and male animals intermittently treated with saline, rapamycin, ethanol or rapamycin plus ethanol. Figure S2 includes the full-length Coomassie blue staining as the loading control. A representative immunoblot of each protein is shown. Values represent mean ± SEM, n = 6 mice/group. $^{*}P < 0.05$ compared to their respective saline-treated group. $^{\#}P < 0.05$ compared to their respective ethanol-treated group.

rapamycin administration was capable of restoring the ERK1/2 phosphorylation levels in the ethanol-treated female [Kruskal–Wallis test = 9.870, $P < 0.05$] and male [Kruskal–Wallis test = 9.242, $P < 0.05$] mice compared to the ethanol-treated mice. No significant difference was observed for either the variable Sex in ERK1/2 phosphorylation ($P = 0.897$) or Akt phosphorylation ($P = 0.176$).

Inhibition of mTOR restores the inflammatory miRNAs alterations induced by adolescent ethanol intermittent treatment

MiRNAs, a class of small noncoding RNAs, are important regulators of gene expression that participate in both physiological and pathological processes (38). We recently demonstrated that both *in vivo* and *in vitro* ethanol treatments alter miRNAs associated with neuroinflammation in mice brains (96) and in glial cells in culture (41). Therefore, we wondered if binge-like ethanol treatment in adolescence could alter the levels of the specific miRNAs associated with neuroinflammation (e.g., miR-155-5p, miR-96-5p and miR-182-5p) (89,96), and whether rapamycin treatment could revert ethanol effects on miRNAs. Figure 6 shows for the ethanol-treated animals a significant increase in the miR-155-5p levels in both female ([$F(2,13) = 8.832$; $P < 0.01$] and male [$F(2,16) = 6.646$; $P < 0.05$] mice, whereas the increase was also observed in male animals compared to their saline group in both miR-96-5p [$F(2,14) = 12.72$; $P < 0.001$] and miR-182-5p [$F(2,15) = 6.120$; $P < 0.05$]. The rapamycin treatment administered prior to ethanol administration was able to restore the alterations observed in the levels of miR-155-5p (females [$F(2,13) = 8.832$; $P < 0.05$] and males [$F(2,16) = 6.646$; $P < 0.05$]), miR-96-5p ([$F(2,14) = 12.72$; $P < 0.05$] and miR-182-5p [$F(2,15) = 6.120$; $P < 0.05$] in male mice compared to the ethanol-treated group. The two-way ANOVA revealed a significant effect for Treatment (miR-155-5p: [$F(2,29) = 6.418$; $P < 0.01$]; miR-96-5p: [$F(2,29) = 12.69$; $P = 0.0001$]), Sex (miR-155-5p: [$F(1,29) = 23.76$; $P < 0.0001$]; miR-96-5p: ([$F(1,29) = 39.66$; $P < 0.0001$]) and their interaction (miR-155-5p: [$F(2,29) = 4.479$; $P < 0.05$]; miR-96-5p: [$F(2,29) = 12.74$; $P = 0.0001$]; miR-182-5p: [$F(2,28) = 7.228$; $P = 0.01$]).

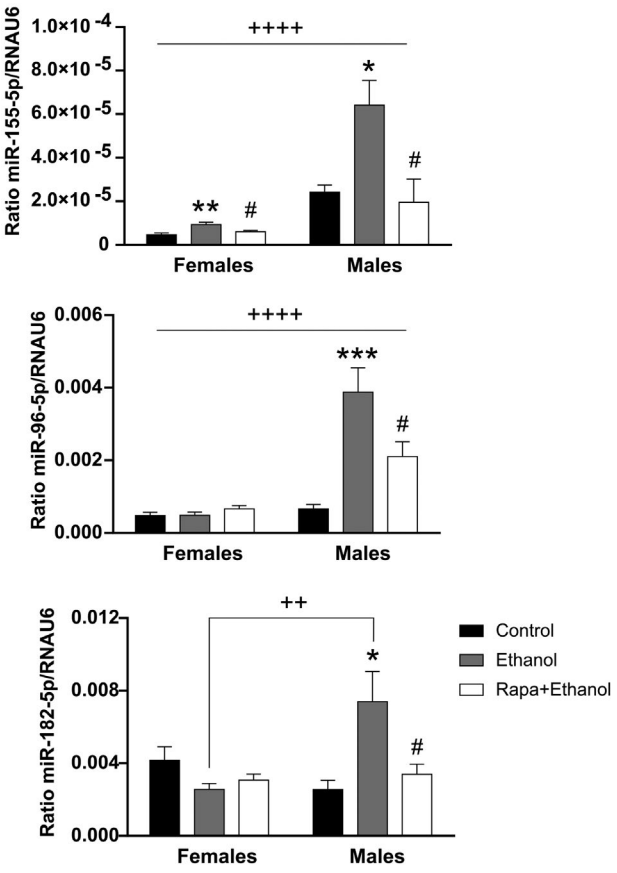

Figure 6. *Rapamycin is able to restore the differential levels of miRNAs induced by binge-like ethanol treatment in adolescent female and male mice*. Bars represent mean ± SEM, n = 6. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$ compared to their respective saline-treated group. $^{\#}P < 0.05$ compared to their respective ethanol-treated group. $^{++}P < 0.01$, $^{++++}P < 0.0001$ sex or interaction differences between females and males.

