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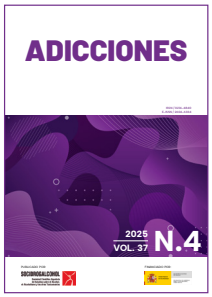

ORIGINAL

Effects of omega-3 fatty acids on CB1 cannabinoid receptor localization in the hippocampal CA1 region following alcohol withdrawal in adolescent male mice

Efectos de los ácidos grasos omega-3 sobre la localización del receptor cannabinoide CB1 en la región CA1 del hipocampo tras la abstinencia de alcohol en ratones machos adolescentes

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Abstract

Adolescent binge drinking has detrimental effects on brain function, leading to long-lasting impairments in synaptic plasticity, cognition, and behavior. These effects are mediated, in part, by disruption of the endocannabinoid system (ECS) and its cannabinoid type-1 (CB1) receptor. Alcohol consumption also depletes omega-3 fatty acids, which are essential for maintaining cell membrane integrity and supporting brain function. This depletion impairs synaptic plasticity by disrupting endocannabinoid signaling and reducing CB1 receptor expression and function. Conversely, enhancement of the ECS can restore brain function and reverse the loss of endocannabinoid-dependent synaptic plasticity associated with omega-3 deficiency. Notably, omega-3 supplementation has been shown to restore CB1 receptor expression in specific brain regions in adult mice following adolescent alcohol exposure. However, despite the established interplay between alcohol, omega-3, and the ECS, the direct impact of omega-3 supplementation on the subcellular localization of CB1 receptors after alcohol exposure remains poorly understood.

In this study, we used immunoelectron microscopy to investigate whether omega-3 supplementation influences CB1 receptor distribution in the hippocampal CA1 region following alcohol withdrawal in adolescent male mice. Our results demonstrate that omega-3 partially restores the excitatory/inhibitory balance disrupted by alcohol, as evidenced by an increased number of excitatory terminals and a significant reduction in inhibitory terminals. However, the distribution and density of CB1 receptors within neuronal and glial compartments remain unchanged following alcohol exposure and omega-3 supplementation.

These findings highlight novel structural effects of omega-3 in mitigating alcohol-induced brain damage.

Keywords: Ethanol, adolescence, endocannabinoid system, neurons, glia, polyunsaturated fatty acids, central nervous system

Resumen

El consumo excesivo de alcohol durante la adolescencia compromete la plasticidad sináptica y las funciones cognitivas, en parte debido a la interferencia con el sistema endocannabinoide (SEC) y su receptor CB1. Asimismo, el alcohol impacta negativamente en los ácidos grasos omega-3, esenciales para la integridad de las membranas celulares y la función cerebral. La deficiencia de omega-3 altera la señalización del SEC y la expresión y funcionalidad del CB1, exacerbando el deterioro inducido por el alcohol. Por el contrario, la activación del SEC contribuye a restaurar las funciones sinápticas dependientes de este sistema, afectadas por la falta de omega-3. De hecho, se ha demostrado que la suplementación con omega-3 normaliza la expresión del CB1 en regiones cerebrales específicas de ratones adultos expuestos al alcohol durante la adolescencia.

A pesar de la estrecha relación entre alcohol, omega-3 y SEC, el efecto directo de los omega-3 sobre la localización subcelular del receptor CB1 tras la exposición al alcohol permanece poco explorado. Para abordar esta cuestión, analizamos la región CA1 del hipocampo en ratones machos adultos en abstinencia después de la ingesta de alcohol durante la adolescencia, utilizando inmunocitoquímica para microscopía electrónica. Nuestros resultados indican que los omega-3 ayudan a restablecer el equilibrio entre excitación e inhibición sináptica, alterado por un aumento en el número de terminales excitadoras y una reducción significativa de las inhibidoras. Sin embargo, ni la distribución ni la densidad del receptor CB1 en compartimentos neuronales y gliales se modificaron tras la exposición al alcohol ni con la suplementación con omega-3.

Estos hallazgos revelan efectos estructurales novedosos de los omega-3 en la protección frente al daño cerebral inducido por el alcohol, aportando nuevas perspectivas sobre los mecanismos neuroprotectores de estos ácidos grasos.

Palabras clave: Etanol, adolescencia, sistema endocannabinoide, neuronas, glía, ácidos grasos poliinsaturados, sistema nervioso central

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According to the 2025 ESTUDES survey conducted by the Spanish Observatory on Drugs and Addictions (Government Delegation for the National Drug Strategy, Ministry of Health, 2025), adolescents constitute a key demographic in alcohol consumption trends, particularly regarding episodic heavy drinking (binge drinking). Nearly one-quarter of individuals aged 14–18 reported engaging in at least one binge-drinking episode in the 30 days preceding the survey. Prevalence was higher in males (26.1%) than in females (23.3%), although both groups exhibited a significant decline compared with the 2023 assessment, with the reduction being more pronounced among females.

Binge drinking during adolescence has significant implications for brain development. This period is marked by extensive neurobiological remodeling, with changes in neurotransmission, synaptic plasticity, and structural maturation, particularly in brain regions associated with learning, memory, and executive function. Consequently, alcohol consumption during this developmental window can result in long-lasting, potentially irreversible alterations in brain function, affecting synaptic transmission and neuroplasticity, ultimately contributing to cognitive, emotional, and motor deficits (Abrahao et al., 2017; Cservenka & Brumback, 2017; Kang et al., 2024; Keshavan et al., 2014; Lovinger & Abrahao, 2018; Lovinger & Alvarez, 2017; Lovinger & Roberto, 2013; Pava & Woodward, 2012; Vetreno & Crews, 2015).

