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Adolescent ethanol intake alters cannabinoid type-1 receptor localization in astrocytes of the adult mouse hippocampus

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ABSTRACT

Cannabinoid type-1 (CB_1) receptors are widely distributed in the brain and play important roles in astrocyte function and the modulation of neuronal synaptic transmission and plasticity. However, it is currently unknown how CB_1 receptor expression in astrocytes is affected by long-term exposure to stressors. Here we examined CB_1 receptors in astrocytes of ethanol (EtOH)-exposed adolescent mice to determine its effect on CB_1 receptor localization and density in adult brain. 4–8-week-old male mice were exposed to 20 percent EtOH over a period of 4 weeks, and receptor localization was examined after 4 weeks in the hippocampal CA1 stratum radiatum by pre-embedding immunoelectron microscopy. Our results revealed a significant reduction in CB_1 receptor immunoparticles in astrocytic processes of EtOH-exposed mice when compared with controls (positive astrocyte elements: 21.50 ± 2.80 percent versus 37.22 ± 3.12 percent, respectively), as well as a reduction in particle density (0.24 ± 0.02 versus 0.35 ± 0.02 particles/μm). The majority of CB_1 receptor metal particles were in the range of 400–1200 nm from synaptic terminals in both control and EtOH. Altogether, the decrease in the CB_1 receptor expression in hippocampal astrocytes of adult mice exposed to EtOH during adolescence reveals a long lasting effect of EtOH on astrocytic CB_1 receptors. This deficiency may also have negative consequences for synaptic function.

Keywords alcohol, endocannabinoid system, glia, immunoelectron microscopy, tripartite synapse.

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INTRODUCTION

The endocannabinoid system (eCBS) is composed of two seven-transmembrane G protein-coupled receptors, known as cannabinoid type-1 (CB_1) and cannabinoid type-2, as well as endogenous eCB lipid ligands (anandamide, 2-AG), enzymes responsible for their synthesis and degradation, and also transportation proteins (Kano *et al.* 2009; Katona & Freund 2012; Gutiérrez-Rodríguez *et al.* 2017). The eCBS participates in a wide range of brain functions and provides important computational properties to brain circuits (Hill *et al.* 2004, 2010; Bellocchio *et al.* 2010; Katona & Freund 2012).

Binge drinking is a significant social issue worldwide, particularly among the youth (Llorens *et al.* 2011). However, how binge drinking affects receptor populations in the brain and impacts the structure of the developing adolescent brain is not well understood. There is considerable evidence for the involvement of the eCBS in alcohol consumption and motivation, reinforcing properties of ethanol (EtOH) and EtOH dependence (Pava & Woodward 2012). For instance, CB_1 receptor agonists stimulate a dose-dependent increase in EtOH intake (Colombo *et al.* 2002), while antagonists reduce voluntary EtOH intake, preference and craving (Economidou *et al.* 2006). Moreover, CB_1 receptor knockout mice show a reduced EtOH preference and intake (Hungund *et al.* 2003). Interestingly, chronic EtOH exposure causes a decrease in CB_1 receptor mRNA expression (Ortiz *et al.* 2004; Mitrirattanakul *et al.* 2007) as well as in CB_1

receptor density and functionality (Basavarajappa, Cooper, & Hungund 1998; Vinod *et al.* 2006) associated with an eCB increase in the hippocampus (Mitrirattanakul *et al.* 2007), but not in amygdala or striatum (Rubio *et al.* 2009), that persists after a long withdrawal period (Mitrirattanakul *et al.* 2007). Furthermore, an increase in anandamide was detected in EtOH animal models (Vinod *et al.* 2006) and ventral striatum of postmortem human alcoholics (Vinod *et al.* 2010) together with a decrease in the anandamide-degrading enzyme fatty acid amide hydrolase (FAAH) and CB_1 receptor expression (Vinod *et al.* 2010). A decrease in CB_1 receptor expression and a reduced G-protein coupling of the receptor was also observed in the striatum, hippocampus, nucleus accumbens and amygdala of FAAH knockout mice (Vinod *et al.* 2008). The changes in hippocampal CB_1 receptor expression have negative consequences on the CB_1 receptor-mediated inhibitory synaptic transmission, despite the recovery of the CB_1 receptor expression after a prolonged withdrawal (Rimondini *et al.* 2002; Mitrirattanakul *et al.* 2007; Vinod *et al.* 2012) probably due to a reduction in eCBs (Vinod *et al.* 2012).

As to the astroglia, prolonged EtOH exposure alters the distribution and content of the glial fibrillary acidic protein (GFAP) that has a negative impact on the astrocytic intermediate filaments and, ultimately, on the astrocyte morphology eventually leading to brain dysfunction (Renau-Piqueras *et al.* 1989). Activation of CB_1 receptors expressed in astrocytes promotes astroglial differentiation, modulates synaptic transmission through the neuron-astrocyte crosstalk (Navarrete & Araque 2010; Han *et al.* 2012; Bosier *et al.* 2013; Araque *et al.* 2014; Gómez-Gonzalo *et al.* 2015; Metna-Laurent & Marsicano 2015; Oliveira da Cruz *et al.* 2016) and regulates leptin receptor expression in cultured cortical and hypothalamic astrocytes (Bosier *et al.* 2013). To our knowledge, however, there is no direct evidence on the effect of chronic EtOH exposure during adolescence on the CB_1 receptor expression in astrocytes of the adult brain. Our results from the CA1 stratum radiatum of the adult mouse hippocampus after binge drinking during adolescence reveal a long-lasting decrease in astroglial CB_1 receptor expression that may compromise the normal functioning of the tripartite synapse.

