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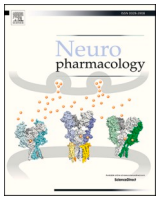

# Adolescent intermittent ethanol exposure decreases perineuronal nets in the hippocampus in a sex dependent manner: Modulation through pharmacological inhibition of RPTPβ/ζ

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ABSTRACT

Adolescence is a critical period for brain maturation in which this organ undergoes critical plasticity mechanisms that increase its vulnerability to the effects of alcohol. Significantly, ethanol-induced disruption of hippocampal neurogenesis has been related to cognitive decline in adulthood. During adolescence, the maturation of perineuronal nets (PNNs), extracellular matrix structures highly affected by ethanol consumption, plays a fundamental role in neurogenesis and plasticity in the hippocampus. Receptor Protein Tyrosine Phosphatase (RPTP) β/ζ is a critical anchor point for PNNs on the cell surface. Using the adolescent intermittent access to ethanol (IAE) model, we previously showed that MY10, a small-molecule inhibitor of RPTPβ/ζ, reduces chronic ethanol consumption in adolescent male mice but not in females and prevents IAE-induced neurogenic loss in the male hippocampus. We have now tested if these effects of MY10 are related to sex-dependent modulatory actions on ethanol-induced effects in PNNs. Our findings suggest a complex interplay between alcohol exposure, neural structures, and sex-related differences in the modulation of PNNs and parvalbumin (PV)-positive cells in the hippocampus. In general, IAE increased the number of PV + cells in the female hippocampus and reduced PNNs intensity in different hippocampal regions, particularly in male mice. Notably, we found that pharmacological inhibition of RPTPβ/ζ with MY10 regulates ethanol-induced alterations of PNNs intensity, which correlates with the protection of hippocampal neurogenesis from ethanol neurotoxic effects and may be related to the capacity of MY10 to increase the gene expression of key components of PNNs.

## 1. Introduction

Ethanol remains the most widely consumed drug among adolescents and young adults, and there has been a concerning reversal in the decreasing trend of alcohol consumption among young people since 2020 (Johnston et al., 2021). Initiation of alcohol use before age 15 increases four times the risk of developing Alcohol Use Disorder (AUD) later in life compared to those who delay alcohol initiation until age 21 or later (Tapert, 2022). Additionally, young people often engage in binge-like alcohol consumption, which differs significantly from adult drinking patterns and confers a considerable risk to neurodevelopment (Tetteh-Quarshie and Risher, 2023). Notably, risky drinking behavior among young women has been progressively increased during the last years (Becker et al., 2017), highlighting the importance of studying sex differences in alcohol-induced harm on the developing brain.

The central nervous system (CNS) undergoes critical plasticity mechanisms during adolescence, which increases its vulnerability to the effects of alcohol and other insults. Ethanol overuse has been observed to affect different brain structures, such as the hippocampus, an area crucial for memory and learning (Crews et al., 2019). Of particular concern is ethanol-induced disruption of neurogenesis, which plays a

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**Abbreviations**

| | |
|---|---|
| PTN | pleiotrophin |
| RPTPβ/ζ | Receptor Protein Tyrosine Phosphatase β/ζ |
| IAE | intermittent access to ethanol |
| PNNs | perineuronal nets |
| DG | dentate gyrus |
| AUD | alcohol use disorder |
| CNS | central nervous systems |
| ALK | anaplastic lymphoma kinase |
| BBB | blood brain barrier |
| WFA | Wisteria floribunda agglutinin |

critical role in developing select brain regions throughout life (Crews et al., 2016). Furthermore, it has been shown that there are significant sex differences in how alcohol affects neurogenesis (Guerri and Pascual, 2019). Considering these differences, identifying mechanisms that underlie ethanol-induced hippocampal injury at stages of neurodevelopment is essential.

Pleiotrophin (PTN) is a neurotrophic factor that regulates behavioral responses to ethanol. It has been previously shown that *Ptn*$^{-/-}$ mice are more susceptible to the rewarding effects of ethanol, whereas mice with transgenic PTN overexpression in the brain are more resistant (Vicente-Rodríguez et al., 2014). PTN is an endogenous inhibitor of Receptor Protein Tyrosine Phosphatase (RPTP) β/ζ (a.k.a. PTPRZ1, RPTPζ, and Phosphacan) (Herradon et al., 2019; Herradón and Pérez-García, 2014). To mimic these PTN actions with drug-like compounds, we designed and synthesized a series of compound inhibitors of RPTPβ/ζ, selecting MY10 as the lead candidate (Pastor et al., 2018). Using the intermittent access to ethanol (IAE) model during adolescence, we recently showed that treatment with MY10 effectively reduced chronic ethanol consumption in adolescent male mice but not in females (Galán-Llario et al., 2023a). In that study, we observed a significant ethanol-induced decrease in hippocampal neurogenesis only in male mice, which correlated with an increased microglial response to this drug. In contrast, this correlation was not observed in mice treated with MY10, in which neurogenic loss was prevented (Galán-Llario et al., 2023a). The modulation of ethanol-induced microglial responses by MY10 may protect hippocampal neurogenesis shown by this compound but does not seem to be the only mechanism involved.

During adolescence, perineuronal nets (PNNs) maturation affects cognitive flexibility and plasticity. PNNs are extracellular matrix structures that form around neurons' soma and proximal dendrites, primarily surrounding GABAergic interneurons expressing the calcium-binding protein parvalbumin (PV) as a marker. PNNs play a significant role in the plasticity of these neurons (Reichelt et al., 2019), influencing the excitatory/inhibitory synaptic balance (Carceller et al., 2022; Carulli and Verhaagen, 2021). Additionally, PNNs have been implicated in alcohol and drug addiction (Lasek, 2016; Lasek et al., 2018). Adolescent mice exposed to alcohol display increased expression of PNN components in the orbitofrontal cortex and prefrontal cortex in adulthood (Coleman et al., 2014; Dannenhoffer et al., 2022). Furthermore, alcohol consumption in periodic binge episodes, using the drinking in the dark (DID) model, intensifies PNNs in the insula, a crucial region for addiction and compulsive drug seeking (Chen et al., 2015; Naqvi et al., 2014). In the hippocampus, PNNs play a fundamental role in neurogenesis and learning (Cope and Gould, 2019; Crapser et al., 2020), and alcohol exposure during adolescence leads to changes in the expression of PV + neurons (Liu and Crews, 2017). Collectively, existing evidence suggests that modulation of ethanol-induced effects on PNNs may contribute to restoring functional connectivity in different brain areas by rebalancing the excitatory/inhibitory ratio (Dannenhoffer et al., 2022).

RPTPβ/ζ has been identified as a critical anchor point for PNNs on the cell surface (Eill et al., 2020; Sinha et al., 2023). Notably, we demonstrated that a single administration of MY10 increases the intensity of PNNs in the hippocampus of male adolescent mice but not in females (Galán-Llario et al., 2023a). This result suggests that the reduction of alcohol consumption and hippocampal neurogenic loss observed only in MY10-treated male mice (Galán-Llario et al., 2023a) is related to sex-dependent modulatory actions on ethanol-induced effects in PNNs. Hence, to test if the pharmacological modulation of RPTPβ/ζ regulates the effects of alcohol on PNNs, we have now analyzed extensively PNNs and PV + cells in the hippocampi from MY10-treated male and female adolescent mice that underwent the IAE procedure (Galán-Llario et al., 2023a).