A growing body of evidence implicates ECS in mediating the effects of alcohol on synaptic function in acute exposure, chronic intake, and withdrawal. The ECS plays a key role in regulating motivation and alcohol consumption and is increasingly recognized as a contributor to the pathophysiology of alcohol use disorder (AUD) (Gonzalez et al., 2002; Kunos, 2020; Navarrete et al., 2022; Peñasco et al., 2020; Sanchez-Marin et al., 2022; Vinod et al., 2006). This system comprises G protein-coupled cannabinoid receptors—primarily CB1 and CB2—endocannabinoids such as 2-arachidonoylglycerol (2-AG) and anandamide (AEA), and the enzymatic machinery responsible for their synthesis, degradation, and transport (Lu & Mackie, 2021).

Multiple studies have demonstrated that chronic alcohol exposure downregulates CB1 receptor mRNA and protein levels, affecting receptor density and function (Basavarajappa et al., 1998; Ortiz et al., 2004; Vinod et al., 2006). In humans, chronic heavy drinking is associated with reduced CB1 receptor availability in several brain regions, often persisting beyond periods of abstinence (Ceccarini et al., 2014; Hirvonen et al., 2013; Wolfe et al., 2022). Conversely, genetic deletion or pharmacological antagonism of CB1 receptors reduces alcohol intake, further underscoring its involvement in alcohol-related behaviors (Maccioni et al., 2010).

While pharmacological interventions hold promise in addressing alcohol-induced cognitive dysfunction, nutritional strategies, such as supplementation with omega-3 polyunsaturated fatty acids (PUFAs), are emerging as effective and accessible alternatives. Prior studies have demonstrated that omega-3 PUFAs can ameliorate the loss of brain plasticity induced by prenatal alcohol exposure, suggesting their potential for preventing and treating alcohol-related neurodevelopmental damage (Joffre et al., 2019; Patten et al., 2013a,b; Serrano et al., 2023). Recent findings indicate that omega-3 restore synaptic plasticity and improve hippocampal-dependent learning and memory following alcohol exposure during critical developmental periods (Haidary et al., 2024; Serrano et al., 2025). Thus, omega-3 supplementation prevents the spatial and associative learning impaired in adolescent male rats exposed to chronic alcohol consumption. The improvement was associated with the reduction in hippocampal oxidative stress and neuroinflammation associated with lower levels of lipid peroxidation and interleukin-6 (Haidary et al., 2024). These findings are in line with previous investigations (Farooqui, 2012; Patten et al., 2013a) and reinforce the potential of omega-3 to exert neuroprotective effects by restoring redox balance and modulating inflammatory pathways. In addition, omega-3 fatty acids, particularly DHA, enhance membrane fluidity ensuring the function of membrane-associated receptors (Bazinet & Layé, 2014; Joffre et al., 2019), which facilitates synaptic transmission and plasticity (Patten et al., 2013a,b). They also influence neurotransmitter systems by modulating receptor expression and signaling cascades involved in long-term potentiation (LTP) and long-term depression (LTD) (Patten et al., 2013b; Serrano et al., 2024, 2025), which are crucial for learning and memory (Peñasco et al., 2020; Serrano et al., 2025).

Adolescent alcohol exposure has been shown to disrupt synaptic plasticity and alter CB1 receptor expression and function in the hippocampus (Peñasco et al., 2020; Risher et al., 2015; Sabeti, 2011; Sanz-Martos et al., 2023), a brain region essential for declarative, spatial, and associative memory. Our previous research has shown that adolescent binge drinking leads to a sustained reduction in CB1 mRNA expression in the mature hippocampus. This downregulation correlates with decreased CB1 receptor density in excitatory terminals of the dentate molecular layer, disrupting endocannabinoid-dependent (LTD) and contributing to memory impairments (Peñasco et al., 2020; Rico-Barrio et al., 2019, 2021). Therefore, understanding the role of the ECS in alcohol-induced synaptic alterations is essential for identifying therapeutic targets aimed at mitigating the long-term effects of adolescent alcohol exposure and treating AUD (Borgonetti et al., 2024; García-Baos et al., 2021; Kunos, 2020; Serrano et al., 2012, 2018; Stopponi et al., 2018; Wolfe et al., 2022). In particular, adolescent binge drinking decreases CB1 receptor expression in the CA1 region of the adult hippocampus after withdrawal, primarily affecting astrocytic localization and, to a lesser extent, excitatory synaptic terminals (Bonilla-Del Río et al., 2019). Similarly, our laboratory has also observed a reduction in CB1

receptors at excitatory synapses in the dentate molecular layer following adolescent alcohol exposure (Peñasco et al., 2020). Interestingly, CB1 receptor density increased at both excitatory and inhibitory synaptic terminals in the dentate molecular layer following omega-3 supplementation (Serrano et al., 2025). However, the effects of an omega-3-enriched diet on CB1 receptor localization in the CA1 hippocampus following alcohol withdrawal remains unexplored. This is important to understand due to the impact of alcohol on CA1 region, which contributes to associative memory formation and play a key role in incremental value learning (Jeong et al., 2018; Takamiya et al., 2021).

In the present study, we investigated whether omega-3 supplementation could modulate alcohol-induced alterations in CB1 receptor distribution in the CA1 region of the hippocampus in male mice. Using immunoelectron microscopy, we provide novel insights into the potential of omega-3 fatty acids to counteract long-term neurobiological consequences of adolescent binge-like alcohol exposure.