MATERIALS AND METHODS

Animals and ethics statement

Three-week-old male C57BL/6J mice (Janvier Labs) were housed in sibling pairs in standard Plexiglas cages (17 cm × 14.3 cm × 36.3 cm). Mice were habituated in their environment for at least 1 week before experimental procedures were initiated. Animals were maintained at 22°C with a 12:12-hour light:dark cycle (red light on at 9:00 hours) and had *ad libitum* access to food throughout all experiments and *ad libitum* access to water except during EtOH access, as noted later. Protocols for animal care and use 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, BOE 08–02-2013). CB_1-KO mice were generated and genotyped as previously described (Marsicano *et al.* 2002).

Drinking in the dark procedure

Adolescent male mice from postnatal day 32 to 56 (4–8 weeks) were randomly assigned to either the water (control) or EtOH experimental group. Mice were subjected to a 4 day binge drinking in the dark procedure (Rhodes *et al.* 2007) over a period of 4 weeks. Each week, animals were weighed 1 hour before lights out on days 1–4. During these days, mice were separated and placed individually in standard Plexiglas cages (17 cm × 14.3 cm × 36.3 cm). Three hours into the dark cycle, mice were either exposed to a single bottle of EtOH [20 percent EtOH (v/v) prepared from 96 percent EtOH and tap water (Alcoholes Aroca S. L., Madrid, Spain)] or a bottle of tap water (control group) for 2 hours and on day 4 for 4 hours. After 4 days of EtOH or water exposure, EtOH bottles were removed and mice had access to only water for 3 days (food was always available). EtOH intake was calculated throughout treatment as grams of EtOH per kilogram of animal per hour (g/kg/hour). The average amount was 2.50 ± 0.15 g/kg/hour (Fig. 1a). At the end of the treatment, blood samples were collected from the lateral tail veins using a capillary tube (Sarstedt, Germany), and EtOH levels were measured with an EtOH assay kit (Sigma-Aldrich). The average blood EtOH concentration was 58.07 ± 6.04 mg/dl (Fig. 1b; $^{***}P < 0.001$).

Brain tissue processing

Four weeks after cessation of EtOH exposure, control, EtOH-treated and CB_1-KO mice (n = 3 each group) were deeply anesthetized by intraperitoneal injection of ketamine/xylazine (80/10 mg/kg body weight) and transcardially perfused at room temperature (RT, 20–25°C) with phosphate buffered saline (0.1 M PBS, pH 7.4) for 20 seconds, followed by infusion of fixative solution (4 percent formaldehyde freshly depolymerized from paraformaldehyde, 0.2 percent picric acid, 0.1 percent glutaraldehyde) in 0.1 M phosphate buffer

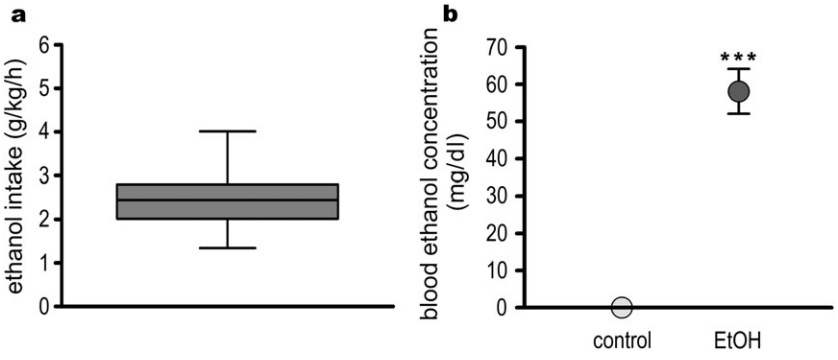

Figure 1 Voluntary oral ethanol consumption and BEC. (a) Total ethanol intake (g/kg/h) and (b) blood ethanol concentration (mg/dl) in C57BL6 mice exposed to 4-day binge drinking in the dark procedure over a period of 4 weeks during adolescence (PND32–56). Data are expressed as mean ± SEM. Data were analyzed by means unpaired *t*-test; ***$P < 0.001$. BEC, blood ethanol concentration; SEM, standard error mean; EtOH, ethanol

(PB) for 10–15 minutes. Brains were then removed from the skull and immersion fixed in the same fixative solution for ~1 week at 4°C. They were then transferred to a 1:10 diluted fixative solution until tissue sectioning.