## 2. Materials & methods

### 2.1. Experimental subjects

In the present study, we characterized the changes in PNNs in the brains of mice exposed to the intermittent access to ethanol (IAE) procedure previously published (Galán-Llario et al., 2023a). In that study, male and female C57BL/6J mice, aged 5–6 weeks at the beginning of the procedure, were used. The IAE protocol involved a 2-bottle choice procedure in which, on alternate days, one of two water bottles was replaced with an ethanol solution for 24 h. During the initial week, ethanol concentration was progressively escalated (3 %, 6 %, 10 % v/v on consecutive drinking days) (Galán-Llario et al., 2023a). Subsequently, mice had 24-h access to a 20 % v/v ethanol solution on Monday, Wednesday, and Friday of each week for 3 weeks. During this period, mice received an administration of MY10 (60 mg/kg, p.o.) (Pastor et al., 2018) or its vehicle (10 % dehydrated ethanol, 20 % polysorbate 80, 70 % PEG-300) 1 h prior to exposure to the ethanol solution during each drinking session. The same protocol was also applied to control mice that exclusively received water throughout the four-week experimental period.

For the gene expression analysis, 5–6 weeks old male and female C57BL/6J mice received a dose of 15, 30, 60, or 120 mg/kg of MY10 or vehicle (p.o.).

Mice were housed in a meticulously controlled environment, maintaining a temperature of 22 ± 1 °C and adhering to a 12-h light and 12-h dark cycle. They were provided unrestricted access to food and water throughout the experimental period.

All procedures involving animals in this study strictly adhered to the guidelines set forth by the European Union Laboratory Animal Care Rules, as outlined in the 2010/63/EU directive. The research protocols used in this study received ethical approval from the Animal Research Committee of USP-CEU (authorization reference: PROEX 76.0/20). These animal studies were conducted in full accordance with the ARRIVE guidelines.

### 2.2. Immunohistochemical analysis

#### 2.2.1. Tissue collection

Following the final drinking session of the IAE procedure (Galán-Llario et al., 2023a), mice were humanely euthanized using pentobarbital and subsequent transcardial perfusion with 4 % paraformaldehyde. Brains were post-fixed overnight and then incubated in 30 % sucrose. Thirty μm sections were obtained using a sliding microtome (Leica SM2010 R). Immunohistochemistry studies were conducted on one slice per 360 μm for the hippocampus, from the bregma −1.94 mm to −2.4 mm.

#### 2.2.2. Staining of perineuronal nets and parvalbumin-positive neurons

For the study of PNNs, sections were blocked in a glycoprotein-free blocking solution diluted to 1x concentration from a 10x commercial solution (Vector Laboratories, CA, USA; ref: SP5040) for 1h at room temperature (RT). Subsequently, they were incubated with biotinylated

Wisteria floribunda agglutinin (WFA, Vector Laboratories, CA, USA; 1:2000) for 24h at 4 °C. Sections were then incubated with Dylight 488-conjugated streptavidin for 1h at RT (Vector Laboratories, CA, USA; 1:200). To visualize PV + neurons, sections were re-blocked with 5 % donkey serum for 1h at RT after WFA staining and then incubated with a PV antibody (Synaptic Systems, Göttingen, Germany; 1:2000) for 24h at 4 °C, followed by incubation with Alexa Fluor 555-conjugated mouse IgG for 1h at RT (Invitrogen, Waltham, MA, USA; 1:200).

Photomicrographs were captured using a digital camera attached to an optical microscope (DM5500B, Leica, Solms, Germany) and subsequently analyzed using the LAS X Core software (Leica Microsystems, Wetzlar, Germany; offline version). Two images at 10x magnification (size, 1600× 1200 pixels) were taken from each animal per hippocampal region (DG, CA1, CA2, CA3). The selection of image field locations was guided by the Paxinos and Franklin mouse atlas (4th edition), and we adhered to the stereotaxic coordinates described in the previous section (2.2.1).

#### 2.2.3. Image analysis

For PNNs analysis, two images per animal (n = 4–6/group) within the corresponding hippocampus area were selected. WFA fluorescence intensity of PNNs for each image was quantified using an established method in ImageJ with the macro plugin "Perineuronal net Intensity Program for the Standardization and Quantification of ECM Analysis" (PIPSQUEAK AI v5.3.9, Rewire Neuro, Inc.) following the "WFA" method. Each data point represented the fluorescent WFA intensity in a single image, calculated as the average fluorescent intensity of all WFA + cells within the image.

To categorize PNNs into low, medium, and high intensity, quartiles were utilized as cutoff values based on the intensity of PNNs in images of vehicle-treated mice within the corresponding area. Low-intensity PNNs were defined as those below the 25th percentile, medium-intensity PNNs were between the 25th and 75th percentile, and high-intensity PNNs were in the 75th percentile. For PV quantification, PV + cells were manually outlined, saved as regions of interest (ROIs), and subsequently analyzed using the PIPSQUEAK software following the "ROI" method. Colocalization of WFA/PV was also assessed using PIPSQUEAK's "Double label analysis" option.

### 2.3. Gene analysis

Two hours after the administrations of the different doses of MY10, mice were euthanized by cervical dislocation and brains were removed. Relative gene expression of aggrecan (*Acan*) and Rptpβ/ζ (also known as protein tyrosine phosphatase receptor type Z1 (*Ptprz1*)) in the hippocampus were analyzed by real-time polymerase chain reaction (RT-PCR). Brains from male and female mice (n = 9/group) treated with different doses of MY10 or its vehicle were removed from the skull and frozen at −80 °C. These samples were cut in a cryostat (−10 °C), obtaining coronal sections of 500 μm. Following the atlas of Paxinos and Franklin, brain nuclei of interest were microdissected following the procedure described by Palkovits and previously performed by our group (Navarrete et al., 2012; Palkovits, 1983). Total ribonucleic acid (RNA) was extracted with TRI Reagent extraction, and reverse transcription was carried out (Applied Biosystems, Madrid, Spain). Quantitative analyses of the relative gene expression of *Acan* (Mm00545794_m1) and *Ptprz1* (Mm00478484_m1) genes were performed on the StepOne Sequence Detector System (Applied Biosystems, Madrid, Spain). Data for each target gene was normalized to the endogenous reference gene 18S (Mm03928990_g1), and the fold change in target gene expression was determined using the 2-ΔΔCt method (Livak and Schmittgen, 2001).