DISCUSSION

We previously showed that binge-like ethanol treatment in adolescence induced an inflammatory immune response through the activation of the TLR4 pathway, which led to synaptic and myelin alterations and caused long-term cognitive and memory dysfunctions (67). These ethanol effects have been associated with an impairment of both excitatory scaffolding synaptic proteins PSD-95 and SHANK3, and the autophagy process (66), events that could underlie structural synaptic plasticity/remodeling and memory storage dysfunctions (84). The present findings evidence that binge-like ethanol treatment increases the density of immature synaptic spines in the hippocampus, impairs CREB phosphorylation and alters the cognitive and memory function in adolescent mice. We further show that effects of ethanol are associated with dysfunctions of mTOR-regulated autophagy as rapamycin, an inhibitor of the mTOR pathway, restores the density and morphology of dendritic spines, along with CREB phosphorylation and plasticity, as well as cognitive and memory dysfunctions in female and male adolescent mice.

The adolescent brain development is a dynamic process in which certain brain regions undergo synaptic pruning, characterized by a reduction in the density of dendritic spines to provide selection and maturation of synapses and neural circuits in adulthood (78). Indeed, during synaptic pruning, the weaker and unstable spines are eliminated, which allows the maintenance of the stablest spines and their connections (20). In some neurological diseases, such as autism spectrum disorders (94), basal dendrite spine density increases and is associated with enhanced local excitatory connectivity (10), which leads to an excitatory/inhibitory imbalance of brain communication (35). Indeed, the density

of dendritic spines and their morphology are the main determinants of neuronal connectivity and signal integration, and alterations in spines are generally viewed as indicators of alterations of neuronal circuits and functional properties (95). We show that binge-like ethanol exposure increases the density of the stubby and thin spines in the hippocampus of female and male adolescent mice, respectively, which indicates an increase in immature synaptic spines that might lead to dysfunctions in synaptic pruning and plasticity, and to alterations in the cognitive function in adolescence. According to our results, an increased number of immature dendritic spines in the pyramidal neurons of the hippocampal CA1 region (83) and the abnormal dendritic structure in layer V of mPFC (50) were observed in adolescent rats exposed to intermittent alcohol exposure and these effects are irreversible. According to the present findings, the knockdown of Rictor, an essential component of mTOR complex 2 (mTORC2) signaling, attenuates binge drinking-induced alterations in spine head size and number of mushroom spines (56).

One major mechanism that participates in synaptic pruning while the brain is developing is autophagy, in which mTOR is the main regulator. Loss of mTOR-dependent autophagy causes autistic-like synaptic pruning deficits (94). We have shown that binge-like ethanol exposure impairs autophagy machinery by altering the levels of excitatory scaffolding synaptic proteins PSD-95 and SHANK3 (66). By inhibiting mTOR with rapamycin in the present study, we were able to restore changes in the density and morphology of dendritic spines induced by binge-like ethanol treatment in female and male adolescent mice. Similarly, Tang *et al*. (2014) demonstrated that rapamycin restores spine pruning defects and behavioral impairments in an autism spectrum disorder animal model (94). In a murine autosomal dominant neurogenetic disorder, rapamycin is also capable of suppressing abnormal spine density maturation and aberrant arborization (23). Inhibition of mTOR with rapamycin also reverses the status epilepticus and improves dendritic branching and spine density in area CA1 (18).

Autophagy is not only the main process in the synaptic pruning function, but it also participates in maintaining the long-term memory function (88), while autophagy regulator mTOR is also involved in the regulation of synaptic plasticity, memory storage and cognition (14,22). Accordingly, we herein show by memory and learning tasks to assess hippocampal function that mTOR inhibition is able to normalize performance in longer times to complete Hebb–Williams mazes, a shorter discrimination index during the object recognition task and the shorter latency during the passive avoidance test, which were induced by binge-like ethanol treatment in both female and male adolescent mice. Notably, we also demonstrate that mTORC1 inhibition by rapamycin during reconsolidation disrupts alcohol-cue associated memories, which leads to a long-lasting suppression of relapse (8). Other studies have also revealed how rapamycin can restore the spatial memory deficits induced by prolonged continuous seizures of status epilepticus (18).