Pre-embedding immunogold/peroxidase electron microscopy

Fifty-µm-thick coronal hippocampal vibrotome sections were cut, collected in 0.1 M PB at RT and incubated in a blocking solution of 10 percent bovine serum albumin (BSA), 0.1 percent sodium azide, and 0.02 percent saponin in Tris–HCl buffered saline (TBS 1X, pH7.4) for 30 minutes at RT. A pre-embedding silver-intensified immunogold was used for the localization of CB_1 receptor protein and an immunoperoxidase method for GFAP protein labeling of astrocytes. Briefly, hippocampal sections were incubated with goat polyclonal anti-CB_1 receptor antibody (2 µg/ml corresponding to a 1:100 dilution, #CB1-Go-Af450, Frontier Institute Co.; RRID: AB_257130) and mouse anti-GFAP antibody (1:1000, #G3893, Sigma-Aldrich; RRID: AB_477010) diluted in 10 percent BSA TBS containing 0.1 percent sodium azide and 0.004 percent saponin on a shaker overnight at 4°C. After three washes in 1 percent BSA TBS, sections were incubated in sequential secondary antibody labeling procedures: horse anti-mouse biotinylated secondary antibody (1:200, BA-2000, Vector Labs) in 1 percent BSA TBS with 0.004 percent saponin for 3 hours followed by a wash in 1 percent BSA TBS overnight at 4°C, and then a 1.4 nm gold-labeled rabbit anti-goat immunoglobulin G antibody (Fab' fragment, 1:100, Nanoprobes Inc.). After several washes in BSA TBS, sections were incubated in avidin-biotin complex (1:50, PK-7100, Vector Labs) for 1.5 hours at RT. Hippocampal sections were washed in BSA TBS overnight at 4°C and postfixed in 1 percent glutaraldehyde in TBS for 10 minutes at RT. After several washes in double-distilled water, gold particles were silver-intensified with a HQ silver enhancement kit (Nanoprobes Inc.) for ~12 minutes in the dark and then washed in 0.1 M PB (pH7.4). To detect GFAP labeling, sections were incubated in 0.05 percent 3,3′-Diaminobenzidine tetrahydrochloride (DAB) and 0.01 percent hydrogen peroxide prepared in 0.1 M PB for 3 minutes at RT. After several washes in 0.1 M PB (30 minutes), stained sections were postfixed in 1 percent osmium tetroxide in 0.1 M PB for 20 minutes, rinsed in distilled water, and dehydrated in graded alcohols (50–100 percent) to propylene oxide, and embedded in Epon resin 812. Ultrathin sections (70 nm) were cut with a diamond knife (Diatome USA), collected on nickel mesh grids, stained with 2.5 percent lead citrate for 20 minutes and examined with a JEOL JEM-1400 electron microscope (JEOL Canada). Sections were imaged with a Gatan SC1000 digital camera (Gatan USA). Minor adjustments in contrast and brightness were made using Adobe Photoshop.

Semi-quantification of CB_1 receptor immunogold staining

To standardize the immunocytochemical conditions to the maximum, six 50-µm-thick hippocampal vibrotome sections obtained from each brain studied ($n = 3$ brains for each group) were simultaneously processed for the pre-embedding immunogold method, as described previously (Gutiérrez-Rodríguez *et al.* 2017). Furthermore, each series of immunocytochemical experiments was independently repeated three times.

The immunogold-labeled hippocampal sections were first examined by light microscopy and portions of the CA1 were identified and trimmed down for ultrathin sectioning. Three to four semi-thin sections (1 µm-thick) were then cut with a histo diamond knife (Diatome USA) and stained with 1 percent toluidine blue. The first 20 ultrathin sections (70 nm each) were collected onto the grids. For quantitation, all electron micrographs were taken at 15 000×. Other images were taken at higher magnification to show detailed CB_1 receptor localization. Sampling was always performed accurately in the same way for all the animals studied. Samples were blinded to experimenters during CB_1 quantification.

To determine the proportion of CB_1 receptor-labeled astrocytes, positive labeling was considered if at least one silver-intensified gold particle was within approximately 30 nm from the plasma membrane. Astrocytic processes were identified by the presence of electron dense DAB reaction product in their cytoplasm.

Silver intensified gold particles on astrocyte membranes were visualized and counted. ImageJ software was used to measure the area and the membrane length. Percentages of CB_1 receptor positive profiles, density (particles/μm membrane) and proportion of CB_1 receptor particles in astrocytes versus total CB_1 receptor expression in cell membranes were determined and displayed as mean ± standard error of the mean (SEM) using a statistical software package (GraphPad Prism 5, GraphPad Software Inc, San Diego, USA). The normality test (Kolmogorov–Smirnov) was applied before running statistical tests, and data were subsequently analyzed using non-parametric or parametric tests (Mann–Whitney U test or unpaired *t*-test).

Semi-quantification of the distance from astrocytic CB_1 receptors to the nearest synapse

The nearby synapses surrounding the CB_1 receptor positive astrocytic process were identified in the electron micrographs. The nearest synapse (excitatory or inhibitory) to the CB_1 receptor particles in astrocytes was selected, and the distance was measured by ImageJ. All data were tabulated, analyzed and displayed as mean ± SEM using a statistical software package (GraphPad Prism 5, GraphPad Software Inc, San Diego, USA).

RESULTS

Subcellular localization of the CB_1 receptor in adult CA1 neurons and astrocytes of control and after adolescent EtOH intake

The DAB immunostaining was used to identify GFAP-containing astrocytes and their processes, which then allowed individual immunogold-labeled CB_1 receptors on astrocytes to be counted. To determine whether EtOH intake during adolescence caused a global change in CB_1 receptor expression in the adult CA1 stratum radiatum, the proportion of CB_1 receptor immunoparticles in different cellular compartments was examined. Metal particles were localized on inhibitory and excitatory axon terminals which formed synapses with dendrites and dendritic spines, respectively (Fig. 2). As expected, the highest proportion of the total CB_1 receptor particles counted in 110 μm^2 (control: 74.59 ± 13.72 particles; EtOH: 68.67 ± 6.72 particles) ($P > 0.05$) was found on inhibitory axon terminal membranes making symmetric synapses with dendrites. Overall, there was no significant difference between the CB_1 receptor expression on inhibitory terminals in control (Figs 2a, a´; Fig. 3a: 52.66 ± 3.59 percent particles) and EtOH (Figs. 2b, b´; Fig. 3a: 53.80 ± 2.89 percent particles) ($P > 0.05$). Furthermore, 82.69 ± 4.28 percent of the inhibitory terminals in control and 76.44 ± 4.49 percent in EtOH-treated mice were CB_1 receptor immunopositive ($P > 0.05$; Fig. 3b). The concentration of immunogold particles was low in the asymmetric (excitatory) synapses in control (11.02 ± 0.75 percent particles) and even lower in EtOH (8.61 ± 0.46 percent particles) ($^*P < 0.05$; Fig. 3a). In this case, 19.48 ± 2.23 percent of the excitatory boutons in control and 15.94 ± 1.67 percent in EtOH animals were CB_1 receptor positive ($P > 0.05$; Fig. 3b).

