Neuropharmacology 227 (2023) 109438

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

# Inhibition of RPTPβ/ζ reduces chronic ethanol intake in adolescent mice and modulates ethanol effects on hippocampal neurogenesis and glial responses in a sex-dependent manner

Milagros Galán-Llario [a], María Rodríguez-Zapata [a], Teresa Fontán-Baselga [a], Esther Gramage [a,c], Marta Vicente-Rodríguez [a,c], José María Zapico [b], Beatriz de Pascual-Teresa [b], Amy W. Lasek [d,1], Gonzalo Herradón [a,c,*]

[a] Departamento de Ciencias Farmacéuticas y de la Salud, Facultad de Farmacia, Universidad San Pablo-CEU, CEU Universities, Urbanización Montepríncipe, 28668, Boadilla del Monte, Spain
[b] Departamento de Química y Bioquímica, Facultad de Farmacia, Universidad San Pablo-CEU, CEU Universities, Urbanización Montepríncipe, 28668, Boadilla del Monte, Madrid, Spain
[c] Instituto de Estudios de las Adicciones, Universidad San Pablo-CEU, CEU Universities, Urbanización Montepríncipe, 28668, Boadilla del Monte, Madrid, Spain
[d] Department of Psychiatry, University of Illinois at Chicago, 1601 West Taylor Street, Chicago, IL, 60612, USA

ARTICLE INFO

Handling Editor: I. Spigelman

*Keywords:*
Pleiotrophin
Perineuronal nets
PTPRZ1
Neuroinflammation

ABSTRACT

Pleiotrophin (PTN) is a cytokine that modulates ethanol drinking and reward and regulates glial responses in different contexts. PTN is an inhibitor of Receptor Protein Tyrosine Phosphatase (RPTP) β/ζ. Inhibition of RPTPβ/ζ reduces binge-like drinking in adult male mice. Whether inhibition of RPTPβ/ζ is effective in reducing ethanol consumption during adolescence and in both sexes remained to be studied. In this work, male and female adolescent mice underwent an intermittent access to ethanol (IAE) 2-bottle choice protocol. Treatment with MY10 (60 mg/kg, i.g.), a small-molecule RPTPβ/ζ inhibitor, reduced chronic 3-week ethanol consumption only in male mice. We detected an ethanol-induced overall decrease in hippocampal GFAPir and Iba1ir, independently of the treatment received, suggesting that RPTPβ/ζ is not key in the regulation of IAE-induced glial responses. However, we found a significant negative correlation between the size of microglial cells and the number of hippocampal neuronal progenitors only in male mice after IAE. This correlation was disrupted by treatment with MY10 before each drinking session, which may be related to the ability of MY10 to regulate the intensity of the perineuronal nets (PNNs) in the hippocampus in a sex-dependent manner. The data show for the first time that inhibition of RPTPβ/ζ reduces chronic voluntary ethanol consumption in adolescent mice in a sex-dependent manner. In addition, we show evidence for sex-specific differences in the effects of IAE on glial responses and hippocampal neurogenesis, which may be related to different actions of the RPTPβ/ζ signalling pathway in the brains of male and female mice.

## 1. Introduction

Adolescence is a critical developmental phase during which insults to the brain can have relevant and long-lasting consequences (Lees et al., 2020). In this regard, alcohol is of particular concern as it remains the substance most widely used by adolescents (Johnston et al., 2021), who tend to consume it in a periodic binge-drinking manner, the most harmful type of drinking for the developing brain. Binge drinking also contributes to an increased risk of developing alcohol use disorder (AUD). Yearly, more adolescent girls report alcohol use and binge

*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; DCx, doublecortin; BBB, blood brain barrier; WFA, Wisteria floribunda agglutinin; BDNF, Brain-derived neurotrophic factor.

* Corresponding author. Lab. Pharmacology, Faculty of Pharmacy, Universidad CEU San Pablo, Urb. Montepríncipe, 28668, Boadilla del Monte, Madrid, Spain.
*E-mail address:* herradon@ceu.es (G. Herradón).
[1] Current affiliation: Department of Pharmacology and Toxicology, Virginia Commonwealth University, 1220 East Broad Street, Box 980613, Richmond, VA 23298, USA.

https://doi.org/10.1016/j.neuropharm.2023.109438
Received 2 October 2022; Received in revised form 9 January 2023; Accepted 23 January 2023
Available online 24 January 2023

drinking than boys and, accordingly, increases in the number of young women suffering from AUD have been observed (Becker, 2016; Becker et al., 2017).

The neurodevelopmental process inherent to adolescence, characterized by highly plastic and dynamic processes in different brain areas such as the hippocampus, is particularly sensitive to external damage. For instance, excessive alcohol consumption has been shown to exert detrimental effects on hippocampal neuronal progenitors (Crews et al., 2006; Macht et al., 2020), which has been proposed to be a consequence of excessive neuroimmune responses resulting in ethanol-induced neuroinflammation and neurodegeneration (Barnett et al., 2022; Coleman and Crews, 2018; Swartzwelder et al., 2019). Thus, there is a pressing need to understand the mechanisms underlying the detrimental effects of alcohol on the adolescent brain to implement strategies during early adolescence to prevent the development of AUD and brain damage.

Receptor Protein Tyrosine Phosphatase β/ζ (RPTPβ/ζ) is mainly expressed in the central nervous system (CNS) in important areas for alcohol effects, such as the prefrontal cortex, insular cortex, amygdala, and hippocampus (Cressant et al., 2017). Pleiotrophin (PTN) is an endogenous inhibitory ligand of RPTPβ/ζ (Meng et al., 2000) that, upon binding to this receptor, causes an increase in the levels of phosphorylation of RPTPβ/ζ substrates, some of which play important roles in ethanol-induced neuroimmune responses and addictive behaviours, such as Fyn kinase (Pariser et al., 2005; Ron and Berger, 2018) and anaplastic lymphoma kinase (ALK) (Dutton et al., 2017). Interestingly, the PTN/RPTPβ/ζ axis regulates glial responses and neuroinflammation induced by different stimuli like amphetamine or LPS administrations (Fernández-Calle et al., 2017; Herradon et al., 2019; Vicente-Rodríguez et al., 2016). It was recently described that RPTPβ/ζ is important in neuronal and microglial viability and plays a role in the modulation of the neuroprotective phenotype of microglia and microglial-neuron communication (del Campo et al., 2021). In addition, RPTPβ/ζ is key for the development of perineuronal nets (PNNs) (Eill et al., 2020), extracellular matrix structures that are highly affected by glial responses and are known to play a role in neurogenesis (Cope and Gould, 2019; Crapser et al., 2020). Whether or not RPTPβ/ζ is involved in the regulation of the neuroimmune response to other insults, such as alcohol consumption, remained to be studied.

Transgenic *Ptn* overexpression in the mouse brain reduces ethanol-conditioned place preference (Vicente-Rodríguez et al., 2014), suggesting that pharmacological inhibition of RPTPβ/ζ may represent a novel therapeutic strategy in AUD. Accordingly, administration of MY10, a blood-brain barrier (BBB)-permeable selective inhibitor of RPTPβ/ζ (Pastor et al., 2018), has been shown to reduce binge drinking and to block the rewarding properties of alcohol in adult mice and rats (Calleja-Conde et al., 2020; Fernández-Calle et al., 2018, 2019). Whether inhibition of RPTPβ/ζ is effective in reducing chronic ethanol consumption, during adolescence and in both sexes, remained to be studied. To fill this gap, we have now tested in male and female mice the effects of RPTPβ/ζ inhibition in an intermittent access to ethanol (IAE) model, which facilitates periodic consumption in a longer term, better reflecting the drinking pattern of adolescents.

## 2. Materials and methods

### 2.1. Animals

For the intermittent access to ethanol (IAE) model, male and female C57BL/6J mice of 5–6 weeks of age at the beginning of the test (15–22 g) were used. Mice were housed under controlled environmental conditions (22 ± 1 °C and a 12-h light/12-h dark cycle) with free access to food and water. For the study of the perineuronal nets (PNNs) in the hippocampus, we used 9–10 weeks old male and female mice.

All the animals used in this study were maintained following the National Institutes of Health Guide for the Care and Use of Laboratory Animals and European Union Laboratory Animal Care Rules (2010/63/EU directive). Protocols were approved by the UIC Animal Care and Use Committee, the Animal Research Committee of USP-CEU and by Comunidad de Madrid (authorization reference: PROEX 76.0/20).