Method

Ethics statement

The protocols for animal care and use were approved by the Committee of Ethics for Animal Welfare of the University of the Basque Country (M20-2020-113; date of approval: 09/29/2020). They were also in agreement with the European Communities Council Directive of September 22, 2010 (2010/63/EU) and Spanish regulations (Real Decreto 53/2013, BOE 08-02-2013). The number of animals and suffering were controlled and minimized.

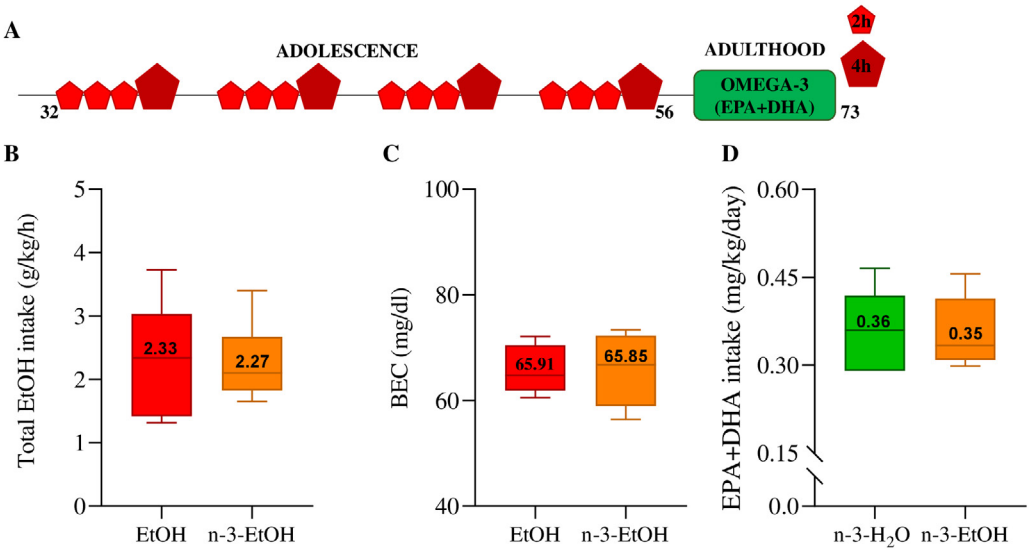
Animal treatment

Four-week-old C57BL/6J male mice (Janvier Labs, Le Genest-Saint-Isle, France) were housed in pairs and randomly distributed in H_2O or alcohol (EtOH) group (n= 12/group). The mice underwent the drinking-in-the-dark (DID) procedure (Rhodes et al., 2005) between postnatal days (PND) 32 and 56 for a period of four weeks, as previously described (Bonilla-Del Río et al., 2019). Briefly, the first four days of each week, mice were individualized with a 10 mL bottle containing tap water or EtOH solution (20% v/v EtOH, prepared from 96% EtOH; Boter S.L., Barcelona, Spain). Mice had access to the bottle for 2 hours on the first three days and the access was extended to 4 hours on the fourth day. During the remaining three days of each week, they were kept resting with food and water *ad libitum* (Figure 1A).

The effectiveness of the DID procedure was evaluated by measuring total EtOH intake (g/kg/h) (Figure 1B). Furthermore, on the last day of DID (PND 56), blood samples were collected from the lateral tail vein 30 minutes after the end of EtOH exposure. Blood ethanol concentration (BEC, mg/dL) was then measured using a commercial ethanol assay kit (Abcam ab65646, Spain). (Figure 1C). In our experimental conditions, BECs were below the threshold associated with adolescent binge drinking (>80 mg/

Figure 1

Schematic representation of the drinking-in-the-dark (DID) model, voluntary ethanol (EtOH) intake, blood ethanol concentration (BEC), and EPA+DHA intake



Note. **A**. C57BL/6J male mice underwent the DID procedure for four weeks (PND 32-56). During this period, they had free access to either H_2O or EtOH for 2 hours on the first three days of each week, and for 4 hours on the fourth day. During the abstinence period (PND 57–73), half of the mice were fed a diet enriched with omega-3 (2% EPA+DHA: 1.2% EPA and 0.8% DHA). **B.** Average EtOH intake per hour (g/kg/h) throughout DID (PND 32-56) (EtOH: 2.33 ± 0.90, n=6; n-3-EtOH: 2.27 ± 0.62, n=6). **C.** BEC (mg/dL) measured after 4 hours of EtOH exposure on the final day of the DID procedure (PND 56) (EtOH: 65.91 ± 4.59, n=6; n-3-EtOH: 65.85 ± 6.96, n=6). **D.** Average daily EPA+DHA intake (mg/kg/day) during the withdrawal period (PND 57–73) (n-3-H_2O: 0.36 ± 0.07, n=6; n-3-EtOH: 0.35 ± 0.06, n=6). All data are expressed as mean ± ***S.E.M.***

dL) (Donovan, 2009). However, these levels have been shown to produce significant neurobiological alterations in adolescent rodents (Bonilla-Del Río et al., 2019; Peñasco et al., 2020; Rico-Barrio et al., 2019; Serrano et al., 2025), thereby supporting the validity of this protocol as binge-like exposure model (Rhodes et al., 2005).

During the withdrawal period (PND 57–73), half of the mice (n = 6/group) were fed a diet enriched with 2% docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (1.2% EPA and 0.8% DHA) (SAFE, Augy, France), corresponding to the n-3-H_2O and n-3-EtOH groups. Mice and food were weighed twice a week to assess EPA and DHA intake (mg/kg/day) (Figure 1D).