In astrocytes, scattered metal particles were observed on thin and thick processes of GFAP-immunoreactive astrocytes in the CA1 stratum radiatum of control mice (Figs. 2a, a´). Immunoparticles were less frequently observed in astrocytic processes following EtOH exposure (Figs. 2b, b´, c). The proportion of the total CB_1 receptor particles found on astrocyte membranes in control (5.72 ± 0.96 percent particles) and EtOH treated mice (2.73 ± 0.44 percent particles) was very low relative to the terminals, mitochondria (control: 13.92 ± 1.57 percent particles; EtOH: 13.95 ± 1.63 percent particles) and other membrane compartments (control: 9.95 ± 1.55 percent particles; EtOH: 12.35 ± 1.70 percent particles) (Fig. 3a). Remarkably, the decrease in CB_1 receptor labeling in astrocytes was statistically significant after EtOH exposure ($^*P < 0.05$; Fig. 3a). Furthermore, the differences between the CB_1 receptor immunopositive astrocytic processes in control (37.22 ± 3.12 percent) and after EtOH exposure (21.49 ± 2.28 percent), as well as the density of receptor labeling (particle/μm of astrocytic membrane) in astrocytes of control and EtOH-exposed mice (control: 0.35 ± 0.02; EtOH: 0.24 ± 0.02), were significantly different ($^{***}P < 0.001$, $^{**}P < 0.01$, respectively; Figs. 3c, d). CB_1 receptor density in the other CB_1 receptor-containing profiles remained statistically unchanged after EtOH ($P > 0.05$; Fig. 3c). Importantly, the CB_1 receptor immunolabeling pattern disappeared in the CA1 stratum radiatum of *CB_1*-KO mice (Fig. 2d) hence demonstrating the specificity of the anti-CB_1 receptor antibody used.

Distance from the astroglial CB_1 receptors to the synapses in adult CA1 stratum radiatum of control and after EtOH exposure during adolescence

First, we assessed the astrocyte morphology. There were fewer processes in astrocytes of EtOH-exposed animals (control: 5.80 ± 0.45; EtOH: 3.93 ± 0.23 astrocytic