### 2.2. Intermittent access to ethanol (IAE)

IAE renders higher levels of ethanol exposure than continuous access as well as introduces short withdrawal periods in between the drinking sessions (Hwa et al., 2011). We used a 2-bottle choice procedure during which every-other-day one of two water bottles was removed and replaced by an ethanol solution for 24 h. The position of the ethanol bottle was alternated between days to prevent side preferences. Mice were individually housed at least 24h prior to the beginning of the protocol, and they had free access to food and water throughout the experiment. The procedure was initiated during late adolescence (5–6 weeks). Both water and ethanol solutions were provided to mice in 10 ml clear polystyrene serological pipets truncated at the end to accommodate connection to a stainless-steel ball-bearing sipper tube. During the first week, the concentration of ethanol gradually increased (3%, 6%, and 10% v/v each drinking day). After this escalation week, mice were distributed in experimental groups in such a way they all started from a similar baseline drinking (n = 11–13/group). For the remainder of the experiment, mice had 24 h of access to 20% v/v ethanol solution on Monday, Wednesday, and Friday of each week over 3 weeks. A schematic representation of this procedure is shown in Fig. 1a.

#### 2.2.1. Treatment

To evaluate the possible effects of the pharmacological inhibition of RPTPβ/ζ on ethanol consumption, mice were treated with the RPTPβ/ζ inhibitor MY10 (Pastor et al., 2018). During the 3 weeks in which animals had access to the 20% ethanol solution, mice received an administration of MY10 (60 mg/kg) or its vehicle (10% dehydrated ethanol, 20% polysorbate 80, 70% PEG- 300) by oral gavage in a volume of approximately 0.1 ml 1 h before being exposed to the ethanol solution at each drinking session. The volume consumed as well as the weight of the mice were recorded at the end of each drinking session.

To test the possibility that MY10 exerts effects on the consumption of water or another natural reward (sucrose), experiments were performed in independent groups of mice. For water consumption, the same IAE protocol was performed with two bottles of water through the whole 4-week procedure (n = 7–10/group). For sucrose consumption, mice had access to one bottle containing water and another containing 2% sucrose solution within 1 week for 24 h on Monday, Wednesday, and Friday. Mice were treated with MY10 1 h prior to each sucrose drinking session (n = 3–7/group).

#### 2.2.2. Immunohistochemistry

Immediately at the end of the last drinking session, mice were anesthetized with pentobarbital and euthanized by transcardial perfusion with 4% paraformaldehyde. Brains were post-fixed overnight and incubated in 30% sucrose. Thirty μm sections of hippocampus were obtained using a sliding microtome (Leica SM2010 R). Immunohistochemistry studies were performed in one slice per 360 μm for dentate gyrus (DG) (from the bregma −2.12 mm to −3.8 mm).

To study neurogenesis, DG sections were subjected to an antigen retrieval procedure in which sections were heated in 10 mM citrate buffer (pH 8.5) at 80 °C for 30 min. Then, they were incubated overnight with rabbit anti-doublecortin (DCx, Cell Signaling Technology, Danvers, MA; 1:1000) antibody. After careful washing, sections were incubated with the biotinylated secondary antisera (Vector, Burlingame, CA) at room temperature. The avidin-biotin reaction was performed using a Vectastain Elite ABC peroxidase kit. Immunoreactivity was visualized using 0.06% diaminobenzidine and 0.03% $H_2O_2$ diluted in PBS. Sections were mounted on gelatin/chrome alum-coated slides and photomicrographs were captured with a Leica SCN400 Scan Scanner (Leica, Solms, Germany).

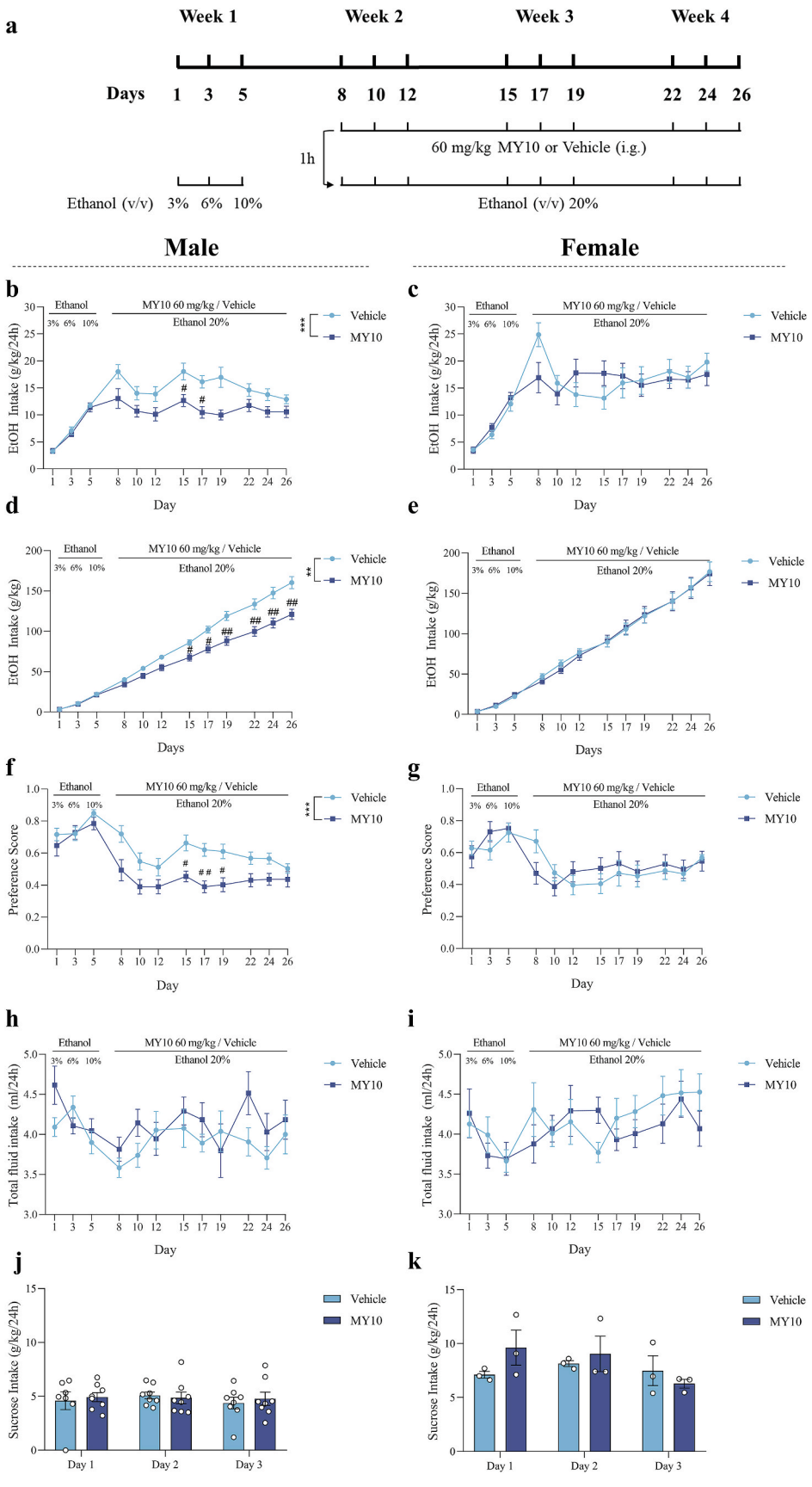


**Fig. 1. MY10 reduces ethanol consumption only in male mice.** (a) Schematic representation of the IAE protocol followed. Graphs are arranged in columns corresponding to male and female data: Ethanol intake (g/kg) by drinking session (day) (b, c), cumulative ethanol intake (g/kg) (d, e), preference score calculated as volume of ethanol consumed/total fluid consumed (f, g), total fluid (ethanol and water) intake (ml/24h) (h, i) and sucrose consumption (g/kg/24h) (j, k). Data are presented as mean ± SEM. ANOVA analyses are shown as $^{**}p < 0.01$, $^{***}p < 0.001$ for differences in the effect of treatment. *Post-hoc* analyses are shown as $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. Vehicle.

To study glial responses, DG sections were incubated overnight at 4 °C with mouse anti-glial fibrillary acidic protein (GFAP; Millipore, Madrid, Spain; 1:1000) and rabbit anti-ionized calcium-binding adaptor molecule 1 (Iba1, Wako, Osaka, Japan; 1:1000) antibodies, following by 30-min incubation with the Alexa-Fluor-555 and Alexa-Fluor- 488 corresponding secondary antibodies (Invitrogen, Waltham, MA, USA; 1:500). Photomicrographs were captured with a digital camera coupled to an optical microscope (DM5500B, Leica, Solms, Germany) using the LAS X Core software (Leica Microsystems, Wetzlar, Germany; offline version).

*2.2.2.1. Image analysis.* Analysis was performed using ImageJ/Fiji software (NIH, Bethesda, MD, Version 1.50f) on three photographs of DG from every subject (n = 4–6/group). For Iba1 and GFAP analysis, after converting the images to 8-bit grayscale, the contrast was automatically enhanced, and images were despeckled and sharpened to better fit the cellular shape. Thresholds were adjusted using an automatic iterative method provided by ImageJ software to generate images with a maximum of Iba1+/GFAP+ cells and a minimum of background artifacts. Total GFAP+ and Iba1+ marked area and Iba1+ cell counts were then analyzed using images acquired at 20× magnification. In addition, the morphology of individual Iba1+ cells was studied using higher magnification images (40×) as previously described (Fernández-Calle et al., 2017).