### 2.4. Statistics

All statistical analyses were performed using GraphPad Prism version 8 (San Diego, CA, USA). Data are presented as mean ± standard error of the mean (SEM). Data from WFA and PV immunohistochemistry analysis were analyzed using three-way ANOVA considering sex, treatment and ethanol drinking as variables. When relevant, to better dissect the effect of each variable, we used a two-way ANOVA, excluding the non-significant variable if the three-way ANOVA results allowed it. Differences were analyzed by post-hoc comparisons with Bonferroni's post-hoc tests. To compare low-, medium-, and high-intensity PNNs, a Chi-squared test ($\chi 2$) was performed to evaluate the distribution among experimental groups. Data from gene expression analysis were analyzed using one-way ANOVA followed by the Student-Newman-Keuls test. $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. IAE decreases PNNs intensity in the male dentate gyrus and increases PV + cells in females

We previously showed that treatment with MY10 prevents the loss of neurogenesis in the DG induced by IAE in adolescent male mice (Galán-Llario et al., 2023a). We now aimed to investigate if this beneficial effect of MY10 is accompanied by a modulation of ethanol-induced changes in PNNs in this area (Fig. 1). Three-way ANOVA revealed a significant reduction in PNNs intensity following ethanol consumption ($F(1,69) = 3.988$, $P = 0.0498$; Fig. 1b) and a significant sex effect on WFA intensity ($F(1,69) = 6.987$, $P = 0.0102$; Fig. 1b), prompting to separate the analysis in both sexes. Then, we found that the decrease in PNNs intensity was primarily attributed to ethanol consumption in the male PNNs, as 2-way ANOVA showed a significant effect of the drinking variable on WFA intensity in the DG of male mice ($F(1,36) = 4.132$, $P = 0.0495$; Fig. 1b). In contrast, we did not observe a significant effect of ethanol drinking in PNNs intensity in females ($F(1,33) = 0.7582$, $P = 0.3902$; Fig. 1b). Interestingly, ethanol increased the proportion of lower-intensity PNNs solely in male mice (d.f. (1), $\chi 2 = 5.704$, $P = 0.0169$; Fig. 1c). In females, ethanol consumption did not significantly affect the distribution of PNNs intensity (d.f. (1), $\chi 2 = 0.4059$, $P = 0.5241$; Fig. 1d); However, pretreatment with MY10 prior to the drinking sessions increased the percentage of high-intensity PNNs compared to vehicle-pretreated female mice (d.f. (1), $\chi 2 = 5.007$, $P = 0.0252$; Fig. 1d). Additionally, when examining PV + cells in the DG, 3-way ANOVA revealed a significant interaction between sex, treatment and drinking variables ($F(1,72) = 9.922$, $P = 0.024$; Fig. 1e). After the subsequent post-hoc analysis, we observed significantly lower PV + cell numbers in females compared to males in basal conditions ($P = 0.0091$; Fig. 1e). Notably, ethanol alone ($P = 0.032$; Fig. 1e), as well as the combination of treatment with MY10 and ethanol consumption ($P = 0.0154$; Fig. 1e), increased the number of PV + cells in the female DG. We did not detect significant changes in PNNs intensity surrounding PV + cells (Fig. 1g). However, a significant effect of the treatment ($F(1,70) = 4.632$, $P = 0.0348$; Fig. 1f) and a significant effect of the sex ($F(1,70) = 8.934$, $P = 0.0061$; Fig. 1f) was observed in PNNs intensity not surrounding PV + neurons (PV- cells). The subsequent ANOVA within each sex revealed a strong trend of ethanol decreasing the intensity of PNNs in males ($F(1,36) = 9.293$, $P = 0.0575$; Fig. 1f). In addition, we observed a 50–60 % colocalization of PV + neurons with PNNs, which was not significantly altered by ethanol or treatment (Supplementary Fig. 1a). However, we detected an increase in the percentage of WFA + cells colocalizing with PV in male and female mice treated with MY10. Ethanol drinking also induced this effect only in the DG of female mice (Supplementary Fig. 1a).

Taken together, our results suggest that IAE reduces PNNs intensity in the DG, particularly in male mice, mainly affecting PNNs surrounding non-PV + neurons. On the other hand, PV + cells in the female DG are more susceptible to the effects of IAE and MY10 treatment.

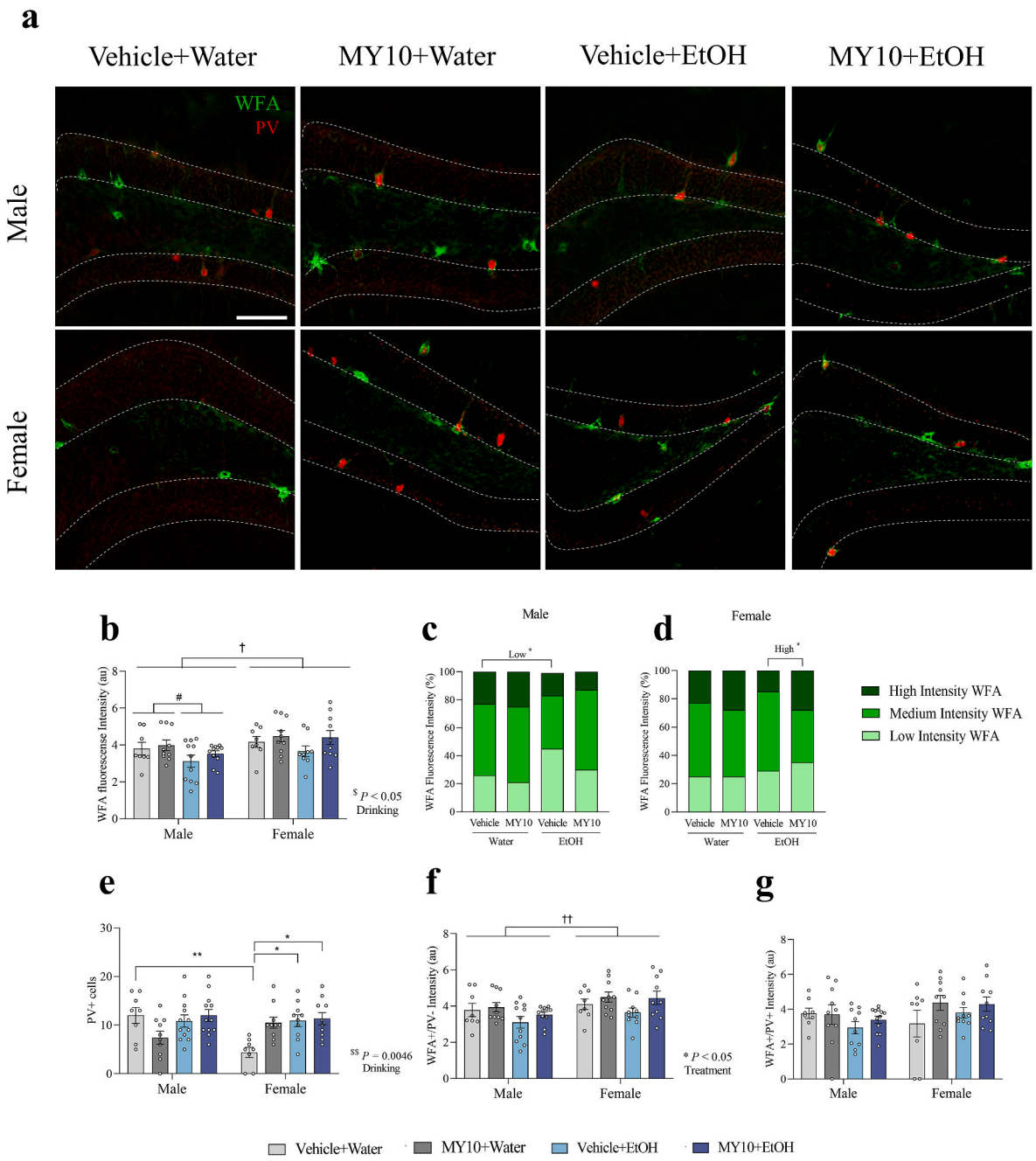


**Fig. 1.** PNNs and PV + cells in dentate gyrus of adolescent mice after IAE. Photomicrographs of WFA (green) and PV fluorescence (red) binding from vehicle- and MY10-treated male and female mice (n = 4–6/group). Dashed lines indicate the outline of the granule cell layer of the hippocampal dentate gyrus (a). Graphs represent data (mean ± S.E.M) from the quantification of WFA intensity (b). The percentages of PNNs corresponding to different WFA intensity levels (c,d), quantification of PV + number cells (e), quantification of WFA intensity of PV- cells (f) and of PV + cells (g) are also shown. *$P < 0.05$; **$P < 0.01$. $^{\#}P < 0.05$. $^{\$}P < 0.05$; $^{\$\$}P < 0.01$ for significant effect of drinking. $^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$ for significant effect of sex. Scale bar = 100 μm.