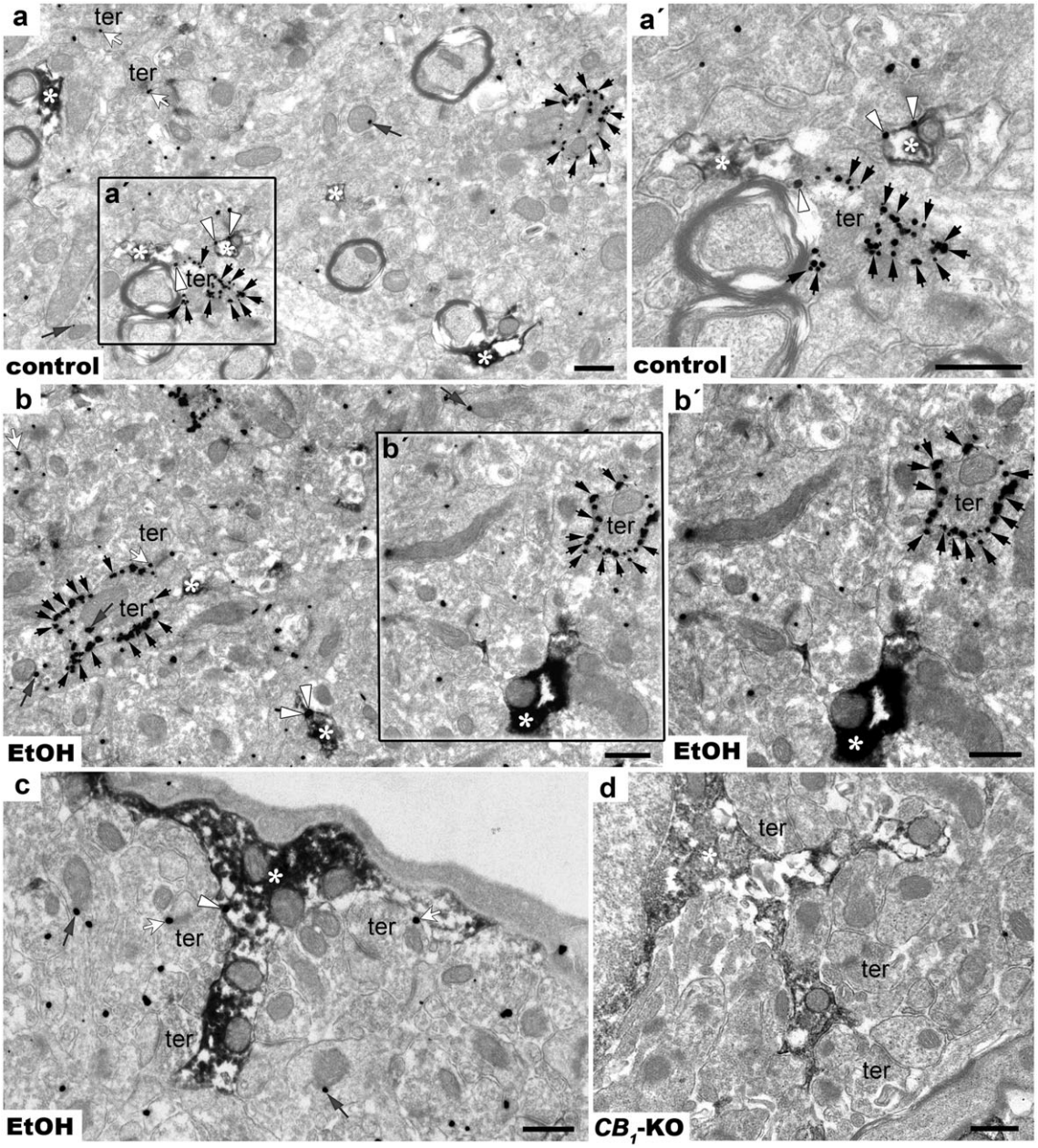

Figure 2 Subcellular CB_1 receptor localization in the adult CA1 stratum radiatum of control and EtOH-treated mice using a combined pre-embedding immunogold/immunoperoxidase labeling methods. (a) In control, CB_1 receptor immunogold labeling is observed on both excitatory (ter, white arrows) and inhibitory terminals (ter, black arrows), and on astrocytic membranes (asterisks, white arrowheads). Note the presence of CB_1 receptor labeling on mitochondria (dark gray arrows). (a´) Higher magnification view showing CB_1 receptor labeling on inhibitory terminals (ter; black arrows) as well as on astrocytic membranes (asterisks, white arrowheads) in control. In EtOH mice (b, c), CB_1 receptor particles are also observed on excitatory terminals (ter, white arrows), inhibitory terminals (ter, black arrows), mitochondrial membranes (dark gray arrows) and astrocytes (asterisks, white arrowheads). (b´) Enlargement of the enclosed area in b. (c) An astrocytic end-foot (asterisk) around a capillary with CB_1 receptor labeling (white arrowhead). Note gold particles on excitatory terminals (ter, white arrows) and mitochondria (dark gray arrows). (d) No CB_1 receptor immunolabeling is detected on terminals (ter), astrocytes (asterisk) and mitochondria in *CB_1-KO* mice, indicating the specificity of the CB_1 receptor antibody used. Scale bars: 1 μm

processes/electron micrograph; $^{***}P < 0.001$; Fig. 4a), however, their area was significantly larger in EtOH (1.06 ± 0.15 μm^2) than in control (0.90 ± 0.15 μm^2; $^{**}P < 0.01$; Fig. 4b). No statistical differences were found in the astrocytic perimeter (control: 4.63 ± 0.26 μm; EtOH: 4.78 ± 0.25 μm; $P > 0.05$; Fig. 4c). Taken together, these data indicate that the astrocytes in the adult hippocampus have a swollen morphology after EtOH exposure during adolescence.