In the case of DCx analysis, images taken at 10× were used to have a complete picture of the DG. Three images per animal were analyzed (n = 4–6/group). Cell shape definition was improved by using the "Band Pass Filter" option before turning the image to 8-bit grayscale and using a "sharp" mask. For quantification, the whole granular cell layer (GCL) was circumscribed to take into analysis the full dendritic complexity of immature neurons. DCx+ staining within the region of interest (ROI) was measured using the "Threshold" option on ImageJ/Fiji software. Thresholds were adjusted using an automatic iterative method provided by ImageJ software. DCx+ density was expressed as a percentage of DCX+ staining in the GCL.

### 2.2.3. Quantitative real-time PCR

Total RNA was isolated from hippocampus using the Total RNA Isolation Kit (Nzytech, Lisbon, Portugal). First-strand cDNA was synthesized using the first-strand cDNA Synthesis Kit (Nzytech). Quantitative real-time PCR analysis was performed using the SYBR green method (Quantimix Easy kit, Biotools, Madrid, Spain) in a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). The relative expression of *Iba1*, *Cd68* and *Bdnf*, in hippocampi from mice of all experimental groups (n = 3–6), was normalized using *Rpl13*, *Rpl37*, and *18S*. The primer sequences are shown in Table 1.

**Table 1**
Primer sets used for qPCR analysis. Rn18s: 18S ribosomal RNA; Cd68: Cluster of differentiation factor 68; Iba1: Ionized calcium binding adapter molecule 1; Bdnf: Brain derived neurotrophic factor; Rpl13: Ribosomal protein L13; Rpl37: Ribosomal protein L37.

| Gene | Primer Forward | Primer Reverse |
|---|---|---|
| *18S* | 5′-CTCAACACGGGAAACCTCAC-3′ | 5′-CGCTCCACCAACTAAGAACG-3′ |
| *Rpl13* | 5′-GGTGCCCTACAGTTAGATACCAC-3′ | 5′-TTTGTTTCGCCTCCTTGGGTC-3′ |
| *Rpl37* | 5′-ACCGCAGATTCAGACATGGATT-3′ | 5′-AGCGTAGGATCCCAGAGCAA-3′ |
| *Cd68* | 5′-TGGCGGTGGAATACAATGTG-3′ | 5′-GATGAATTCTGCGCCATGAA-3′ |
| *Iba1* | 5′-GTCCTTGAAGCGAATGCTGG-3′ | 5′-CATTCTCAAGATGGCAGATC-3′ |
| *Bdnf* | 5′- CGACATCACTGGCTGACACT -3′ | 5′- ATGTTTGCGGCATCCAGGTA -3′ |

### 2.3. Perineuronal nets (PNNs)

To study the effects of RPTPβ/ζ inhibition on the PNNs in the DG, we performed an analysis of these nets after the administration of MY10. Ethanol-naïve mice were treated with MY10 or vehicle at 9–10 weeks of age, thus matching the age at which mice from the IAE protocol were sacrificed. To test the effects of the administration of MY10, mice (n = 3/sex/group) received one MY10 (60 mg/kg) or vehicle i.g. administration and were sacrificed 24h after the treatment.

### 2.3.1. Immunohistochemistry

Mice were anesthetized with pentobarbital and euthanized by transcardial perfusion with 4% paraformaldehyde. Brains were post-fixed overnight and incubated in 30% sucrose. Thirty μm coronal sections were obtained using a cryostat (Thermo Scientific™ CryoStar™ NX50). Immunohistochemistry studies were performed in one slice per 360 μm for DG (from the bregma −2.12 mm to −3.8 mm).

Sections were blocked in glycoprotein-free blocking solution and incubated with biotinylated *Wisteria floribunda* agglutinin (WFA, Vector Laboratories, CA, USA, 1:2000). Sections were then incubated with Dylight 488-conjugated streptavidin (Vector Laboratories, CA, USA; 1:200). Photographs of the sections were taken with the fluorescence microscope Axio Imager.M2 (Carl Zeiss, Thornwood, NY) at 20× magnification.

### 2.3.2. Image analysis

For analysis of PNNs, two images per animal (n = 3/group) were used. WFA fluorescence intensity of PNNs for each image was quantified using an established method in ImageJ by 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. For PNN intensity, each data point represents the fluorescent WFA intensity in a single image, obtained by calculating the average fluorescent intensity of the total number of WFA+ cells per image. For categorizing PNNs into low, medium, and high intensity, quartiles were used as the cutoff based on the intensity of PNNs in vehicle-treated male mice. Low-intensity PNNs were below the 25th percentile (lower than 1.46 raw intensity units); medium-intensity PNNs were between the 25th and 75th percentile and high-intensity PNNs were in the 75th percentile (greater than 2.54 raw intensity units).

*2.3.2.1. 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 (S.E.M.). The Shapiro-Wilk test was used for normalities of sample distribution (Suppl. Table 1). Ethanol intake (g/kg), total fluid intake (ml), ethanol preference (%), and sucrose intake (g/kg) from the IAE assays were analyzed using a three-way repeated measures (RM) ANOVA considering sex, treatment and drinking session as variables. For the analysis of ethanol intake and ethanol preference, only the data from the maintenance weeks (20% ethanol solution) were used. All references to statistical significance made to the three-way ANOVA test's individual factors or their interaction, together with effect sizes, are shown in Supplementary Table 2. Sex differences in similar paradigms of ethanol consumption have been consistently reported and are confirmed in our studies. Since the main purpose of this study was to test the effects of MY10 on chronic ethanol consumption, once confirmed the significant difference between sexes, we performed two-way RM ANOVAs within each sex to better discern treatment effects.

Data from GFAP, Iba1 and DCx immunohistochemistry analysis were analyzed using three-way ANOVA considering sex, treatment and ethanol drinking as variables (Suppl. Table 2). Again, when relevant, to better dissect the treatment effect (MY10 vs. vehicle), we used a two-way ANOVA within each sex if the three-way ANOVA revealed a significant sex effect. Differences were analyzed by post-hoc comparisons

with Bonferroni's post-hoc tests. The correlation between Iba1+ cell size and DCx+ staining was analyzed by Pearson correlation coefficient (r). $P < 0.05$ was considered statistically significant.

For PNN studies of WFA fluorescence intensity, data were analyzed by two-way ANOVA with sex and treatment as between-subject factors. References to statistical significance are made to the two-way ANOVA test's individual factors or their interaction and are provided, together with effect sizes, in Supplementary Table 3. Relevant 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. $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Inhibition of RPTPβ/ζ reduces ethanol intake only in male mice

During the first week of the IAE procedure, male and female mice increased ethanol (3, 6 and 10% v/v) intake in a similar manner (Suppl. Fig. 1; Fig. 1b and c). For the next 3 weeks, mice received an administration of MY10 (60 mg/kg) or its vehicle 1 h before having access to 20% ethanol. The 3-way ANOVA revealed significant effects of the drinking session, sex and treatment on ethanol intake (Suppl. Table 2A). This analysis also showed a significant session x sex × treatment interaction (Suppl. Table 2A). To further explore the effect of the treatment and its interaction with drinking session, we employed two-way ANOVAs within each sex and found a significant effect of treatment in male mice (Fig. 1b; $F(1,24) = 16.21$, $P = 0.0005$). Treatment with MY10 reduced ethanol consumption with a maximum effect during the second week of access to 20% ethanol (days 15–19). In contrast, we did not detect a significant effect of the treatment on ethanol intake in female mice (Fig. 1c; $F(1,22) = 0.0681$, $P = 0.7965$). Accordingly, MY10 significantly reduced the cumulative ethanol consumed by males (Fig. 1d; $F(1,24) = 13.92$, $P = 0.0010$), not by female mice (Fig. 1e). MY10-treated male mice showed a lower ethanol preference score (Fig. 1f, $F(1,24) = 21.32$, $P = 0.0001$), whereas MY10 did not exert significant effects in female mice (Fig. 1g). MY10 treatment did not alter total fluid consumption in male mice (Fig. 1h; $F(1,24) = 1.598$, $P =$