### *3.2. IAE decreases PNNs intensity in the male hippocampal CA1 region and increases PV + cells in females*

We also investigated other hippocampus areas to determine whether ethanol and MY10 specifically target regions with different proportions of immature neurons. In the CA1 region (Fig. 2), we observed that ethanol drinking reduced the intensity of PNNs ($F(1,73) = 4.492$, $P = 0.0471$; Fig. 2b) and detected a significant difference between sexes ($F(1,73) = 9.878$, $P = 0.0038$; Fig. 2b). The subsequent ANOVA performed within each sex revealed that ethanol consumption selectively decreased WFA intensity in the CA1 of male mice ($F(1,38) = 5.375$, $P = 0.0259$; Fig. 2b). In contrast, we did not observe a significant effect of ethanol drinking in PNNs intensity in females ($F(1,35) = 0.7566$, $P = 0.393$; Fig. 2b–d). Interestingly, ethanol decreased the percentage of high-intensity PNNs only in the male CA1 region (d.f. (1), $\chi2 = 3.107$, $P = 0.001$; Fig. 2c). Notably, pretreatment with MY10 during the IAE protocol reversed this effect of ethanol on the high intensity PNNs (d.f. (1), $\chi2 = 3.979$, $P = 0.0461$; Fig. 2c) and reduced the percentage of low-intensity nets (d.f. (2), $\chi2 = 2.205$, $P = 0.0275$; Fig. 2c).

The 3-way ANOVA demonstrated a significant interaction between sex and drinking on PV + cells ($F(1,70) = 7.648$, $P = 0.0118$; Fig. 2e). Post-hoc analysis revealed that ethanol significantly increased the number of PV + cells in the female CA1 region ($P = 0.0223$; Fig. 2e), an effect that was not altered by pretreatment with MY10. In the

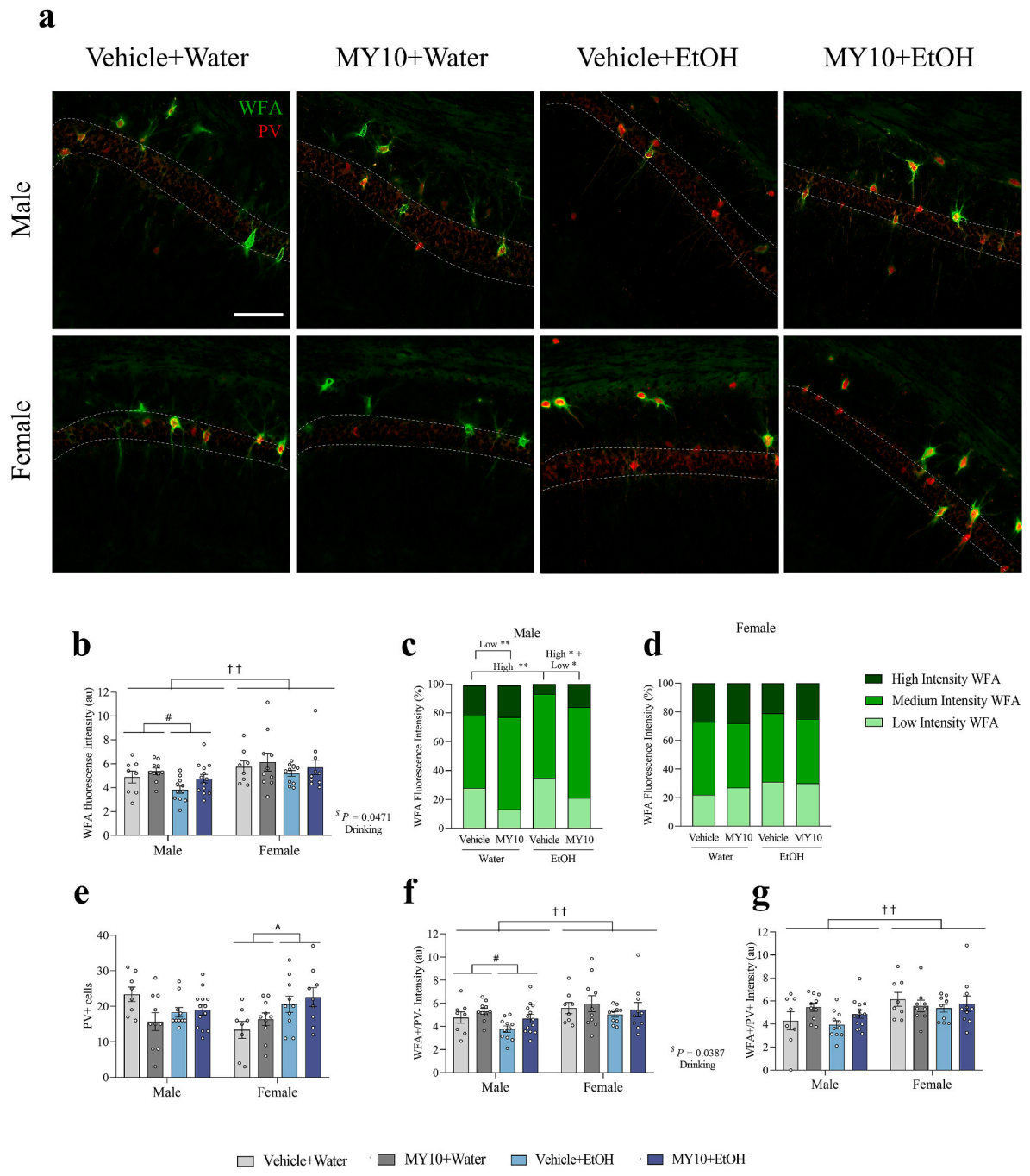


**Fig. 2.** PNNs and PV + cells in CA1 of mice after adolescent IAE. Photomicrographs of WFA (green) and PV fluorescence (red) binding from vehicle and MY10 treated male and female mice (n = 4–6/group). Dashed lines indicate the outline of the granule cell layer of the hippocampal CA1 (a). Graphs represent data (mean ± S.E.M) from the quantification of WFA intensity (b). The percentages of PNNs corresponding to different WFA intensity levels (c,d), quantification of PV + number cells (e), quantification of WFA intensity of PV- cells (f) and of PV + cells (g) are also shown. $^{*}P < 0.05$; $^{**}P < 0.01$. $^{\#}P < 0.05$. $^{\wedge}P < 0.05$. $^{\$}P < 0.05$ for significant effect of drinking. $^{\dagger\dagger}P < 0.01$ for significant effect of sex. Scale bar = 100 μm.

colocalization analysis, ANOVA indicated a significant effect of ethanol ($F(1,72) = 5.000$, $P = 0.0387$; Fig. 2f) and a significant effect of the sex ($F(1,72) = 8.501$, $P = 0.0076$; Fig. 2f) on PNNs intensity not surrounding PV + cells. The subsequent ANOVA within each sex revealed that ethanol decreased the intensity of PNNs surrounding non-PV + neurons in the male CA1 region ($F(1,38) = 5.307$, $P = 0.0268$; Fig. 2f). In the case of PNNs intensity surrounding PV + neurons, we detected a significant effect of the sex ($F(1,71) = 9.462$, $P = 0.0030$; Fig. 2g), but we did not observe significant effects of ethanol consumption or MY10 treatment. In addition, we observed that treatment with MY10 induced a decrease in the percentage of PV + cells colocalizing with WFA only in the CA1 of female mice (Supplementary Fig. 1b). We did not detect significant changes in the percentage of WFA + cells colocalizing with PV (Supplementary Fig. 1b).