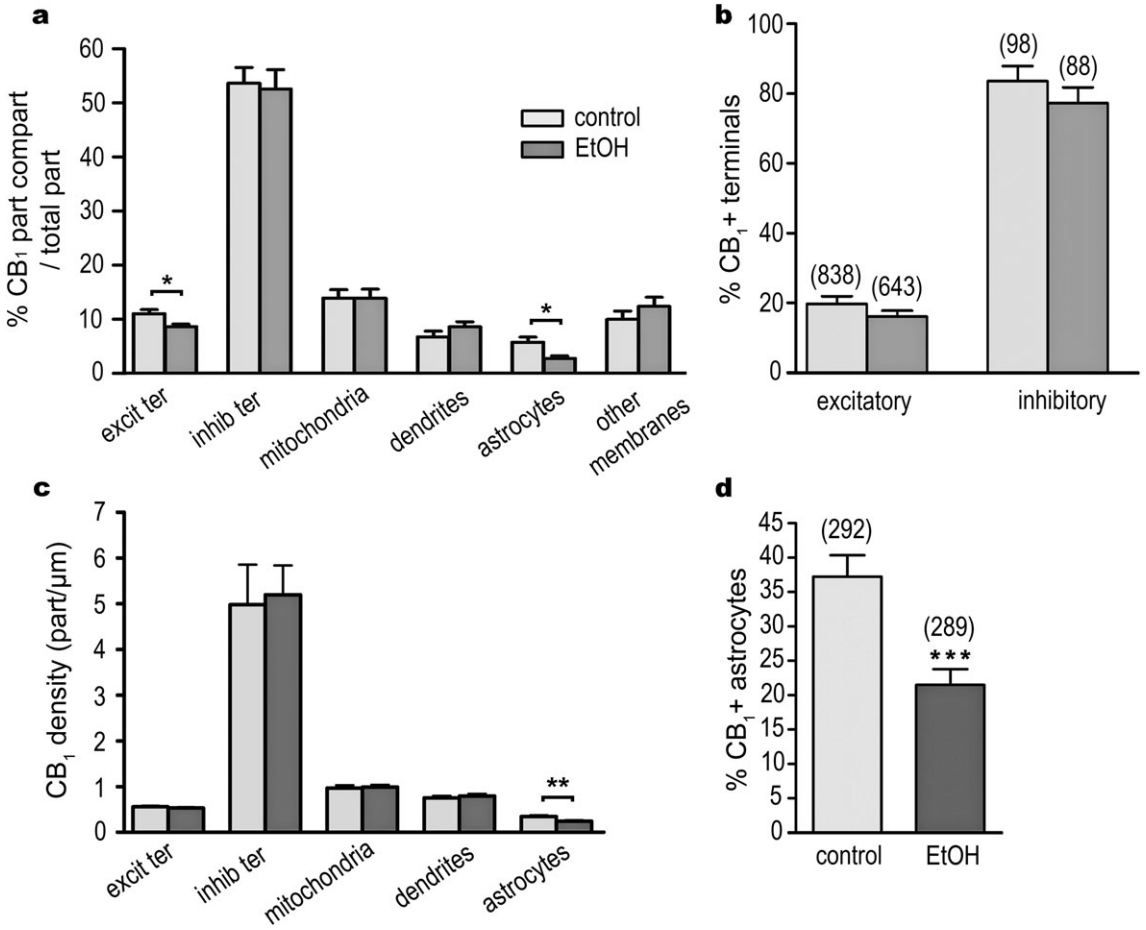

Figure 3 CB_1 receptor distribution in the adult CA1 stratum radiatum of control and EtOH-treated mice. (a) Proportion of CB_1 receptor labeling in different compartments normalized to the total CB_1 content in control and EtOH mice (analyzed area: ~2000 μm^2). (b) Percentage of CB_1 receptor-immunopositive excitatory and inhibitory synaptic terminals in control and EtOH mice. The number of synaptic terminals studied is in parentheses on the top of each column. (c) CB_1 receptor density (particles/µm) in CB_1 receptor-positive profiles in control versus EtOH treated mice. (d) Percentage of labeled astrocytic processes in control and EtOH mice. The number of astrocytic portions studied is in parentheses on the top of the columns. Data are expressed as mean ± SEM. Data were analyzed by means of non-parametric or parametric tests (Mann–Whitney U test or unpaired *t*-test $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$)

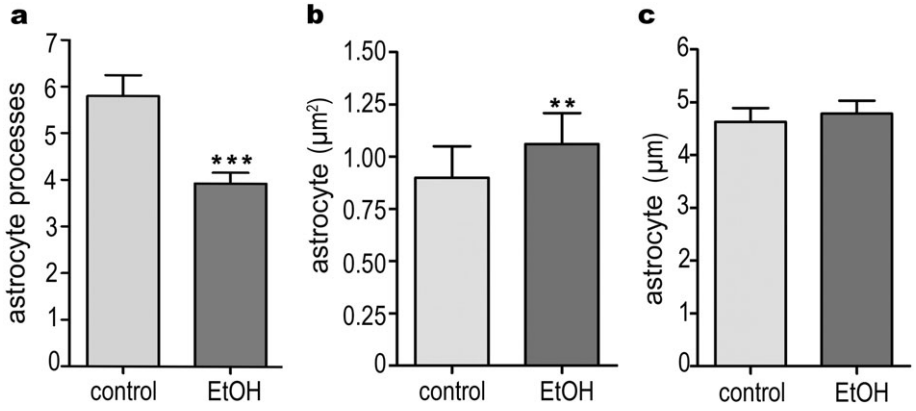

Figure 4 Ultrastructural assessment of the astrocyte morphology in the adult CA1 stratum radiatum of control and EtOH-treated mice. (a) Number of astrocytes per 110 μm^2 in control and EtOH mice. (b) Area (in μm^2) of the astrocytes in control (n = 292) and EtOH (n = 289) mice. (c) Perimeter (µm) of the astrocytes in control (n = 292) and EtOH (n = 289) mice. Data are expressed as mean ± SEM. Data were analyzed by means of non-parametric or parametric tests (Mann–Whitney U test or unpaired *t*-test $^{**}P < 0.01$; $^{***}P < 0.001$)

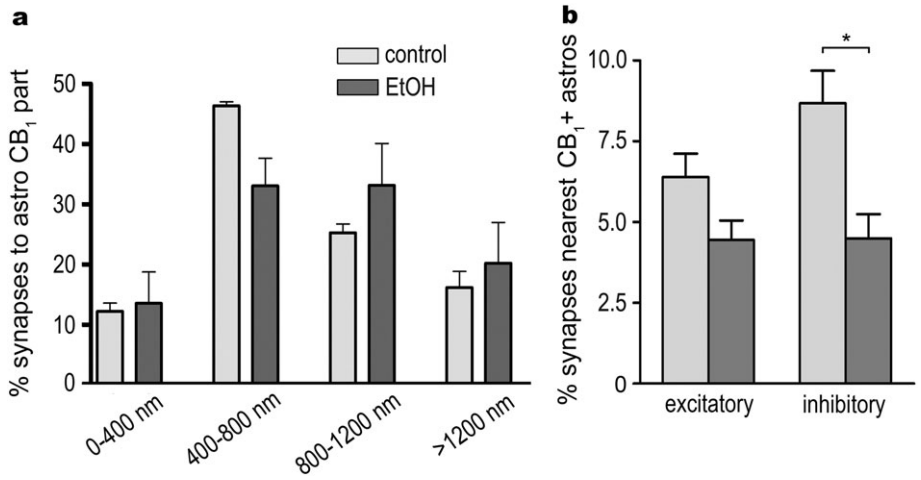

Figure 5 Distance from the astrocytic CB_1 receptors to the nearest synapse in the adult CA1 stratum radiatum of control and EtOH-treated mice. (a) Proportion of synapses in 400 nm bin distances from the astrocytic CB_1 receptor particles to the midpoint of the nearest synapse in control (n = 123 synapses) and EtOH (n = 73 synapses) mice. (b) Percentage of synapses (asymmetric/excitatory versus symmetric/inhibitory synapses) surrounding CB_1 receptor positive astrocytes in control (n = 936 synapses) and EtOH (n = 731 synapses) mice. Data are expressed as mean ± SEM. Data were analyzed by means of non-parametric or parametric tests (Mann–Whitney U test or unpaired t-test; *$P < 0.05$)