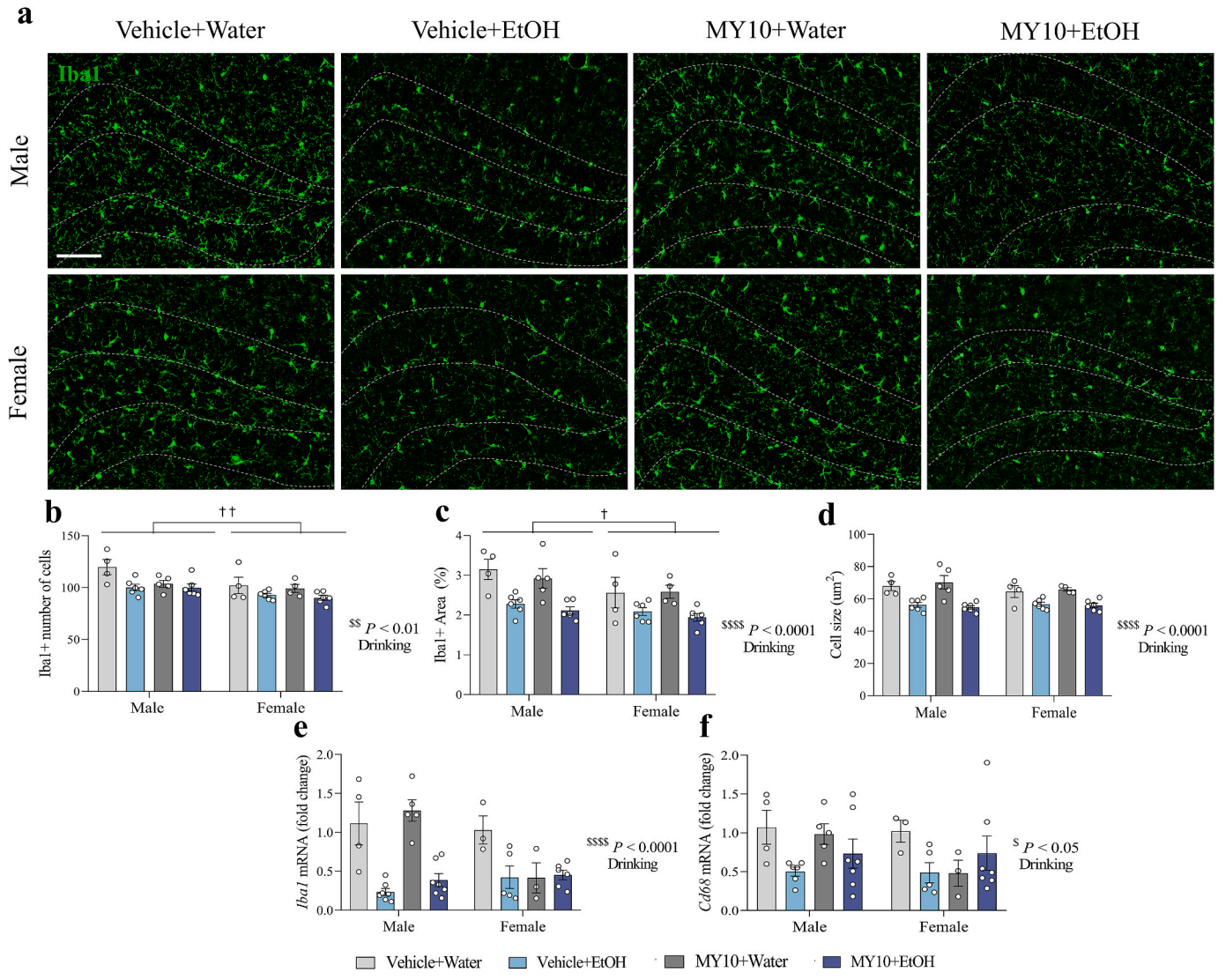


**Fig. 2. Effects of IAE and MY10 on microglial responses in the adolescent hippocampus.** (a) Photomicrographs are from Iba1-immunostained hippocampal sections of Vehicle+Water, Vehicle+EtOH, MY10+Water and MY10+EtOH-treated animals. Dashed lines indicate the outline of the granule cell layer of the hippocampal dentate gyrus. Graphs represent quantification of the counts of Iba1-positive cells (b), percentage of Iba1+ stained area (c) and microglia cell size in dentate gyrus sections (d). *Iba1* (Ionized calcium binding adapter molecule 1) mRNA (e) and *Cd68* (Cluster of differentiation factor 68) mRNA (f) were measured in the hippocampi of mice from the different experimental groups. Data are presented as mean ± SEM of n = 3–6 mice/group. Three-way ANOVA analysis is shown as $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ for significant effect of sex; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$\$}p < 0.0001$ for significant effect of ethanol drinking. Scale bar = 100 μm.

0.2184) or in female mice (Fig. 1i; F (1,22) = 0.3539, $P$ = 0.5580). Moreover, to test if palatable-substances preference was altered by RPTPβ/ζ inhibition, we tested 2% sucrose intake after MY10 treatment. MY10 did not alter sucrose intake in male mice (Fig. 1j, F (1,14) = 0.1044, $P$ = 0.7514) nor in female mice (Fig. 1k; F (1,4) = 0.4268, $P$ = 0.5492). In additional control experiments, MY10 treatment did not alter water consumption when male and female mice had access to two bottles of water for 4 weeks (Supplementary Fig. 2). Taken all together, these data suggest that pharmacological inhibition of RPTPβ/ζ reduces ethanol consumption in a sex-specific manner.

### 3.2. Inhibition of RPTPβ/ζ does not regulate the effects of IAE on glial responses in the dentate gyrus

First, we analyzed the astrocytic response in the DG through GFAP staining (Supplementary Fig. 3). The 3-way ANOVA of the data from GFAP+ area (Suppl. Fig. 3b) revealed a significant effect of ethanol drinking (Suppl. Table 2B). In both sexes, we observed a decrease in the GFAP+ area in the animals with access to ethanol, independently of the treatment received (Suppl. Fig. 3). The analysis of the data from Iba1+ staining (Fig. 2) revealed a significant effect of sex on the number of Iba1+ cells and on Iba1 marked area (Suppl. Table 2C). In addition, we also found a significant effect of ethanol drinking on Iba1+ cell count, marked area and on cell size (Suppl. Table 2C). This analysis also showed a significant sex x treatment × drinking interaction on Iba1+ cell count (Suppl. Table 2C). The overall decrease in Iba1+ cells induced by IAE was exacerbated in the DG of male mice (Fig. 2b), although similar trends of Iba1+ area and cell size decreases were observed in males and females (Fig. 2c and d). None of the effects of chronic ethanol exposure on Iba1ir was altered by MY10 treatment (Fig. 2b–d). In addition, we found a significant effect of ethanol drinking on *Iba1* mRNA and significant sex x treatment and sex × drinking interactions (Fig. 2e; Suppl. Table 2C). A similar pattern was observed with the marker of activated microglia *Cd68* mRNA, which rendered a significant effect of ethanol drinking and a significant interaction treatment x ethanol drinking (Fig. 2f; Suppl. Table 2C). Taking together, the data suggest that *RPTPβ/ζ* is not key in the regulation of IAE-induced glial responses.

### 3.3. Inhibition of RPTPβ/ζ prevents sex-dependent differences in the effect of IAE on neuronal progenitors in the dentate gyrus

The 3-way ANOVA of the DCX+ staining (Fig. 3) revealed a significant effect of sex and of the treatment (Suppl. Table 2D) on the percentage of neural progenitors in the GCL (Fig. 3b). Importantly, we also found a significant interaction treatment x ethanol drinking (Suppl. Table 2D). The sex-dependent effect of chronic voluntary ethanol drinking was prevented in male mice treated with MY10 before each ethanol drinking day (Fig. 3b). The data from the control experimental groups similarly treated with MY10 but having only access to water during the whole procedure (MY10 + Water) did not show any effect of MY10 treatment on neural progenitors of male or female mice (Fig. 3b). In addition, we found that ethanol drinking had a significant effect on hippocampal *Bdnf* mRNA levels (Fig. 3c; Suppl. Table 2D). Ethanol intake decreased the expression of *Bdnf* in the hippocampus of male and female mice, but MY10 did not exert any modulatory action on this effect of ethanol (Fig. 3c).