These results suggest that IAE reduces PNNs intensity in the CA1 of male mice, mainly affecting PNNs surrounding non-PV + neurons, an effect of ethanol consumption that seems to depend on RPTPβ/ζ activity. Furthermore, ethanol appears to increase the number of PV + cells in the CA1 of female adolescent mice, while MY10 does not seem to modulate this effect.

### 3.3. IAE and MY10 modulate both PNNs and PV + cells in CA2 and CA3 areas of adolescent mice

To further investigate the impact of IAE in the hippocampus and its possible modulation by the pharmacological inhibition of RPTPβ/ζ, we examined PNNs and PV + cells in the CA2 and CA3 regions. In the CA3 region (Fig. 3), the 3-way ANOVA analysis of WFA intensity revealed a significant difference between sexes ($F(1,70) = 8.562$, $P = 0.0046$; Fig. 3b) and a significant interaction between sex and drinking ($F(1,70) = 7.849$, $P = 0.0066$, Fig. 3b). Post-hoc analysis indicated that ethanol drinking decreased the intensity of PNNs in male mice ($P = 0.0307$, Fig. 3b). In addition, the PNNs of male mice were significantly less intense than those of female mice after ethanol consumption ($P = 0.0010$, Fig. 3b). However, when dividing the PNNs based on intensity percentile, no significant difference between groups was found in male mice (Fig. 3c). In females, low-intensity nets were decreased in the ethanol drinking group pretreated with MY10 compared to the group pretreated with vehicle (d.f. (1), $\chi 2 = 6.349$, $P = 0.0117$, Fig. 3d). We also detected a significant reduction of high-intensity PNNs in female mice only treated with MY10 (d.f. (1), $\chi 2 = 4.678$, $P = 0.0305$, Fig. 3d). Unlike the other hippocampal regions, we did not observe differences in the number of PV + cells in the CA3 region (Fig. 3e). Colocalization

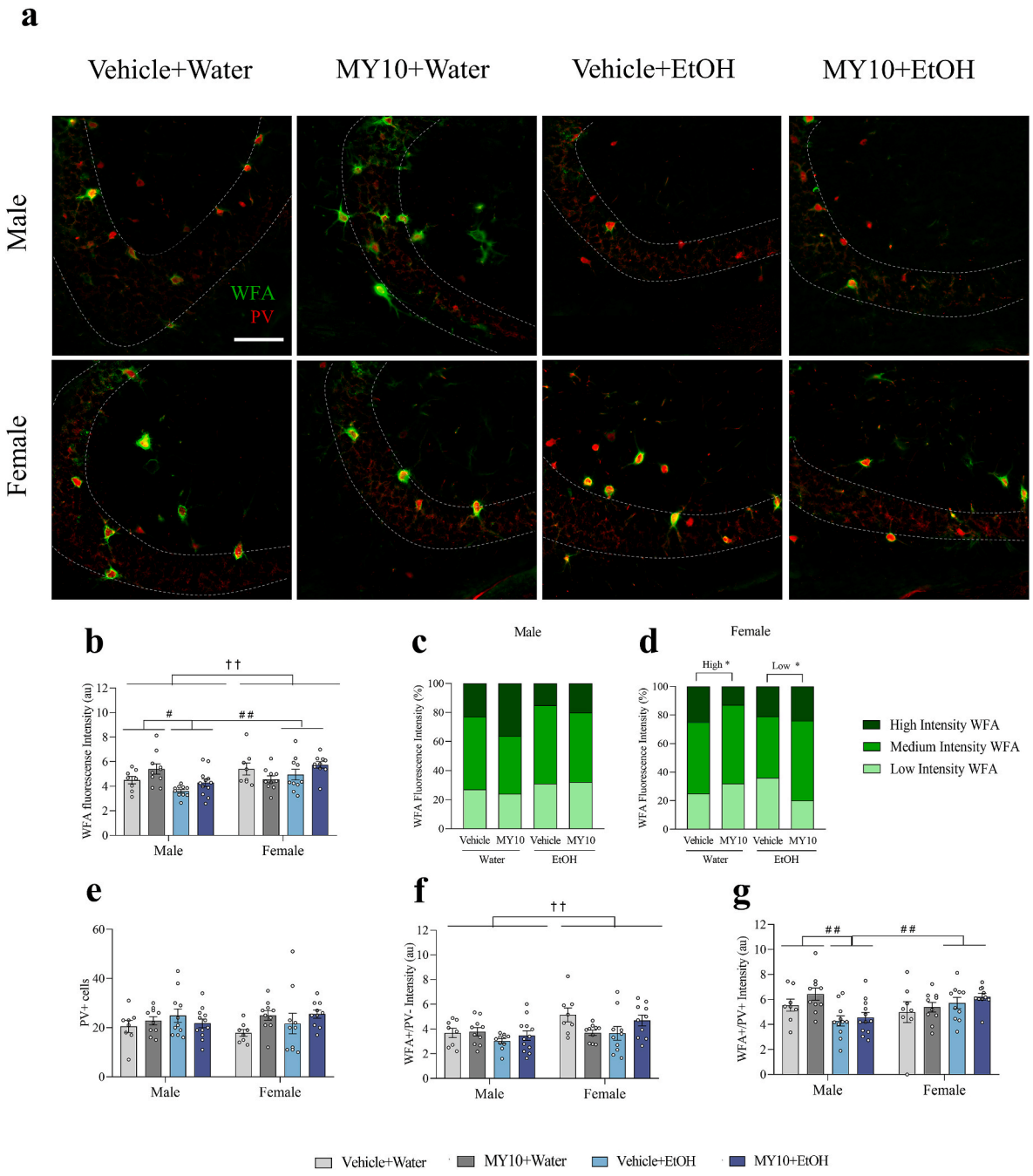


**Fig. 3.** PNNs and PV + cells in CA3 of mice after adolescent IAE. Photomicrographs of WFA (green) and PV fluorescence (red) binding from vehicle and MY10 treated male and female mice (n = 4–6/group). Dashed lines indicate the outline of the granule cell layer of the hippocampal CA3 (a). Graphs represent data (mean ± S.E.M) from the quantification of WFA intensity (b). The percentages of PNNs corresponding to different WFA intensity levels (c,d), quantification of PV + number cells (e), quantification of WFA intensity of PV- cells (f) and of PV + cells (g) are also shown. $^{*}P < 0.05$. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$. $^{\dagger\dagger}P < 0.01$ for significant effect of sex. Scale bar = 100 μm.

analysis revealed that ethanol only affected the intensity of PNNs surrounding PV + cells (Fig. 3f and g). ANOVA revealed an interaction between sex and drinking (F (1,71) = 12.58, $P = 0.0007$; Fig. 3g), with ethanol decreasing the intensity of PNNs surrounding PV + cells in male mice ($P = 0.0042$; Fig. 3g), which were also significantly less intense than those in female mice ($P = 0.0051$; Fig. 3g). In addition, we did not detect significant changes in the percentage of PV + cells colocalizing with WFA (Supplementary Fig. 1c). However, we detected that ethanol drinking induced an increase in the percentage of WFA + cells colocalizing with PV only in the CA3 region of female mice (Supplementary Fig. 1c).