Brain tissue processing

On PND 73, three mice from each group were deeply anesthetized using 4% chloral hydrate (10 mL/kg body weight, i.p.) and perfused through the left ventricle with 30 mL of phosphate-buffered saline (PBS, 0.1 M, pH 7.4). This was followed by perfusion with 80 mL of a fixative solution (4% formaldehyde, 0.2% picric acid, and 0.1% glutaraldehyde) prepared in PBS at room temperature (RT). The brains were removed and post-fixed in the same fixative solution at 4°C for one week before being transferred to a 1:10 diluted fixative. Coronal sections of 50 µm thickness were then cut using a vibratome (Leica VT 1000s, Wetzlar, Germany) and collected in phosphate buffer (PB, 0.1 M, pH 7.4) at RT.

Double pre-embedding immunogold and immunoperoxidase method for electron microscopy

The method was conducted based on our previously described protocol (Puente et al., 2015). In brief, 50 µm thick coronal sections of the hippocampus were pre-incubated at RT for 30 minutes in a solution of 10% bovine serum albumin (BSA) and Tris-HCl buffered saline (TBS) (pH 7.4) containing 0.1% sodium azide and 0.02% saponin. Then, the sections were incubated with primary antibodies highly specific against the CB1 receptor and glutamate aspartate transporter (GLAST) (Table 1) in 10% BSA/TBS with 0.1% sodium azide and 0.004% saponin. This step was performed with gentle agitation over two days at 4°C. After extensive washing, sections were incubated with secondary antibodies (Table 1), followed by an incubation in an avidin-biotin complex solution (1:50, PK-7100, Vector Labs) for 1.5 hours at RT. Sections were then washed overnight and post-fixed for 10 minutes in 1% glutaraldehyde prepared in TBS at RT.

Gold particles were then silver-enhanced using an HQ Silver Enhancement Kit (Nanoprobes Inc.) for 12 minutes in the dark. Following this, sections were treated with 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 0.1 M PB for 3 minutes at RT. After multiple washes, stained sections were post-fixed in 1% osmium tetroxide in 0.1 M PB for 20 minutes, dehydrated in a graded series of alcohols (50–100%) followed by propylene oxide, and embedded in Epon resin 812. Finally, ultrathin sections (50 nm) were cut with a diamond knife (Diatome USA), collected on nickel mesh grids, and stained with 2.5% lead citrate for 20 minutes. These sections were examined using an electron microscope (JEOL JEM 1400 Plus, Tokyo, Japan) and imaged with a digital camera (Morada, sCMOS, Olympus, Tokyo, Japan). Images were captured at 8,000X magnification, covering a total area of approximately 1,100 µm² per mouse. The number of excitatory and inhibitory terminals, as well as GLAST-stained astrocytes, was consistent across experimental groups (Table 2).

Statistical analysis

It was performed using GraphPad Prism 8 (GraphPad Software; RRID: SCR_002798). Data normality was assessed with the Shapiro–Wilk test, followed by a one-way ANOVA and either parametric or nonparametric multiple comparison tests, as appropriate. All values are presented as mean ± S.E.M.

Table 1

Primary and secondary antibodies used in pre-embedding immunocytochemistry for electron microscopy

Antibody	Concentration [µg/mL]	Species and clonality	Immunizing antigen	Source, Cat.
Anti-CB1 receptor	2	Guinea pig polyclonal	mouse CB1, C-terminal 31 aa (NM007726)	Nittobo Medical Co., CB1-GP-Af530
Anti-GLAST	0.3	Rabbit polyclonal	mouse GLAST, C-terminal 41 aa (NM148938)	Nittobo Medical Co., GLAST-Rb-Af660
1.4 nm gold-conjugated anti-guinea pig	0.8	Goat (polyclonal)	-	Nanoprobes, #2055
Biotinylated anti-rabbit	7.5	Goat (polyclonal)	-	Vector Labs, BA-1000

Table 2

Number of excitatory and inhibitory terminals, and GLAST-stained astrocytes in mice from each experimental group

	H_2O				EtOH				n-3-EtOH				n-3-H_2O			
	n 1	n 2	n 3	**Total**	n 1	n 2	n 3	**Total**	n 1	n 2	n 3	**Total**	n 1	n 2	n 3	**Total**
Exc.ter	458	396	283	**1137**	387	467	430	**1284**	228	375	276	**879**^^	356	291	372	**1019**
Inh.ter	35	54	63	**152**	38	38	17	**93***	37	33	45	**115**	45	75	52	**172**^^†
Astros	115	279	238	**632**	102	214	126	**442**	129	135	343	**607**	350	382	179	**911**^^

Note. Statistical significance was analyzed using a one-way ANOVA with either parametric (Bonferroni) or non-parametric (Dunn) multiple comparison tests, as appropriate. *p<0.05 compared to H_2O; ^^p<0.01 compared to EtOH and †p<0.05 compared to n-3-EtOH.

Results

CB1 receptor localization in excitatory and inhibitory synaptic terminals in CA1 *stratum radiatum* two weeks after withdrawal from adolescent binge drinking