Since it was previously shown that inhibition of RPTPβ/ζ may interfere with the microglia-neuron communication (del Campo et al., 2021), we aimed to test its impact on a possible correlation between microglial responses and IAE-induced effects on neural progenitors in the DG (Fig. 4). This analysis revealed a negative correlation between Iba1+ cell size and DCx+/GCL staining in male mice treated with vehicle before each ethanol-drinking day, but not in female mice with the same treatment (Fig. 4a,c). Interestingly, treatment with MY10 prevented this negative correlation between Iba1+ cell size and percentage of neural progenitors in the GCL of the DG in male mice

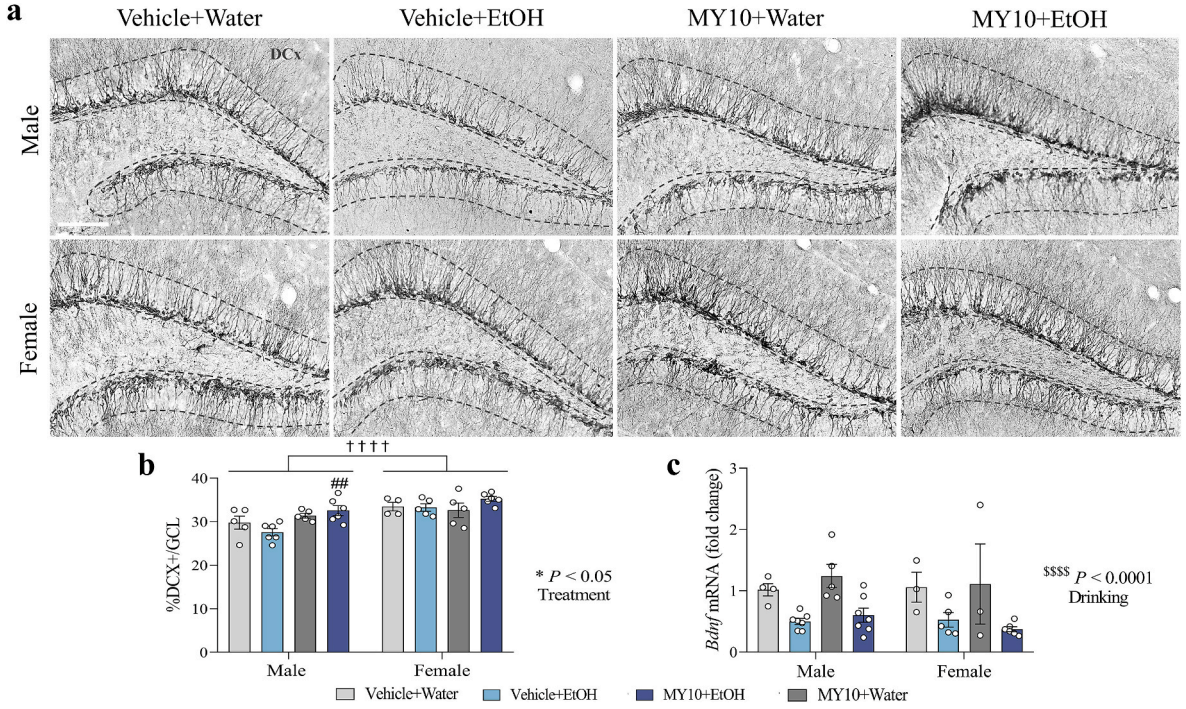


**Fig. 3. Effects of IAE and MY10 on neural progenitor cells in the adolescent hippocampus.** (a) Photomicrographs are from DCX-immunostained hippocampal sections of Vehicle+Water, Vehicle+EtOH, MY10+Water, and MY10+EtOH-treated animals. Dashed lines indicate the outline of the granule cell layer of the hippocampal dentate gyrus. (b) Graph represents quantification of the percentage DCX+ staining in the granular cell layer (%DCx+/GCL) in dentate gyrus sections. (c) *Bdnf* (Brain-derived neurotrophic factor) mRNA was measured in the hippocampi of mice from the different experimental groups. Data are presented as mean ± SEM of n = 3–6 mice/group. 3-Way ANOVA analyses are shown as $^{††††}p < 0.0001$ for significant effect of sex; $^{\$\$\$\$}p < 0.0001$ for significant effect of ethanol drinking; $^{*}p < 0.05$ for significant effect of treatment. *Post-hoc* analyses of 2-way ANOVA within each sex are shown as $^{\#\#}p < 0.01$ vs. Vehicle+EtOH. Scale bar = 200 μm.

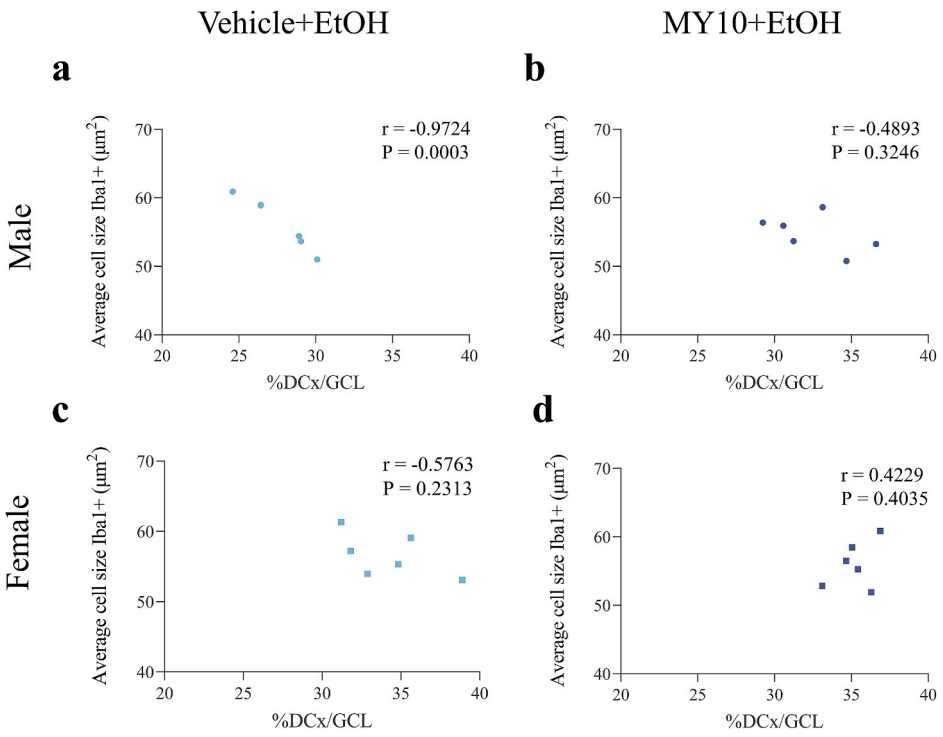


**Fig. 4. Treatment with MY10 prevents the negative correlation between microglial cell size and neurogenesis after IAE only in male mice.** Graphs show the correlation between average Iba1 + cell size (μm$^2$) in the dentate gyrus (DG) and the percentage of DCx+ staining in the granular cell layer (GCL) (%DCx+/GCL) of the DG in vehicle-treated males (a), MY10-treated males (b), vehicle-treated females (c) and MY10-treated females (d) with IAE. Pearson correlation coefficients (r) and p values are shown in all correlation graphs.

(Fig. 4b). We did not find any significant correlation in the control animals having only access to two bottles of water during the whole IAE procedure (data not shown).

### *3.4. PNN intensity in the dentate gyrus is regulated by RPTPβ/ζ in a sex-dependent manner*

To shed some light on the mechanisms triggered by MY10 with a possible impact on neurogenesis, we evaluated the actions of a single dose of this compound on PNNs in the DG (Fig. 5). RPTPβ/ζ is an important anchor for several PNN components (Eill et al., 2020) and these structures have been previously shown to play a role in neurogenesis (Cope and Gould, 2019) and to be highly affected by microglial responses (Crapser et al., 2020). The ANOVA of the WFA intensity in the DG revealed a significant effect of the treatment and a significant interaction sex x treatment (Suppl. Table 3). We found that WFA intensity was significantly higher only in male mice treated with MY10 compared with vehicle treatment (Fig. 5b). This MY10-induced change in WFA intensity was not due to differences in the number of WFA+ cells in the DG (Fig. 5c). We also performed an analysis of WFA intensity by dividing PNN-wrapped neurons into three categories: low-, medium- and high-intensity level. The analysis of distribution of the nets in these categories demonstrated that MY10 treated-male mice exhibited a significantly larger proportion of DG neurons with high WFA intensity than that observed in vehicle-treated males (Fig. 5d; d.f. (2), $\chi 2 = 20.23$, $P < 0.0001$) and in MY10-treated females (Fig. 5d; d.f. (2), $\chi 2 = 16.23$, $P < 0.001$). On the contrary, MY10 did not alter the proportion of high WFA intensity PNNs in females when compared to vehicle-treated females. These results indicate that RPTPβ/ζ inhibition regulates the intensity of PNNs in the mouse DG in a sex-dependent manner.

## 4. Discussion

Adolescent drinking is a global health problem. It is characterized by a high alcohol intake, typically in a periodic pattern, which is considered the most harmful drinking for the developing brain. Thus, it is of critical importance to adopt measures to reduce alcohol consumption during adolescence and to prevent its long-lasting deleterious effects on the brain. Previously, we found that treatment with MY10, a selective inhibitor of RPTPβ/ζ, significantly reduced binge-like ethanol drinking in male adult mice and rats (Calleja-Conde et al., 2020; Fernández-Calle et al., 2018). In the present work, we aimed to test this novel pharmacological strategy in chronic ethanol intake using a mouse model that reproduces some of the most important characteristics of the typical human adolescent alcohol consumption pattern, the IAE model (Hwa et al., 2011). The most important finding of this study provides the first pre-clinical evidence that inhibition of RPTPβ/ζ significantly reduces ethanol intake in male adolescent mice during the 3-weeks testing phase of the IAE paradigm, without altering the consumption of either water or sucrose intake, suggesting that the effects are specific for ethanol. It is important to note that these effects of MY10 on chronic ethanol consumption should not be automatically extended to other models. Particularly, additional studies should be performed to test the effects of MY10 on compulsive drinking. Remarkably, treatment with MY10 did not exert any effect on ethanol consumption in female mice, suggesting relevant sex differences in the action of RPTPβ/ζ in the CNS.