In the CA2 region (Fig. 4), the 3-way ANOVA of PNNs intensity showed a significant decrease in WFA intensity after ethanol consumption (F (1,71) = 19.27, $P < 0.0001$; Fig. 4b) and a significant sex difference (F (1,71) = 6.961, $P = 0.0102$, Fig. 4b). The 2-way ANOVA within each sex revealed that, in contrast to other hippocampal areas, IAE significantly decreased WFA intensity in CA2 of both males (F (1,37) = 10.73, $P = 0.0023$, Fig. 4b) and females (F (1,34) = 8.895, $P = 0.005$, Fig. 4b). Additionally, ethanol reduced the percentage of high-intensity nets in males (d.f. (1), $\chi 2 = 4.065$, $P = 0.0438$; Fig. 4c) and females (d.f. (1), $\chi 2 = 12.47$, $P = 0.0004$; Fig. 4d), and significantly increased the percentage of low-intensity nets in females (d.f. (1), $\chi 2 = 4.147$, $P = 0.0417$; Fig. 4d). MY10 did not seem to exert any modulatory actions on these ethanol effects. No significant changes were observed in

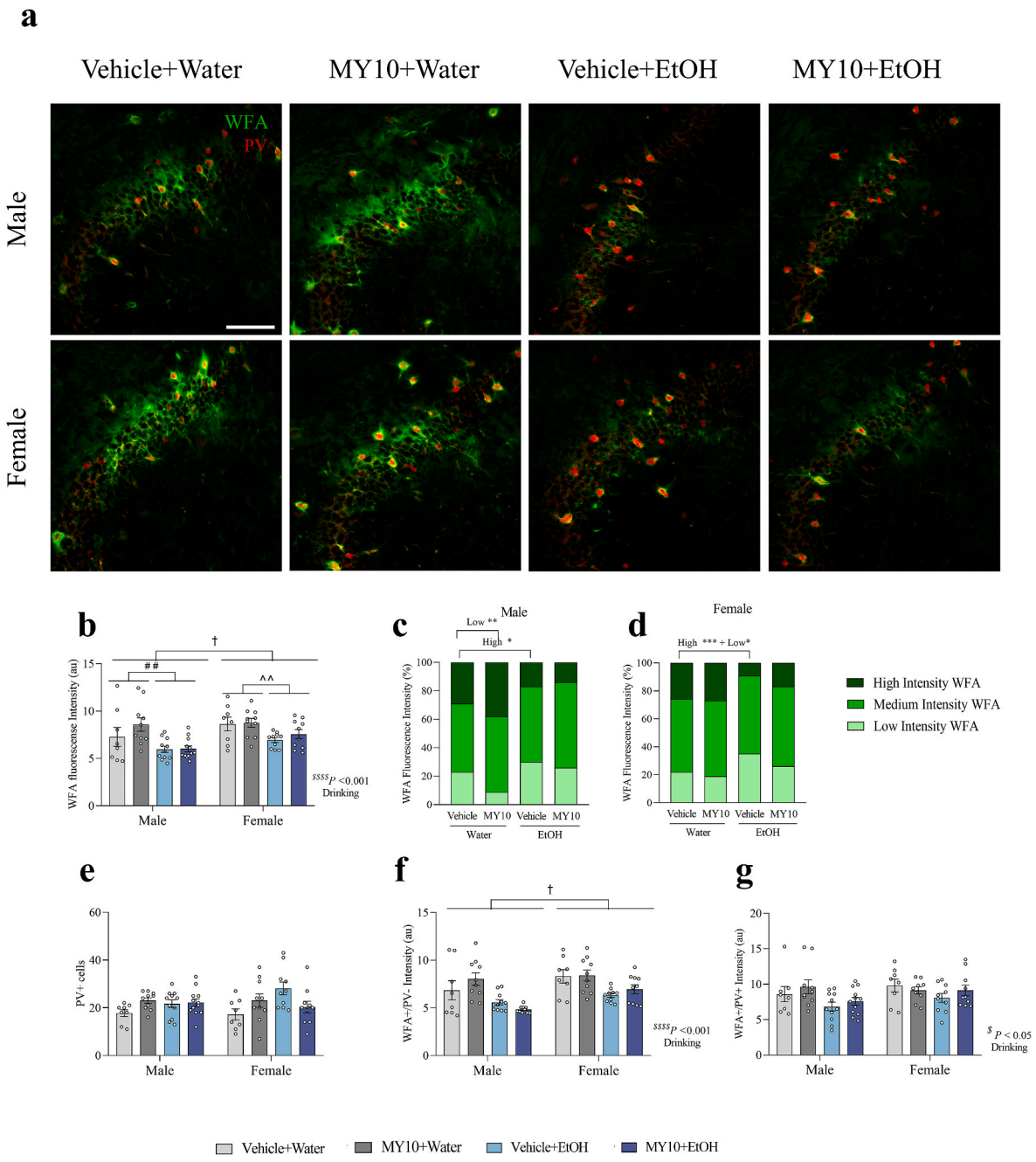


**Fig. 4.** PNNs and PV + cells in CA2 of mice after adolescent IAE. Photomicrographs of WFA (green) and PV fluorescence (red) binding from vehicle and MY10 treated male and female mice (n = 4–6/group) (a). Graphs represent data (mean ± S.E.M) from the quantification of WFA intensity (b). The percentages of PNNs corresponding to different WFA intensity levels (c,d), quantification of PV + number cells (e), quantification of WFA intensity of PV- cells (f) and of PV + cells (g) are also shown. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. $^{\#\#}P < 0.01$ $^{\wedge\wedge}P < 0.01$ $^{\$}P < 0.05$; $^{\$\$\$\$}P < 0.0001$ for significant effect of drinking. $^{\dagger}P < 0.05$ for significant effect of sex. Scale bar = 100 μm.

the number of PV + cells in this region (Fig. 4e). Colocalization analysis indicated that the intensity of PNNs not surrounding PV + cells were reduced by ethanol drinking in both sexes ($F(1,69) = 27.15$, $P < 0.0001$; Fig. 4f), whereas the PNNs intensity surrounding PV + cells were unaffected (Fig. 4g). In the case of the CA2 region we did not detect any relevant changes in the percentage of PV + cells colocalizing with WFA or in the percentage of WFA + cells colocalizing with PV (Supplementary Fig. 1d).