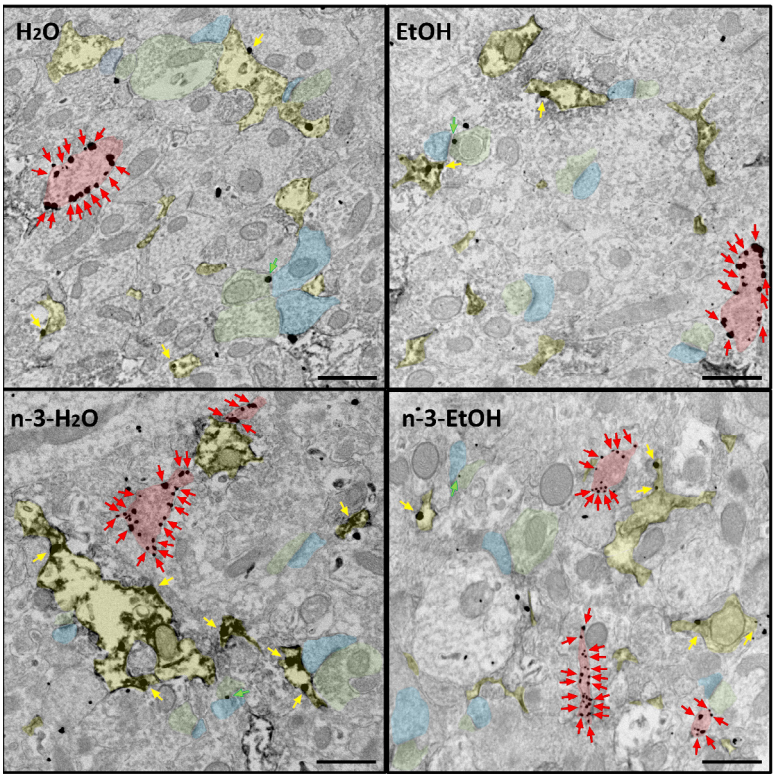
As expected, gold particles were localized in both excitatory and inhibitory axon terminals, forming asymmetric and symmetric synapses with dendritic spines and dendrites, respectively, in all experimental groups. Analyses were performed on three animals per group (Figure 2).

The total number of excitatory terminals in EtOH (~1,100 µm² per animal) was significantly decreased in n-3-EtOH mice (EtOH: 1,284; n-3-EtOH: 879; EtOH vs. n-3-EtOH **p<0.01) (Figure 3A) (Table 2). Moreover, the number of inhibitory terminals was significantly lower in EtOH mice compared to both H_2O and n-3-H_2O mice (H_2O: 152; n-3-H_2O: 172; EtOH: 93; EtOH vs. H_2O *p<0.05 and n-3-H_2O **p<0.01). There was no statistically significant difference between EtOH and n-3-EtOH mice; however, n-3-H_2O mice exhibited a significantly higher number of inhibitory terminals compared to n-3-ETOH (n-3-EtOH: 115; n-3-H_2O: 172; n-3-EtOH vs. n-3-H_2O *p<0.05) (Figure 3A) (Table 2). Hence, the ratio of excitatory/inhibitory synaptic terminals was significantly increased in the EtOH mice relative to the other experimental groups (H_2O: 8.36 ± 1.12; n-3-H_2O: 6.83 ± 0.88; EtOH: 25.57 ± 8.11, n-3-EtOH: 8.21 ± 0.88; EtOH vs. H_2O and n-3-EtOH *p<0.05, and vs. n-3-H_2O **p<0.01) (Figure 3B).

Both the percentage of CB1-positive excitatory terminals and the density of CB1 particles were similar across the four experimental conditions (n-3-H_2O, EtOH, n-3-EtOH vs. H_2O p>0.05) (Figure 3C and D) (Table 3). In addition, not significant differences were found in the percentage of CB1-positive inhibitory terminals (n-3-H_2O, EtOH, n-3-EtOH vs. H_2O p>0.05). However, receptor density increased significantly following omega-3 supplementation in H_2O control mice (H_2O: 6.54 ± 0.24, n-3-H_2O: 7.67 ± 0.23; n-3- H_2O vs. H_2O **p<0.01) (Figure 3C and D) (Table 3).

Figure 2

Immunoelectron localization of CB1 receptors in the CA1 stratum radiatum of H_2O, n-3-H_2O, EtOH, and n-3-EtOH male mice



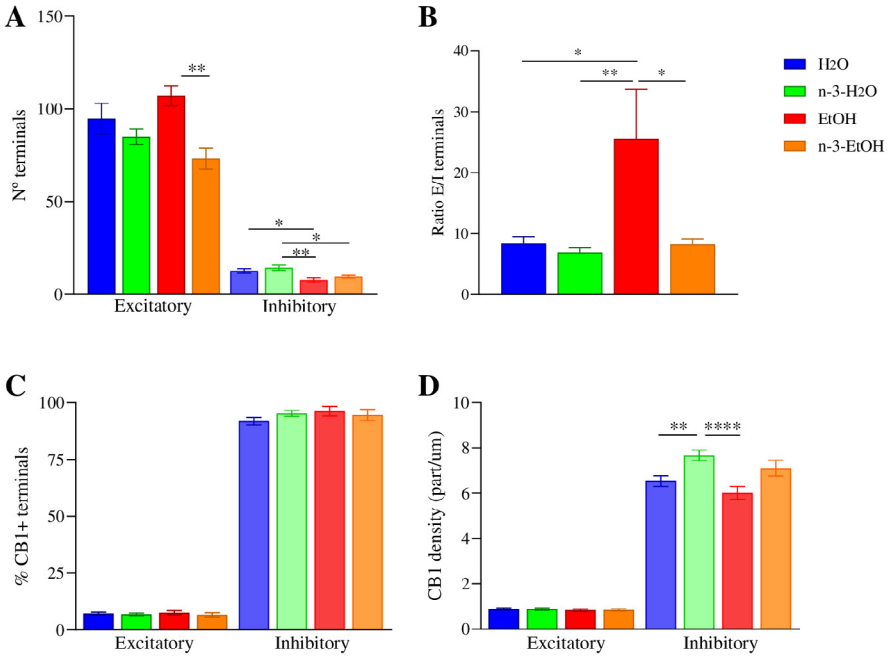
Note. CB1 receptors are localized to axon terminals forming symmetric (red shading and arrows) and asymmetric (green shading and arrows) synapses—the latter with dendritic spines (blue shading)—as well as to astrocytic membranes (yellow shading and arrows). Scale bars = 1 µm.