The RPTPβ/ζ signalling pathway is involved in the regulation of the neuroimmune response to different stimuli (Herradon et al., 2019). Accordingly, we have shown that inhibition of RPTPβ/ζ with MY10 potentiates LPS-induced microglial responses (Fernández-Calle et al., 2020). Thus, in the present study we aimed to test the possibility that RPTPβ/ζ inhibition modulates IAE-induced changes in glial responses in the dentate gyrus. Furthermore, we aimed to extend our study to hippocampal neurogenesis since ethanol-induced alterations of immune responses have been related to the effects of this drug on neural progenitors in the hippocampus (Coleman and Crews, 2018). We found that IAE reduced GFAPir, Iba1ir, *Iba1* mRNA and *Cd68* mRNA levels in the DG of both female and male adolescent mice and that these effects were not apparently regulated by treatment with MY10. The data are in agreement with previous studies showing that glial cells may be more susceptible to ethanol damage, which may, in turn, affect trophic and metabolic support for neurons (Crews and Nixon, 2009). Furthermore, the lack of more exacerbated changes in morphology induced by IAE is not surprising according to previous studies that showed limited effects

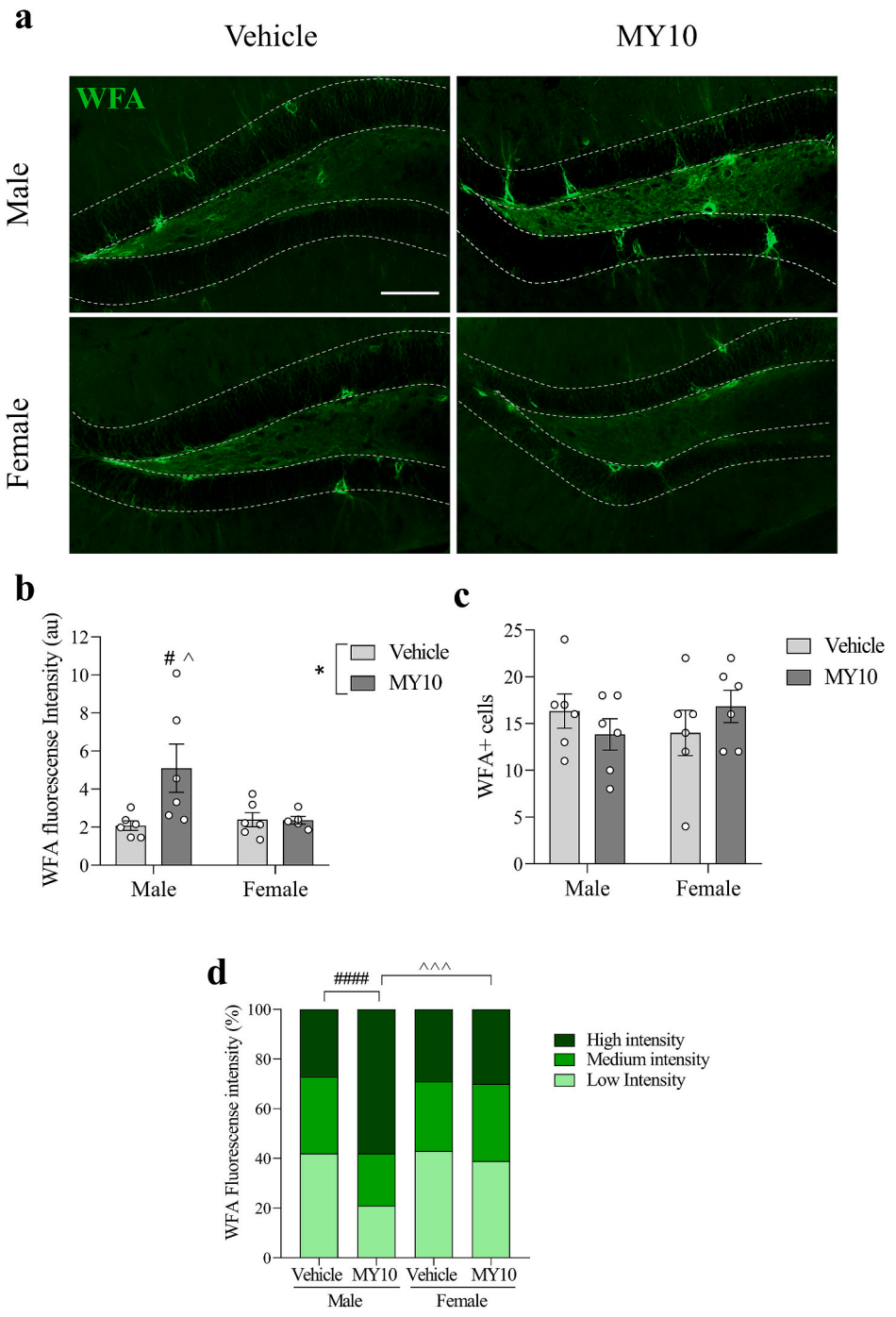


**Fig. 5. MY10 increased PNN intensity in the dentate gyrus of male mice.** Photomicrographs of WFA fluorescence binding from vehicle and MY10 treated male and female animals (a) (n = 3/group). Dashed lines indicate the outline of the granule cell layer of the hippocampal dentate gyrus. Graphs represent data (mean ± S.E.M) from the quantification of WFA intensity (b), quantification of WFA+ number cells (c) in the dentate gyrus. Graphs representing the percentage of PNNs corresponding to different WFA intensity levels are also shown (d). ANOVA analyses are shown as * $p < 0.05$ for differences in the effect of treatment. ^ $p < 0.05$; ^^^ $p < 0.001$ vs. same treatment in the opposite sex. # $p < 0.05$; #### $p < 0.0001$ vs. vehicle within same sex. Scale bar = 100 μm.

of this kind of chronic moderate ethanol consumption on glial morphology despite the capacity of this drug to facilitate the glial release of cytokines and other inflammatory mediators (Crews et al., 2017).

The adolescent brain is highly plastic, with processes like neurogenesis being very active and affected by ethanol consumption (Broadwater et al., 2014). In the present work, we found that hippocampal DCXir is affected by the treatments and that neurogenesis is overall enhanced in the DG of female mice compared to males, which was found particularly exacerbated in the groups treated with vehicle before each ethanol drinking session. However, male mice treated with MY10 before each drinking session showed similar levels of neuronal progenitors to those of their female counterparts. Intriguingly, we only found a significant negative correlation between the size of microglial cells and the number of neuronal progenitors in the case of vehicle-treated male mice with intermittent access to ethanol. This correlation was prevented by treatment with MY10 before each drinking session. The data point again to sex-specific differences in the actions of RPTPβ/ζ in the brain and suggest a beneficial modulation by MY10 of ethanol-induced effects on hippocampal neurogenesis that could be related to the ability of MY10 to regulate ethanol-induced microglial responses. Recently, we have shown that acute treatment with MY10 fully prevents the loss of hippocampal neurogenesis induced by a single administration of ethanol (6 g/kg) in both male and female mice (Galán-Llario et al., 2022). The data indicate that acute effects of MY10 on brain alterations induced by a single administration of ethanol do not differ between males and females (Galán-Llario et al., 2022), but the effects of repeated administrations of MY10 on chronic ethanol consumption are sex-dependent. It has been shown that estrogen induces the expression of RPTPβ/ζ in mice. In fact,

the treatment with estrogen in males results in a change in RPTPβ/ζ that is equivalent to females (Xi et al., 2020). Thus, the sexual dimorphism in response to MY10 may be due in part to the ability of estrogen to induce RPTPβ/ζ. All in all, one limitation of this study is that we only tested one dose of MY10 (60 mg/kg), whereas in the chronic drinking procedure, the dose of MY10 in females may need to be increased to achieve similar effects as in males.