In conclusion, intermittent alcohol exposure during adolescence had differential effects on PNNs and PV + cells in the CA2 and CA3 regions of the hippocampus. Ethanol consumption decreased PNNs intensity, and these effects were influenced by sex in both areas but were only modulated by MY10 treatment in the female CA3 region. These findings suggest a complex interplay between alcohol exposure, neural structures, and sex-related differences in the modulation of PNNs and PV + cells in the hippocampus.

### 3.4. Real-time PCR of Acan and Prptz1 in the hippocampus of male and female mice treated with MY10

In male mice, acute administration of MY10 significantly increased gene expression levels of *Acan* at doses of 60 and 120 mg/kg compared to the control group ($F(4,40) = 4.719$, $P < 0.003$, Fig. 5a), whereas no significant effects were observed at the doses of 15 and 30 mg/kg. Curiously, MY10 induced opposite changes in females, since a significant reduction of *Acan* levels was detected at all doses tested in comparison to vehicle-treated group. Indeed, ANOVA followed by Student-Newman-Keuls revealed that changes induced by the doses of 30, 60 and 120 mg/kg were significantly different compared to the dose of 15 mg/kg ($F(4,40) = 13.051$, $P < 0.001$; Fig. 5b) (n = 9/group).

*Prptz1* gene expression was significantly increased in males after acute administration of MY10 at doses of 60 and 120 mg/kg, whereas no effect was observed at doses of 15 and 30 mg/kg compared to the control group. Interestingly, the up-regulation of *Prptz1* induced by the 120 mg/kg dose of MY10 was significant compared to the 15 and 30 mg/kg doses ($F(4,40) = 8.166$, $p < 0.001$; Fig. 5c). However, MY10 did not alter *Prptz1* gene expression in females at any dose tested compared to the vehicle group ($F(4,40) = 0.392$, $P = 0.813$; Fig. 5d) (n = 9/group).

## 4. Discussion

Adolescence represents a pivotal period for CNS maturation, characterized by the closure of critical periods of neural plasticity (Tapert, 2022). Among the brain regions crucial for neuroplasticity, the hippocampus stands out. Its function is often compromised by substance abuse, leading to cognitive and learning impairments (Tetteh-Quarshie

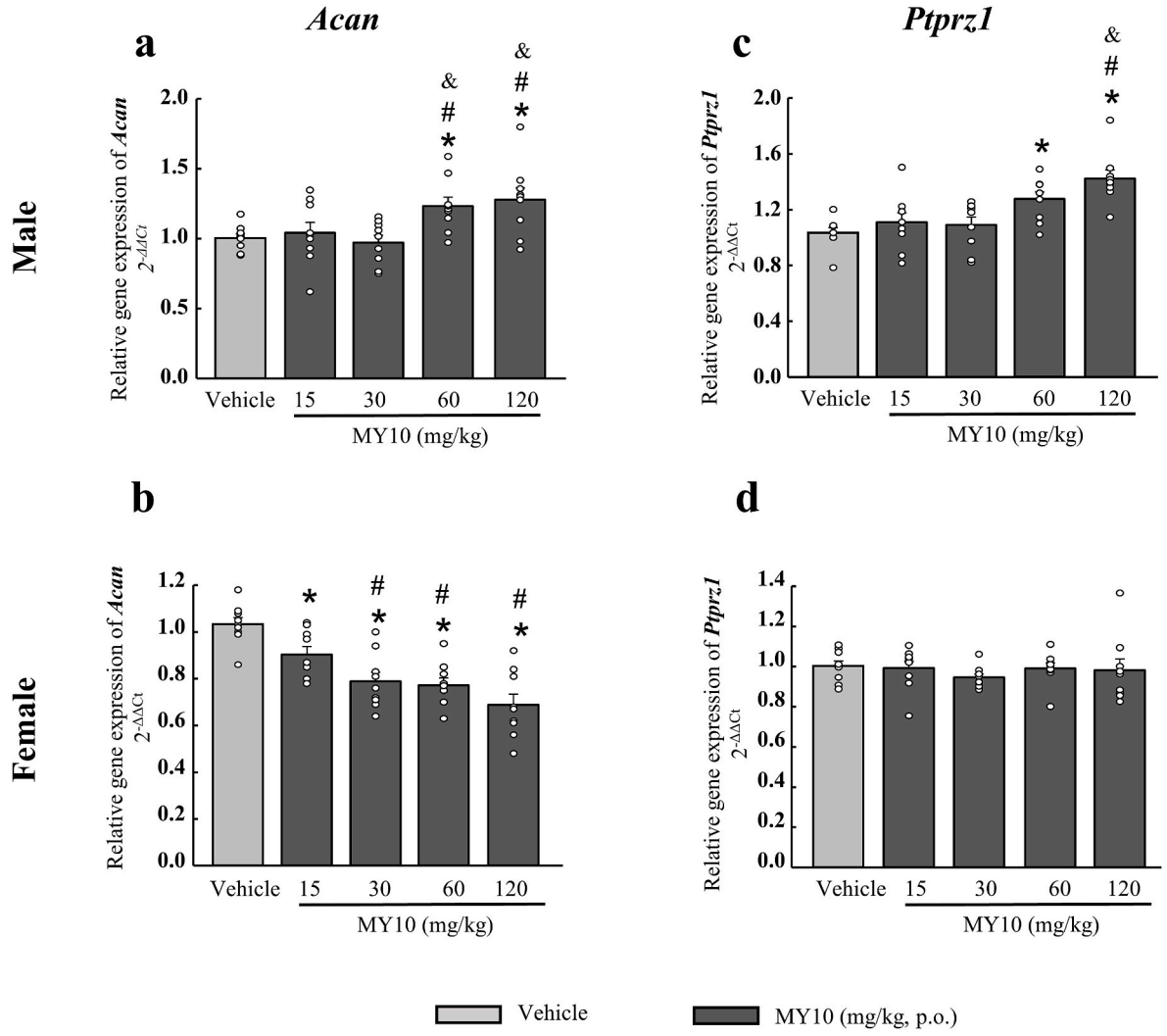


**Fig. 5.** Relative gene expression analysis by RT-PCR in male (a, c) and female (b, d) mice acutely treated with different doses of MY10 (15, 30, 60 and 120 mg/kg, p.o.). Aggrecan (*Acan*, a and b), and protein tyrosine phosphatase receptor type Z1 (*Ptprz1*, c and d) relative gene expression changes were evaluated in the hippocampus. $^{*}P < 0.05$ vs Vehicle. $^{\#}P < 0.05$ vs MY10-15 mg/kg $^{\&}P < 0.05$ vs MY10-30 mg/kg.

and Risher, 2023). The maturation of neural plasticity is closely associated with forming PNNs around specific neurons (Carulli and Verhaagen, 2021). Recent research has underscored alterations in these nets following alcohol consumption, which is particularly relevant given the prevalence of alcohol use among adolescents (Lasek, 2016). Sex-specific variations in how drugs impact PNNs have also been reported (de Carvalho et al., 2023).