CB1 receptor localization in GLAST-stained astrocytes in the CA1 stratum radiatum two weeks after withdrawal from adolescent binge drinking

Gold particles were also localized to astrocytic membranes in all four experimental groups (Figure 2). The total number of GLAST-positive astrocytes in the analyzed area (~1,100 µm² per animal) was significantly higher in n-3-H_2O mice, in contrast to the trend toward reduction

Figure 3

Ultrastructural localization of the CB1 receptor in neuronal compartments in the CA1 stratum radiatum of H_2O, n-3-H_2O, EtOH and n-3-EtOH adult male mice



Note. **A.** Total number of excitatory (H_2O: 1,137; n-3-H_2O: 1,019; EtOH: 1,284; n-3-EtOH: 879, **p<0.01 vs. EtOH) and inhibitory terminals (H_2O: 152; n-3-H_2O: 172; EtOH: 93, *p<0.05 vs. H_2O, **p<0.01 vs. n-3-H_2O; n-3-EtOH: 115, *p<0.05 vs. n-3-H_2O) in the analyzed area (~1,100 µm²) of the four experimental groups. **B.** Ratio of total excitatory and inhibitory terminals (H_2O: 8.36 ± 1.12; n-3-H_2O: 6.83 ± 0.88; EtOH: 25.57 ± 8.11, *p<0.05 vs. H_2O, **p<0.01 vs. n-3-H_2O; n-3-EtOH: 8.21 ± 0.88, *p<0.05 vs. EtOH). **C.** Percentage of CB1-positive excitatory (H_2O: 7.18 ± 0.58; n-3-H_2O: 6.86 ± 0.54; EtOH: 7.57 ± 0.91; n-3-EtOH: 6.59 ± 0.91) and inhibitory terminals (H_2O: 91.89 ± 1.68; n-3-H_2O: 95.30 ± 1.29; EtOH: 96.30 ± 2.09; n-3-EtOH: 94.64 ± 2.41). **D.** CB1 receptor density (particles/µm) in excitatory (H_2O: 0.89 ± 0.05; n-3-H_2O: 0.89 ± 0.05; EtOH: 0.84 ± 0.05; n-3-EtOH: 0.84 ± 0.05) and inhibitory terminals (H_2O: 6.54 ± 0.24; n-3-H_2O: 7.67 ± 0.23, **p<0.01 vs. H_2O; EtOH: 6.02 ± 0.29, ****p<0.0001 vs. n-3-H_2O; n-3-EtOH: 97.11 ± 0.35). All data are expressed as mean ± S.E.M. The Shapiro–Wilk test was used to assess normality, followed by one-way ANOVA with either parametric (Bonferroni) or non-parametric (Dunn) multiple comparison tests, as appropriate.

Table 3

Percentage of CB1-positive excitatory terminals, inhibitory terminals, GLAST-stained astrocytes, and receptor density in the CA1 stratum radiatum across the four experimental conditions (n = 3 mice per group)

	H_2O	EtOH	n-3-EtOH	n-3-H_2O
Excitatory terminals				
% CB1+	7.18 ± 0.58	7.57 ± 0.91	6.59 ± 0.91	6.86 ± 0.54
Density	0.89 ± 0.05	0.84 ± 0.05	0.84 ± 0.05	0.89 ± 0.05
Inhibitory terminals				
% CB1+	91.89 ± 1.68	96.30 ± 2.09	94.64 ± 2.41	95.30 ± 1.29
Density	6.54 ± 0.24	6.02 ± 0.29	7.11 ± 0.35	7.67 ± 0.23**††††
Astrocytes				
% CB1+	26.92 ± 2.97	19.26 ± 2.67	22.48 ± 2.53	16.65 ± 1.38*
Density	0.64 ± 0.03	0.64 ± 0.05	0.70 ± 0.06	0.66 ± 0.04

Note. Values are expressed as mean ± S.E.M. Statistical significance was assessed using one-way ANOVA with either parametric (Bonferroni) or non-parametric (Dunn) multiple comparison tests, as appropriate. *p<0.05, **p<0.01 compared to H_2O; and ††††p<0.0001 compared to n-3-EtOH.

observed in EtOH mice (n-3-H_2O: 991; EtOH: 442; n-3-H_2O vs. EtOH **p<0.01) (Figure 4A) (Table 2).

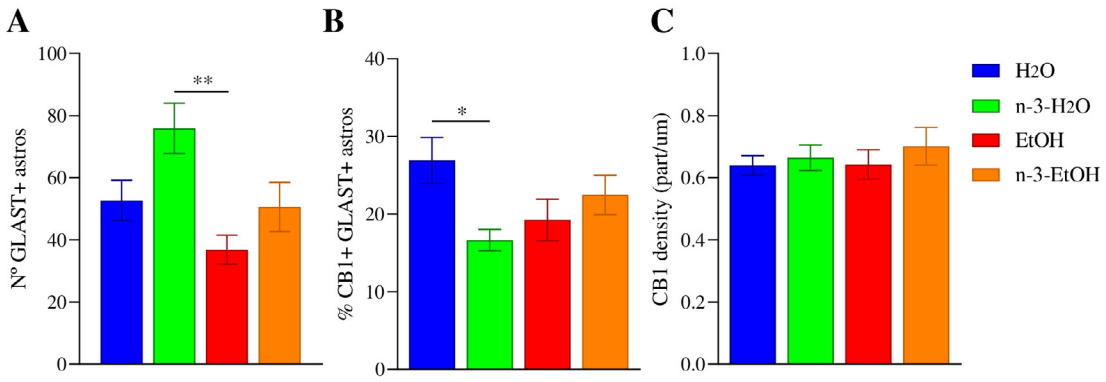
Accordingly, the percentage of CB1-positive, GLAST-stained astrocytes was reduced in n-3-H_2O mice compared to H_2O controls (H_2O: 26.92 ± 2.97; n-3-H_2O: 16.65 ± 1.38; n-3-H_2O vs. H_2O *p<0.05) (Figure 3B). However, CB1 receptor density was similar across the four conditions (n-3-H_2O, EtOH, n-3-EtOH vs. H_2O p>0.05) (Figure 3C) (Table 3).