Evidence indicates that hippocampus plays a critical role in spatial learning and memory consolidation (30) by participating in several biological components of different pathways. For instance, transcription factor CREB is a regulatory target for both protein kinase Akt/PKB (27) and MAPK/ERK (61) by contributing to memory consolidation (77) and, therefore, to long-term potentiation and memory consolidation (43). Impairment in the hippocampal PI3K/Akt/mTOR signaling pathway induced by ethanol might also be involved with cognitive deficits in male mice (100). We herein show that rapamycin is able to restore the low CREB levels induced by ethanol treatment in adolescence, which supports the role of the mTOR pathway in synaptic plasticity and molecular pathways, which lead to the maturation of memory systems. The upregulation of CREB has been correlated with the induction of prosurvival genes in preventing neuronal death (93). Alcohol-induced neurotoxicity in the hippocampus has also been associated with the downregulation of CREB (73). Our results suggest that rapamycin, by blocking mTOR, increases CREB and these effects could be associated with the restoration of the long-term cognitive and behavioral impairments previously demonstrated in Montesinos *et al* (2015, 2016) (67,68) in adult mice treated with ethanol in adolescence.

Emerging evidence reveals that the CNS is highly enriched with miRNAs to play essential roles in neurogenesis, neural development and synaptic plasticity (81). However, the deregulation of miRNAs can lead to neurodegeneration and neurological disorders (28). Recent studies have demonstrated the dysregulation of miRNA by ethanol treatment. For instance, the dysregulation of both the TLR4-dependent miR-183 cluster (miR-182-miR-183-miR-96) and miR-200a/b expression takes place in the cerebral cortices of chronic ethanol-treated WT mice (96). Similarly, extracellular vesicles/exosomes from ethanol-treated cortical astroglial cells are enriched with inflammatory mediators and different inflammatory miRNAs (miR-182, miR-146a and miR-200b), which are capable of being transported to neurons to spread neuroinflammation. These effects have also been mediated by the TLR4 response (41). In the present study, we show that rapamycin is able to restore the upregulation of miR-96-5p, miR-182-5p and miR-155-5p in the hippocampus of ethanol-treated adolescent male mice. MiR-96-5p and miR-182-5p have been associated with the depletion of the pathways related to the immune system (86,96,102), while miR-155-5p is associated with a proinflammatory immune response (7). However, miR-155 has also been associated with not only the innate immune response and cognitive functions (44,59), but also with neurogenic deficits associated with neuroinflammation during hippocampal development (99). These results suggest that rapamycin, by downregulating miR-155, would contribute to mitigate the neuroinflammatory effects of ethanol.

Our findings further evidence that ethanol-treated male mice have higher levels of anti-inflammatory miRNAs than ethanol-treated females, which suggests that the innate immune response in males is less activated than in female mice. Accordingly, our previous studies in human adolescents

with binge drinking also demonstrate that, at the same blood alcohol levels (76), inflammatory cytokines are higher in females than in males, which indicates a sex difference in the inflammatory effects of alcohol (76). Although the sex differences in the immune response remain unclear, variations in hepatic alcohol metabolism, as well as hormonal changes and differences in alcohol distribution, such as the body/fat ratio, differ in females and males (31,98), which might explain some of the observed sex differences. Interestingly, sex differences in the immune response, with lower innate and adaptive immune responses in males vs. females, have also been observed in several species of insects, lizards, birds and mammals (49).

Taken together, these novel results support the role of the mTOR-regulated autophagy system in the synaptic spines alterations and cognitive dysfunction induced by binge-like ethanol treatment in adolescence. They also suggest that transcriptional factor CREB and inflammatory-related microRNAs might also be involved in these events. Finally, the use of rapamycin opens up new pharmacological venues to restore the structural synaptic plasticity dysfunctions and behavioral alterations associated with binge alcohol in adolescence.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web site:

Figure S1. Body weights in mice treated intermittently with saline, rapamycin, ethanol and rapamycin plus ethanol during 2 weeks (PND30 to PND43). Body weights were measured previously to the intraperitoneal injection at the indicated PND. Brain weights were quantified after sacrificing the animals at PND44. Values represent mean ± SEM, n=11-13 mice/group.

Figure S2. Whole membranes of P-mTOR, P-CREB, P-ERK and P-Akt are shown. These membranes were stripped and reincubated with Coomassie Brilliant Blue staining (CBB) as total protein loading controls. S: saline; R: rapamycin; E: ethanol; R+E: rapamycin plus ethanol.