In this study, we investigated the impact of intermittent exposure to ethanol on hippocampal PNNs in adolescent male and female mice. Recent studies have shown that adolescent mice exposed to alcohol exhibit PNNs increase in other areas, such as the orbitofrontal cortex and prefrontal cortex in adulthood (Coleman et al., 2014; Dannenhoffer et al., 2022). In contrast, our findings reveal, for the first time, a general decrease in PNNs intensity in the hippocampus following ethanol consumption during adolescence. Intriguingly, this ethanol-induced reduction in WFA intensity was more pronounced in the different regions studied in hippocampi from male mice. Previous studies have employed WFA intensity to indicate PNNs maturation (Vazquez-Sanroman et al., 2017), suggesting that chronic alcohol exposure during adolescence may render hippocampal PNNs more labile. Notably, diminished PNNs intensity has been linked to heightened plasticity, albeit at the cost of increased vulnerability to oxidative stress (Cabungcal et al., 2013). This may render hippocampal neurons more susceptible to reactive oxygen species following ethanol consumption. Importantly, our previous work showed that IAE reduces neuronal progenitors in the DG of adolescent male mice (Galán-Llario et al., 2023a), suggesting that ethanol-induced decrease of PNNs contributes to this neurogenic loss. In contrast, female doublecortin-positive (DCX+) neurons were unaffected by IAE, corresponding to the observed resilience of female hippocampal PNNs to ethanol (Galán-Llario et al., 2023a).

Our study shows a more pronounced decrease in PNNs intensity in the hippocampus of male subjects following ethanol exposure; however, it is noteworthy that females also exhibited PNNs alterations, particularly in the CA2 region. The PNNs of this region are known to be relatively more abundant and diffuse compared to other brain regions (Fawcett et al., 2022), a characteristic often associated with reduced neuronal plasticity. Moreover, the CA2 region is involved in social memory processes and is implicated in disorders such as autism (Reichelt et al., 2019). Therefore, investigating sex-specific variations in the effects of ethanol on PNNs within the CA2 region may offer valuable insights into the interplay between ethanol exposure, neural plasticity, and social cognition. It has to be noted that the stage of the estrous cycle was not assessed in this study; however, protocols extending far beyond the timeframe of the estrous cycle are deemed capable of encompassing all variations in the estrous cycle (Bloodgood et al., 2021), what is considered to prevent the need for assessment of the estrous cycle in the IAE procedure.

A wealth of literature underscores the potential of PNNs modulation to treat various disorders (Reichelt et al., 2019), yet there remains a pressing need for specific therapeutic molecules. In this context, our research group previously demonstrated that MY10, a selective small-molecule inhibitor of RPTPβ/ζ, increases PNNs intensity in the hippocampus of adult male mice (Galán-Llario et al., 2023a). RPTPβ/ζ is a pivotal component of the PNNs structure (Eill et al., 2020), binding to tenascin-C through its FNIII domain (Sinha et al., 2023). Here, we demonstrate that treatment with MY10 prior to ethanol exposure prevents the decrease in PNNs intensity in the CA1 and DG regions of male mice, which is consistent with our previous work showing that MY10 mitigates IAE-induced neurogenic loss in the male DG (Galán-Llario et al., 2023a). MY10 binds to the active D1 domain of RPTPβ/ζ, causing the inhibition of its phosphatase activity (Pastor et al., 2018). The results suggest that pharmacological inhibition of RPTPβ/ζ prevents ethanol-induced changes in hippocampal PNNs and, possibly through this action, preserves neurogenesis in the DG of the adolescent male mice. In the case of CA1 region, the relevance of these data needs to be further analyzed, since we did not detect evidence of the presence of immature neurons in this region when we conducted the study focused in the DG (Galán-Llario et al., 2023a).

To unravel the molecular mechanism underlying these actions of RPTPβ/ζ inhibition, we examined the effects of MY10 administration on the expression of genes encoding for two crucial components of the PNNs in the hippocampus of male and female mice, including RPTPβ/ζ (*Ptprz1*). Interestingly, we found a dose-response increase in the expression of both *Acan* and *Ptprz1* genes in male mice 2 h after the administration of MY10. In contrast, female mice showed a dose-response decrease of *Acan* expression in the hippocampus, while *Ptprz1* expression remained unchanged after MY10 administration, underscoring the sex-dependent nature of RPTPβ/ζ action, as previously suggested in different contexts (Galán-Llario et al., 2023a; Galán-Llario et al., 2023b). Both aggrecan and Ptprz1 (RPTPβ/ζ) are critical contributors to the formation and stabilization of PNNs (Sinha et al., 2023; Morawski et al., 2014). Thus, we hypothesize that the observed prevention of ethanol-induced decreases in the hippocampal PNNs of male mice treated with MY10 in our IAE paradigm may be related to the capacity of MY10 to increase the gene expression of these components of PNNs. All in all, a limitation of the present study is that the gene expression data have been obtained in the whole hippocampus and the precise regulatory mechanism governing these changes in gene expression necessitates further investigation.

PNNs primarily enshroud fast-spiking neurons, aiding in the rapid ionic exchange required for their prompt response (Tewari et al., 2022). Among these, PV + interneurons, crucial for regulating principal neuron activity in the hippocampus, are often surrounded by PNNs (Jakovljević et al., 2021). In the control animals used in our studies, we found that females had fewer PV + cells than males. However, in contrast to males, female mice that drank ethanol were treated with MY10, or the combination of both, and showed a substantial increase in PV + cell numbers in the hippocampus. This suggests that PV + interneurons in females may be more sensitive to external stimuli, potentially modulated by locally produced 17-beta-estradiol (Martins de Carvalho et al., 2023). However, in the hippocampus, it has been found that apart from PV + cells, other neurons like glutamatergic CAMKII + cells are also surrounded by PNNs (Lensjø et al., 2017). Intriguingly, the PNNs most affected by IAE and MY10 appeared to be those not surrounding PV + neurons. This effect was more pronounced in male mice, which may be relevant in light of recent evidence highlighting the importance of the hippocampus in drug reward processes (Belmer et al., 2022), a role that is not yet fully understood. Using this IAE paradigm, we previously demonstrated that MY10 treatment selectively reduced ethanol consumption in adolescent male mice. It is conceivable that the restorative role of MY10 in ethanol-induced alterations in PNNs may, as a result, regulate the modifications in hippocampal network connections that influence drug consumption patterns. However, further investigations are needed to elucidate this mechanism.

## 5. Conclusion

This study represents the first examination of chronic ethanol consumption's gender-specific effect on PNNs during adolescence. Pharmacological inhibition of RPTPβ/ζ regulates ethanol-induced alterations of PNNs intensity, which correlates with the protection of hippocampal neurogenesis from ethanol neurotoxic effects.

**Declaration of interest:** None.

The datasets used the current study will be available from the corresponding author on reasonable request.

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## CRediT authorship contribution statement

**Milagros Galán-Llario:** Formal analysis, Investigation, Methodology, Writing – original draft. **Esther Gramage:** Conceptualization, Data curation, Formal analysis, Methodology, Supervision, Writing – review & editing. **Alba García-Guerra:** Investigation. **Abraham B. Torregrosa:** Investigation. **Ani Gasparyan:** Investigation, Writing – review & editing. **Daniela Navarro:** Investigation, Writing – review & editing. **Francisco Navarrete:** Investigation, Writing – review & editing. **María Salud García-Gutiérrez:** Investigation, Writing – review & editing. **Jorge Manzanares:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Writing – review & editing. **Gonzalo Herradón:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft.

## Declaration of competing interest

The authors do not have any conflict of interest to declare.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2024.109850.

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