Discussion

We analyzed the subcellular distribution of the CB1 receptor in excitatory and inhibitory terminals, as well as in astrocytes, in the *stratum radiatum* of the CA1 region of the hippocampus. Using electron microscopy, we achieved high-resolution localization of CB1, allowing detailed insights into the long-term synaptic effects of adolescent binge drinking following withdrawal, and the modulatory influence of an omega-3-supplemented diet. Omega-3 fatty acids, particularly DHA and EPA, are essential for maintaining brain health. As major components of neuronal membranes, omega-3 fatty acids influence cell membrane

Figure 4

Ultrastructural localization of CB1 receptors in astrocytes of the CA1 stratum radiatum in H_2O, n-3-H_2O, EtOH, and n-3-EtOH adult male mice



Note. **A.** Total number of GLAST-stained astrocytes in the analyzed area (~1,100 µm²) for the four experimental groups (H_2O: 632; n-3-H_2O: 991; EtOH: 442, **p<0.01 vs. n-3-H_2O; n-3-EtOH: 607) in the analyzed area (~1,100 µm²). **B.** Percentage of CB1-positive astrocytes (H_2O: 26.92 ± 2.97; n-3-H_2O: 16.65 ± 1.38, *p<0.05 vs. H_2O; EtOH: 19.26 ± 2.67; n-3-EtOH: 22.48 ± 2.53). **C.** CB1 receptor density (particles/µm) in GLAST-stained astrocytes (H_2O: 0.64 ± 0.03; n-3-H_2O: 0.66 ± 0.04; EtOH: 0.64 ± 0.05; n-3-EtOH: 0.70 ± 0.06). All data are expressed as mean ± S.E.M. The Shapiro–Wilk test was used to assess normality, followed by one-way ANOVA with either parametric (Bonferroni) or non-parametric (Dunn) multiple comparison tests, as appropriate.

fluidity, and thus, membrane-associated receptor activity, and synaptic plasticity (Calder, 2016). DHA is especially abundant in the brain and plays a critical role in synapse pruning and neuroprotection (Aguilera García et al., 2010). However, alcohol consumption impairs DHA absorption, leading to reduced brain DHA levels, which exacerbates neural damage, particularly during adolescence (Akbar et al., 2006; Bondi et al., 2014). Conversely, omega-3 supplementation can restore DHA levels in the brain, promoting synaptogenesis, glutamatergic activity, and CB1 receptor expression (Hashimoto et al., 2015; Kim, 2008).

The results of this investigation demonstrate a significant increase in the ratio of excitatory to inhibitory synaptic terminals in alcohol-exposed mice. This imbalance in the stability between excitation and inhibition—critical for normal behavior and cognition—supports previous findings that adolescent alcohol exposure impairs synaptic organization and plasticity, particularly in the hippocampus (Peñasco et al., 2020; Spear, 2018). Such disruption may underlie cognitive deficits observed in adulthood (Serrano et al., 2025; Vetreno & Crews, 2015). In this sense, a persistent excitation/inhibition imbalance in the CA1 region of the mouse hippocampus following a single postnatal binge-like alcohol exposure has been associated with dysregulation of synaptic and mitochondrial gene expression, including Cnr1, which encodes CB1 receptors. This dysregulation contributes to long-lasting synaptic dysfunction, impaired learning and memory, and increased anxiety-like behaviors (Arzua et al., 2024). Remarkably, our data show that omega-3 supplementation significantly reduced the number of excitatory terminals in the adult CA1 hippocampus following adolescent binge alcohol exposure, while having minimal effects on inhibitory terminals. As a result, the dietary intervention effectively restored the balance between excitatory and inhibitory synaptic terminals, highlighting the role of omega-3 fatty acids in maintaining synaptic integrity.

Furthermore, our findings fit with previous reports on the neuroprotective effects of omega-3 against alcohol-induced neurotoxicity (Patten et al., 2013b), and suggest that omega-3 may engage compensatory mechanisms to re-establish homeostatic synaptic plasticity disrupted by binge-like alcohol exposure. Such restoration may involve modulation of membrane composition and fluidity—key factors influencing synaptogenesis, synaptic pruning, and receptor trafficking (Calder, 2016; Kim & Spector, 2018)—as well as the preservation of synaptic networks (Cao et al., 2009; Feltham et al., 2020; Lafourcade et al., 2011).

Given the known role of CB1 receptors in regulating synaptic transmission, functional recovery may also involve changes in CB1 receptor activity or signaling, even in the absence of overt alterations in expression levels. Nevertheless, we observed that omega-3 supplementation increased CB1 receptor density in inhibitory terminals in water-control conditions, while leaving CB1 levels in excitatory terminals unchanged in all conditions examined. Previous studies have shown that omega-3 fatty acids enhance CB1 expression (Kim et al., 2016), and our earlier work demonstrated that omega-3 increased CB1 receptor expression by approximately 30% in hippocampal synaptosomes compared to water-treated controls (Serrano et al., 2024). This upregulation was associated with endocannabinoid-dependent LTP at excitatory medial perforant path–granule cell synapses in the dentate molecular layer, reduced anxiety-like behavior, and significantly improved novel object discrimination

(Serrano et al., 2024). Mechanistically, the potentiation of synaptic plasticity required group I metabotropic glutamate receptors, 2-AG, and CB1 receptors (Serrano et al., 2024).