It has been shown that 3-week chronic free-choice ethanol consumption in mice induces the activation of brain-derived neurotrophic factor (BDNF) signaling pathway in the hippocampus (Stragier et al., 2015), which may underlie the moderate effects of this pattern of ethanol consumption on neurogenesis, compared to short-term binge--like drinking episodes (Coleman and Crews, 2018; Crews et al., 2006; Swartzwelder et al., 2019). Several studies using different chronic ethanol consumption paradigms (6 weeks–6 months) have shown that ethanol reduces the levels of *Bdnf* in different brain areas including the hippocampus (Popova et al., 2020; Yao et al., 2021), and therapeutic strategies that restore *Bdnf* levels also counteract long-term ethanol behavioural and neural adaptations (Akbari et al., 2023). Interestingly, in other situations of brain damage (i.e., oxygen-glucose deprivation), the endogenous inhibitor of RPTPβ/ζ, PTN, has been shown to stimulate the secretion of BDNF from microglia (Miao et al., 2019). RPTPβ/ζ plays a role in microglia-neuron communication (Del Campo et al., 2021) suggesting that MY10 may regulate IAE-induced changes in BDNF hippocampal expression to enhance neurogenesis in males. However, the data suggest that this is not a key mechanism involved in our studies since treatment with MY10 did not affect ethanol-induced changes in hippocampal *Bdnf* levels.

It has been described that microglia regulate ECM and PNN remodeling (Bosiacki et al., 2019). Our results show that a single administration of MY10 does not alter the number of WFA+ cells but significantly enhances high-intensity PNNs in the hippocampus of male mice, but not in female mice. These data confirm and emphasize the importance of RPTPβ/ζ in PNN structure (Eill et al., 2020), but suggest relevant differences in this function of RPTPβ/ζ between sexes. PNN remodeling is known to be involved in the development of new neurons and synaptic plasticity in different pathological conditions (Bosiacki et al., 2019). Moreover, neurons ensheathed by PNNs have been shown to be less vulnerable to damaging insults because of the neuroprotective function of these structures against oxidative stress (Morawski et al., 2004), which is compatible with the increased proportion of DCX+ cells in the hippocampus of male mice treated with MY10 before each drinking session compared to vehicle-treated male mice. From these separate lines of evidence, we suggest that through direct actions of RPTPβ/ζ in PNNs or through glial-regulated remodeling of ECM and PNNs, MY10 may modulate the effects of chronic ethanol in hippocampal neurogenesis. To test this hypothesis, further studies are needed to assess how MY10 may regulate the effects of ethanol exposure on PNNs maturation during adolescence.

## 5. Conclusion

In summary, the data presented here show for the first time that pharmacological inhibition of RPTPβ/ζ with MY10 significantly reduces chronic voluntary ethanol consumption only in male adolescent mice. In addition, we show evidence for sex-specific differences in the effects of IAE on glial responses and hippocampal neurogenesis, which may be related to different actions of the RPTPβ/ζ signalling pathway in the brains of male and female mice.

## Funding

This work was supported by National Plan on Drug abuse, Ministerio de Sanidad of Spain (MSSSI, grant PNSD2019I015 to G.H.) and by ISCIII-Redes de Investigación Cooperativa Orientadas a Resultados en Salud (RICORS), Red de Investigación en Atención Primaria de Adicciones (RIAPAd; grant RD21/0009/0013 to G.H.) and the United States National Institutes of Health, National Institute on Alcohol Abuse and Alcoholism (grant R01 AA027231 to A.W.L.)

## CRediT authorship contribution statement

**Milagros Galán-Llario:** Investigation, Data curation, Formal analysis, Data Interpretation, Writing – original draft, Writing – review & editing. **María Rodríguez-Zapata:** Investigation, Data curation, Formal analysis, Data Interpretation. **Teresa Fontán-Baselga:** Investigation, Data curation. **Esther Gramage:** Conceptualization, Methodology, Investigation, Data curation, Formal analysis. **Marta Vicente-Rodríguez:** Methodology, Investigation. **José María Zapico:** Investigation. **Beatriz de Pascual-Teresa:** Data Interpretation, Supervision, Writing – review & editing. **Amy W. Lasek:** Conceptualization, Methodology, Funding acquisition, Data Interpretation, Supervision, Project administration, Writing – review & editing. **Gonzalo Herradón:** Conceptualization, Methodology, Funding acquisition, Data Interpretation, Supervision, Project administration, Writing – original draft, Writing – review & editing, All authors have read and agreed to the published version of the manuscript.

## Declaration of competing interest

None.

## Data availability

Data will be made available on request.

## Acknowledgments

We thank our colleagues in the Animal facility and the “Instituto de Medicina Molecular Aplicada” (IMMA) in the Universidad CEU San Pablo. We also thank Dr. Luana Martins de Carvahlo and Dr. Hu Chen for advice on fluorescent WFA staining and PNN quantification.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2023.109438.

## References

Akbari, E., Hossaini, D., Amiry, G.Y., Ansari, M., Haidary, M., Beheshti, F., Ahmadi-Soleimani, S.M., 2023. Vitamin B12 administration prevents ethanol-induced learning and memory impairment through re-establishment of the brain oxidant/antioxidant balance, enhancement of BDNF and suppression of GFAP. Behav. Brain Res. 438, 114156. https://doi.org/10.1016/j.bbr.2022.114156.

Barnett, A., David, E., Rohlman, A., Nikolova, V.D., Moy, S.S., Vetreno, R.P., Coleman, L. G., 2022. Adolescent binge alcohol enhances early alzheimer's disease pathology in adulthood through proinflammatory neuroimmune activation. Front. Pharmacol. 13 (884170) https://doi.org/10.3389/fphar.2022.884170.

Becker, J.B., 2016. Sex differences in addiction. Dialogues Clin. Neurosci. 18 (4), 395–402. https://doi.org/10.31887/DCNS.2016.18.4/jbecker.

Becker, J.B., Mcclellan, M.L., Reed, B.G., 2017. Review sex differences, gender and addiction. J. Neurosci. Res. 95 (1–2), 136–147. https://doi.org/10.1002/jnr.23963.

Bosiacki, M., Assowska-Dobrowolska, M.G., Kojder, K., Fabiá Nska, M., Je˙Zewski, D., Gutowska, I., Lubkowska, A., 2019. Molecular sciences perineuronal nets and their role in synaptic homeostasis. Int. J. Mol. Sci. 20 (17) https://doi.org/10.3390/ijms20174108.

Broadwater, M.A., Liu, W., Crews, F.T., Spear, L.P., 2014. Persistent loss of hippocampal neurogenesis and increased cell death following adolescent, but not adult, chronic ethanol exposure. Dev. Neurosci. 36, 297–305. https://doi.org/10.1159/000362874.

Calleja-Conde, J., Fernández-Calle, R., Zapico, J.M., Ramos, A., de Pascual-Teresa, B., Bühler, K.M., Echeverry-Alzate, V., Giné, E., Rodríguez de Fonseca, F., López-Moreno, J.A., Herradón, G., 2020. Inhibition of receptor protein tyrosine phosphatase β/ζ reduces alcohol intake in rats. Alcohol Clin. Exp. Res. 44 (5), 1037–1045. https://doi.org/10.1111/acer.14321.

Coleman, L.G., Crews, F.T., 2018. Innate immune signaling and alcohol use disorders. Handbook of Experimental Biology 248, 369–396. https://doi.org/10.1007/164_2018_92.

Cope, E.C., Gould, E., 2019. Adult neurogenesis, glia, and the extracellular matrix. Cell Stem Cell 24 (5), 690–705. https://doi.org/10.1016/j.stem.2019.03.023.

Crapser, J.D., Spangenberg, E.E., Barahona, R.A., Arreola, M.A., Hohsfield, L.A., Green, K.N., 2020. Microglia facilitate loss of perineuronal nets in the Alzheimer's disease brain. EBioMedicine 58. https://doi.org/10.1016/J.EBIOM.2020.102919.

Cressant, A., Dubreuil, V., Kong, J., Kranz, T.M., Lazarini, F., Launay, J.-M., Callebert, J., Sap, J., Malaspina, D., Granon, S., Harroch, S., 2017. Loss-of-function of PTPR γ and ζ, observed in sporadic schizophrenia, causes brain region-specific deregulation of monoamine levels and altered behavior in mice. Psychopharmacology 234, 575–587. https://doi.org/10.1007/s00213-016-4490-8.

Crews, F.T., Lawrimore, C.J., Walter, T.J., Coleman, L.G., 2017. The role of neuroimmune signaling in alcoholism. Neuropharmacology 122, 56–73. https://doi.org/10.1016/j.neuropharm.2017.01.031.

Crews, F.T., Mdzinarishvili, A., Kim, D., He, J., Nixon, K., 2006. Neurogenesis in adolescent brain is potently inhibited by ethanol. Neuroscience 137 (2), 437–445. https://doi.org/10.1016/j.neuroscience.2005.08.090.

Crews, F.T., Nixon, K., 2009. Mechanisms of neurodegeneration and regeneration in alcoholism. Alcohol Alcohol 44 (2), 115–127. https://doi.org/10.1093/alcalc/agn079.

del Campo, M., Fernández-Calle, R., Vicente-Rodríguez, M., Martín Martínez, S., Gramage, E., María Zapico, J., Haro, M., Herradon, G., 2021. Molecular sciences role of receptor protein tyrosine phosphatase β/ζ in neuron-microglia communication in a cellular model of Parkinson's disease. Int. J. Mol. Sci. 22 https://doi.org/10.3390/ijms22136646.