To determine how CB_1 receptor distribution is affected in the swollen astrocytes surrounding the synapses following adolescent EtOH exposure, the distance between the astroglial CB_1 receptors and the midpoint of the nearest synapse was measured in adult CA1 stratum radiatum. In control, while only 12.21 ± 1.41 percent of the synapses from the total analyzed (n = 123) were localized at a distance 0–400 nm from the closest astrocytic CB_1 receptor particles (Fig. 5a), the majority of them (46.34 ± 0.69 percent) were at 400–800 nm. The second most frequent distance occurred between 800 and 1200 nm (25.27 ± 1.46 percent of the synapses), while numbers were again reduced (16.18 ± 2.66 percent of the synapses) at distances more than 1200 nm from the nearest astrocytic CB_1 receptor labeling. In EtOH-treated mice, 13.57 ± 5.22 percent of the total synapses (n = 73) was within 400 nm from the astroglial CB_1 receptor particles. The distribution was fairly equivalent between 400–800 nm (33.04 ± 4.61 percent), and 800–1200 nm (33.16 ± 6.92 percent), while 20.23 ± 6.73 percent of the synapses were localized beyond 1200 nm (Fig. 5a). No statistical differences were observed between control and EtOH-treated mice in the astrocytic CB_1 receptor distribution with respect to neighboring synapses ($P > 0.05$; Fig. 5a) nor in the proportion of asymmetric/excitatory versus symmetric/inhibitory synapses relative to the CB_1 receptor positive astrocytes (control: excitatory 6.38 ± 0.72 percent, inhibitory 8.66 ± 1.00 percent. EtOH: excitatory 4.44 ± 0.61 percent, inhibitory: 4.50 ± 0.74 percent) ($P > 0.05$; Fig. 5b). However, the proportion of inhibitory synapses closely related to the astrocytic CB_1 receptor labeling was significantly lower in EtOH-exposed mice than in control (*$P < 0.05$; Fig. 5b) with no differences detected at the excitatory synapses ($P > 0.05$; Fig. 5b).

DISCUSSION

We have examined the effect of the adolescent intermittent binge drinking in the dark, a model of chronic EtOH intake, on the CB_1 receptor expression in adult brain astrocytes for two main reasons. First, the adolescent brain is highly vulnerable to EtOH having a tremendous impact on the brain's cellular structure and function, including the astrocytes (Pascual *et al.* 2007; Vetreno & Crews 2015; Adermark & Bowers 2016). Second, the astroglial CB_1 receptors at the tripartite synapse play a key role in brain functions such as synaptic plasticity, memory and behavior (Navarrete & Araque 2010; Han *et al.* 2012; Araque *et al.* 2014; Gómez-Gonzalo *et al.* 2015; Metna-Laurent & Marsicano 2015; Oliveira da Cruz *et al.* 2016) that are altered upon EtOH intake (Lovinger & Roberto 2013; Lovinger & Alvarez 2017). Our results show that adolescent mice subjected to a 4 day model of binge EtOH drinking have a 40 percent decrease in astroglial processes expressing CB_1 receptors and a 30 percent drop in receptor density relative to control in adult CA1 stratum radiatum astrocytes. In addition, the proportion of total CB_1 receptor particles found on astrocytes in EtOH mice is much lower than in control. Lastly, the measurements taken (perimeter, area) indicate that astrocytes remain swollen in the adult CA1 upon cessation of EtOH intake. Because of the disrupted cell morphology, the astroglial CB_1 receptor expression was analyzed on a similar number of astroglial processes which corresponded to about a 30 percent larger area in

EtOH than in control. Astrocytic swelling seems to be a phenomenon associated with EtOH consumption that leads to astroglial dysfunction (Adermark & Bowers 2016) and disruption of GFAP found in the astrocyte intermediate filaments (Renau-Piqueras *et al.* 1989). Furthermore, long-term behavioral and cognitive impairments, synaptic plasticity disturbance, late alcohol abuse and addiction related to binge drinking during adolescence have been associated with neuroinflammatory mechanisms (Nestler 2001; Montesinos *et al.* 2016). Astrocytes participate in the inflammatory response through their capacity to release pro-inflammatory molecules (Farina, Aloisi, & Meinl 2007) that can be diminished by anti-inflammatory reactions mediated by eCBs acting on astroglial CB_1 receptors (Metna-Laurent & Marsicano 2015). Hence, because of the drastic reduction in CB_1 receptors in adult astrocytes, it is reasonable to expect an impairment of the astroglial anti-inflammatory reaction in response to adolescent EtOH intake. Furthermore, the altered astroglial morphology should affect the extracellular matrix components and the perineuronal nets between astrocytes and synapses, thus impairing the homeostasis at the tripartite synapse. The supposedly resulting disturbance of neurotransmitter clearance and gliotransmission may lead to deficits in synaptic plasticity (Dzyubenko, Gottschling, & Faissner 2016) that underlie brain dysfunction after chronic EtOH consumption (Pava & Woodward 2012; Lovinger & Roberto 2013; Lovinger & Alvarez 2017). Interestingly, the astroglial glutamate transporter GLAST (EAAT1) appears to be up-regulated upon EtOH exposure (Rimondini *et al.* 2002) that should favor glutamate clearance from the synaptic cleft. However, this compensation seems to not be relevant for EtOH effects, as mice lacking GLAST but equipped with functional presynaptic CB_1 receptors show less EtOH consumption, motivation and reward (Karlsson *et al.* 2012). It remains to be determined in our model whether the reduction in CB_1 receptors in astrocytes correlates with changes in GLAST expression.