We have demonstrated recently that omega-3 supplementation during alcohol withdrawal increased CB1 receptor density in presynaptic terminals of the hippocampus in male mice, and restored receptor-stimulated [^{35}S]GTPγS binding to Gαi/o proteins. These changes were also associated with LTP at the medial perforant path excitatory synapses, which depended on anandamide, transient receptor potential vanilloid 1 (TRPV1), and NMDA receptors, and were linked to the partial alleviation of cognitive deficits, as assessed using the Barnes maze (Serrano et al., 2025). Based on our current findings, it is plausible that omega-3 fatty acids also influence inhibitory synaptic transmission and plasticity in both the dentate molecular layer and the CA1 *stratum radiatum*; however, further investigation is needed to confirm this hypothesis.

In male mice exposed to adolescent binge-like alcohol consumption, CB1 receptor density significantly increased in inhibitory terminals of the dentate molecular layer two weeks after withdrawal, with an even greater increase observed following omega-3 supplementation (Serrano et al., 2025). This was not the case in the CA1 *stratum radiatum*, where neither alcohol exposure nor omega-3 supplementation after alcohol intake altered CB1 density in either inhibitory or excitatory terminals. Interestingly, the CB1 density increase observed in inhibitory terminals of the CA1 *stratum radiatum* in omega-3-fed mice was not detected in the dentate molecular layer (Serrano et al., 2025). Together, these findings indicate that the long-term effects of adolescent binge-like alcohol exposure on CB1 receptors and the modulatory influence of omega-3 supplementation vary among hippocampal regions. Therefore, both the pathophysiological consequences of alcohol and the potential therapeutic benefits of omega-3 should be considered region- and subregion-specific within the hippocampus.

Regarding CB1 receptor expression in astrocytes, our laboratory previously demonstrated that adolescent binge drinking alters astrocytic morphology characterized by swollen branches, and reduces the proportion of CB1-positive astrocytic processes by 40% and CB1 receptor density by 30% in astrocytes in the CA1 *stratum radiatum* of adult male mice (Bonilla-Del Río et al., 2019)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. In contrast, the present study found no changes in CB1 expression in GLAST-labeled astrocytes under similar alcohol exposure conditions. A potential explanation for this discrepancy roots in the different astrocytic markers used in the studies. In our earlier work, we employed antibodies against glial fibrillary acidic protein (GFAP), a cytoskeletal protein primarily localized to astrocytic cell bodies. In contrast, GLAST is more abundantly expressed in astrocytic processes, covering a larger astroglial area and membrane surface than GFAP (Achicallende et al., 2022). Remarkably, CB1 receptors are more accurately localized in GLAST-stained astrocytes, with up to 12% of total CB1 receptor labeling found in GLAST-positive astrocytes—twice the amount observed with GFAP—in the CA1 region of the hippocampus. Given these characteristics, GLAST appears to be a more suitable marker than GFAP for detailed studies of CB1 receptor distribution in astrocytes (Achicallende et al., 2022).

Given the absence of CB1 alterations in GLAST-labeled astrocytes, it seems that the anti-inflammatory signaling via astrocytic CB1 receptors (Metna-Laurent & Marsicano, 2015) remains intact two weeks after withdrawal from adolescent binge drinking. Similarly, CB1 receptor expression in astrocytes in the dentate molecular layer remained unchanged under the same conditions (Serrano et al., 2025), reinforcing the idea that CB1 receptors in GLAST-labeled astrocytes in the hippocampus are not significantly affected in adulthood by adolescent alcohol exposure.

Interestingly, omega-3 supplementation alone led to notable changes in both GLAST-labeled astrocytes and CB1 receptor distribution within astroglial compartments in the CA1 region. Thus, we observed a significant increase in the number of GLAST-positive astrocytic compartments in omega-3-supplemented mice compared to the alcohol group, together with a reduction in the proportion of CB1-positive, GLAST-labeled astrocytes relative to the H_2O control group, without any change in receptor density. These findings suggest that omega-3 may influence astrocytic morphology, potentially increasing the complexity of astrocytic processes in n-3-H_2O mice, as previously reported in other models of neuroinflammation and neuroprotection (Champeil-Potokar et al., 2016). DHA, in particular, is known to promote astrocyte differentiation, enhancing the complexity and ramification of astrocytic processes—features associated with the anti-inflammatory properties of omega-3 PUFAs (Das & Das, 2019; Joffre et al., 2019). Therefore, the reduced proportion of CB1-positive astrocytes in n-3-H_2O mice may result from extensive astrocytic branching. Taken together, these results suggest that omega-3 may modulate astrocyte structure and indirectly influence CB1 receptor localization in astrocytes through morphological remodeling, rather than through direct changes in receptor expression.

In conclusion, our results contribute to the growing body of evidence that dietary interventions can modulate the endocannabinoid system, highlighting a promising non-pharmacological approach to mitigate the adverse effects of adolescent alcohol exposure on brain function. Future

studies are required to clarify the underlying mechanisms, particularly how omega-3 fatty acids influence the excitatory-inhibitory balance through CB1 receptors on inhibitory terminals, and modulate astrocyte function via these receptors. Moreover, future research should include female subjects to determine the extent to which adolescent alcohol intake affects CB1 receptor localization and the impact of omega-3 supplementation.

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Conflict of interests

The authors declare no conflict of interests.

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