We observed that adolescent binge drinking does not alter the distribution of the remaining CB_1 receptors in astrocytes relative to neighboring synapses, however, the proportion of inhibitory synapses closely related to the astrocytic CB_1 receptors was significantly lower in EtOH-exposed mice. The drastic decrease in astrocytic CB_1 receptors distributed on swollen astrocytes may not be effective in sensing the endocannabinoids produced on demand by neural activity, compromising gliotransmitter availability elicited by cannabinoids at the synapses (Han *et al.* 2012; Araque *et al.* 2014). The CB_1 receptor expression on glutamatergic synaptic terminals was affected too, as a slight but significant decrease in CB_1 receptor immunolabeling (but not the proportion of CB_1 receptor positive terminals nor the labeling density) was noticed in EtOH adult CA1 stratum radiatum. This is in line with our recent observations in the dentate gyrus molecular layer which revealed that EtOH consumption during adolescence negatively impacts adult CB_1 receptor-dependent long-term depression of excitatory synaptic transmission, a form of synaptic plasticity (Peñasco *et al.* 2015). In contrast, the expression and localization of CB_1 receptors in GABAergic synaptic terminals was unaffected and coincided with the CB_1 receptor distribution pattern described for rodent interneurons in CB_1-WT mice (Nyíri *et al.* 2005; Gutiérrez-Rodríguez *et al.* 2017). However, the percentage of CB_1 receptor-immunopositive excitatory terminals in this and in our preceding study (Gutiérrez-Rodríguez *et al.* 2017) was to a certain extent lower than the values formerly reported (Katona *et al.* 2006; Uchigashima *et al.* 2011), most likely attributable to the different immunocytochemical protocols and antibodies used (Katona *et al.* 2006; Uchigashima *et al.* 2011). Furthermore, the proportion of CB_1 receptor particles on mitochondria in control (13.92 ± 1.57 percent) was similar to our previous reports (Bénard *et al.* 2012; Hebert-Chatelain *et al.* 2016), and no changes in the CB_1 receptor expression could be detected in this organelle upon EtOH exposure (13.95 ± 1.63 percent).

The eCBS participates in EtOH behaviors (Economidou *et al.* 2006), and, reciprocally, EtOH has effects on the eCB-dependent neural activity and behavior (Pava & Woodward 2012; Talani & Lovinger 2015). This system prevents the EtOH-induced potentiation of GABA release (Roberto *et al.* 2010; Talani & Lovinger 2015) and suppresses the glutamatergic transmission elicited by EtOH (Basavarajappa, Ninan, & Arancio 2008). Furthermore, CB_1 receptor antagonism reduces EtOH self-administration and seeking while CB_1 receptor activation has opposite effects during the relapse and maintenance phase of EtOH drinking (Getachew *et al.* 2011). Sardinian alcohol preferring rats, a genetic model of alcoholism, exhibit a higher CB_1 receptor density, CB_1 receptor-mediated G-protein coupling and eCB levels which was associated with lower expression of FAAH (Vinod *et al.* 2012). Upon alcohol consumption, however, CB_1 receptor-mediated G-protein coupling was reduced. This effect was attenuated during alcohol withdrawal and reversed by CB_1 receptor antagonism, and was associated with reduced alcohol intake (Vinod *et al.* 2012). In this line, the CB_1 receptor expression tends to normalize after a prolonged withdrawal (Rimondini *et al.* 2002; Mitrirattanakul *et al.* 2007; Vinod *et al.* 2012) probably due to a reduction in eCB levels (Vinod *et al.* 2012). However, the rapid CB_1 receptor internalization and lysosomal degradation of CB_1 receptors due to the eCB increase upon EtOH intake, and the ultimate

membrane reinsertion upon alcohol intake cessation (Pava & Woodward 2012), seem to operate differently in neuronal and astroglial compartments. In our model of adolescent intermittent alcohol intake, a drastic decrease in CB_1 receptor immunoparticles, proportion of CB_1 receptor-expressing profiles and receptor density were only observed in adult CA1 astrocytes, suggesting that some differences should exist between CB_1 receptors in neuronal and astroglial compartments. Actually, CB_1 receptors signal in neurons via coupling to Gαi/o proteins (Kano *et al.* 2009), and mitochondrial CB_1 receptors have been shown to signal through Gαi proteins, since pertussis toxin blocks the decrease in mitochondrial cAMP, protein kinase A, complex I activity and respiration induced by cannabinoids (Hebert-Chatelain *et al.* 2016). Interestingly, no changes in the mitochondrial CB_1 receptors were observed in our study. In astrocytes, there is evidence indicating that CB_1 receptors, in addition to Gαi/o proteins, also signal through Gαq proteins enabling astroglial CB_1 receptors to couple to different intracellular signaling pathways (Metna-Laurent & Marsicano 2015). These biochemical differences might also have consequences on CB_1 receptor-binding proteins, like the G-protein-associated sorting protein 1 responsible for linking CB_1 receptors to degradation, or the cannabinoid receptor associated protein 1a involved in CB_1 receptor function modulated by antagonists (Vinod *et al.* 2012). Taken together, coupling of the CB_1 receptor to different G-proteins might be behind the distinct effect of adolescent EtOH exposure on the CB_1 receptor expression in astrocytes of adult brain.

The long-lasting effects of adolescent binge drinking on astroglial CB_1 receptors and astroglial morphology suggest the existence of an architectural disruption of the neuron-astrocyte crosstalk at the tripartite synapse of the adult brain. Yet, the effects of these drastic changes on adult synaptic function and behavior remain to be elucidated. Lastly, the reciprocal interactions between the eCBS and the acute and chronic effects of EtOH have been implicated as targets for treatment of EtOH addiction. Thus, the changes observed in astroglial CB_1 receptors might represent a novel target of interest to palliate the structural, functional and behavioral consequences of adolescent binge drinking later in life.

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Authors Contribution

IBDR, NP, SP, IR and AGR performed the experiments and contributed to the acquisition of animal data. IE, AR, LR and IG assisted with data analysis and interpretation of findings. PG was responsible for the study concept and design and wrote the manuscript. BC and PN provided critical revision of the manuscript. All authors critically reviewed content and approved final version for publication.

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