Dutton, J.W., Chen, H., You, C., Brodie, M.S., Lasek, A.W., 2017. Anaplastic lymphoma kinase (ALK) regulates binge-like drinking and dopamine receptor sensitivity in the ventral tegmental area. Addiction Biol. 22 (3), 665–678. https://doi.org/10.1111/adb.12358.

Eill, G.J., Sinha, A., Morawski, M., Viapiano, M.S., Matthews, R.T., 2020. The protein tyrosine phosphatase RPTPξ/phosphacan is critical for perineuronal net structure. J. Biol. Chem. 295 (4), 955–968. https://doi.org/10.1074/jbc.RA119.010830.

Fernández-Calle, R., Galán-Llario, M., Gramage, E., Zapatería, B., Vicente-Rodríguez, M., Zapico, J.M., de Pascual-Teresa, B., Ramos, A., Ramos-Álvarez, M.P., Uribarri, M., Ferrer-Alcón, M., Herradón, G., 2020. Role of RPTPβ/ζ in neuroinflammation and microglia-neuron communication. Sci. Rep. 10, 1–12. https://doi.org/10.1038/s41598-020-76415-5.

Fernández-Calle, R., Gramage, E., Zapico, J.M., de Pascual-Teresa, B., Ramos, A., Herradón, G., 2019. Inhibition of RPTPβ/ζ blocks ethanol-induced conditioned place preference in pleiotrophin knockout mice. Behav. Brain Res. 369 https://doi.org/10.1016/J.BBR.2019.111933.

Fernández-Calle, R., Vicente-Rodríguez, M., Gramage, E., Pita, J., Pérez-García, C., Ferrer-Alcón, M., Uribarri, M., Ramos, M.P., Herradón, G., 2017. Pleiotrophin regulates microglia-mediated neuroinflammation. J. Neuroinflammation 14 (1), 1–10. https://doi.org/10.1186/s12974-017-0823-8.

Fernández-Calle, R., Vicente-Rodríguez, M., Pastor, M., Gramage, E., di Geronimo, B., Zapico, J.M., Coderch, C., Pérez-García, C., Lasek, A.W., de Pascual-Teresa, B., Ramos, A., Herradón, G., 2018. Pharmacological inhibition of Receptor Protein Tyrosine Phosphatase β/ζ (PTPRZ1) modulates behavioral responses to ethanol. Neuropharmacology 137, 86–95. https://doi.org/10.1016/j.neuropharm.2018.04.027.

Galán-Llario, M., Rodríguez-Zapata, M., Gramage, E., Vicente-Rodríguez, M., Fontán-Baselga, T., Ovejero-Benito, M.C., Pérez-García, C., Carrasco, J., Moreno-Herradón, M., Sevillano, J., Ramos-Álvarez, M.P., Zapico, J.M., de Pascual-Teresa, B., Ramos, A., Herradón, G., 2022. Receptor protein tyrosine phosphatase β/ζ regulates loss of neurogenesis in the mouse hippocampus following adolescent acute ethanol exposure. Neurotoxicology 94, 98–107. https://doi.org/10.1016/j.neuro.2022.11.008. Advance online publication.

Herradon, G., Pilar Ramos-Alvarez, M., Gramage, E., 2019. Connecting metainflammation and neuroinflammation through the PTN-MK-RPTPβ/ζ axis: relevance in therapeutic development. Front. Pharmacol. 10, 1–13. https://doi.org/10.3389/fphar.2019.00377.

Hwa, L.S., Chu, A., Levinson, S.A., Kayyali, T.M., DeBold, J.F., Miczek, K.A., 2011. Persistent escalation of alcohol drinking in C57BL/6J mice with intermittent access to 20% ethanol. Alcohol Clin. Exp. Res. 35 (11), 1938–1947. https://doi.org/10.1111/j.1530-0277.2011.01545.x.

Johnston, L.D., Miech, R.A., O'malley, P.M., Bachman, J.G., Schulenberg, J.E., Patrick, M.E., 2021. Monitoring the Future National Survey Results on Drug Use, 1975-2020: Overview, Key Findings on Adolescent Drug Use.

Lees, B., Meredith, L.R., Kirkland, A.E., Bryant, B.E., Squeglia, L.M., Biochem, P., Author, B., 2020. Effect of alcohol use on the adolescent brain and behavior HHS Public Access Author manuscript. Pharmacol. Biochem. Behav. 192, 172906 https://doi.org/10.1016/j.pbb.2020.172906.

Macht, V., Crews, F.T., Vetreno, R.P., 2020. Neuroimmune and epigenetic mechanisms underlying persistent loss of hippocampal neurogenesis following adolescent intermittent ethanol exposure. Curr. Opin. Pharmacol. 50, 9–16. https://doi.org/10.1016/j.physbeh.2017.03.040.

Meng, K., Rodríguez-Peña, A., Dimitrov, T., Chen, W., Yamin, M., Noda, M., Deuel, T.F., 2000. Pleiotrophin signals increased tyrosine phosphorylation of beta-catenin through inactivation of the intrinsic catalytic activity of the receptor-type protein tyrosine phosphatase. Proc. Natl. Acad. Sci. U.S.A. 97 (6), 2603–2608. https://doi.org/10.1073/pnas.020487997.

Miao, J., Wang, F., Wang, R., Zeng, J., Zheng, C., Zhuang, G., 2019. Pleiotrophin regulates functional heterogeneity of microglia cells in EAE animal models of multiple sclerosis by activating CCr-7/CD206 molecules and functional cytokines. Am. J. Tourism Res. 11 (4), 2013–2027. www.ajtr.org.

Morawski, M., Brückner, M.K., Riederer, P., Brückner, G., Arendt, T., 2004. Perineuronal nets potentially protect against oxidative stress. Exp. Neurol. 188 (2), 309–315. https://doi.org/10.1016/j.expneurol.2004.04.017.

Pariser, H., Ezquerra, L., Herradon, G., Perez-Pinera, P., Deuel, T.F., 2005. Fyn is a downstream target of the pleiotrophin/receptor protein tyrosine phosphatase β/ζ-signaling pathway: regulation of tyrosine phosphorylation of Fyn by pleiotrophin. Biochem. Biophys. Res. Commun. 332 (3), 664–669. https://doi.org/10.1016/j.bbrc.2005.05.007.

Pastor, M., Fernández-Calle, R., di Geronimo, B., Vicente-Rodríguez, M., Zapico, J.M., Gramage, E., Coderch, C., Pérez-García, C., Lasek, A.W., Puchades-Carrasco, L., Pineda-Lucena, A., de Pascual-Teresa, B., Herradón, G., Ramos, A., 2018. Development of inhibitors of receptor protein tyrosine phosphatase β/ζ (PTPRZ1) as candidates for CNS disorders. Eur. J. Med. Chem. 144, 318–329. https://doi.org/10.1016/j.ejmech.2017.11.080.

Popova, N.K., Ilchibaeva, T.V., Antonov, E.V., Pershina, A.V., Bazovkina, D.V., Naumenko, V.S., 2020. On the interaction between BDNF and serotonin systems: the effects of long-term ethanol consumption in mice. Alcohol (Fayetteville, N.Y.) 87, 1–15. https://doi.org/10.1016/j.alcohol.2020.04.002.

Ron, D., Berger, A., 2018. Targeting the intracellular signaling "STOP" and "GO" pathways for the treatment of alcohol use disorders. Psychopharmacology 235, 1727–1743. https://doi.org/10.1007/s00213-018-4882-z.

Stragier, E., Martin, V., Davenas, E., Poilbout, C., Mongeau, R., Corradetti, R., Lanfumey, L., 2015. Brain plasticity and cognitive functions after ethanol consumption in C57BL/6J mice. Transl. Psychiatry 5, 696. https://doi.org/10.1038/tp.2015.183.

Swartzwelder, H.S., Healey, K.L., Liu, W., Dubester, K., Miller, K.M., Crews, F.T., 2019. Changes in neuroimmune and neuronal death markers after adolescent alcohol exposure in rats are reversed by donepezil. Sci. Rep. 9, 12110 https://doi.org/10.1038/s41598-019-47039-1.

Vicente-Rodríguez, M., Pérez-García, C., Ferrer-Alcón, M., Uribarri, M., Sánchez-Alonso, M.G., Ramos, M.P., Herradón, G., 2014. Pleiotrophin differentially regulates the rewarding and sedative effects of ethanol. J. Neurochem. 131 (5), 688–695. https://doi.org/10.1111/jnc.12841.

Vicente-Rodríguez, M., Rojo Gonzalez, L., Gramage, E., Fernández-Calle, R., Chen, Y., Pérez-García, C., Ferrer-Alcón, M., Uribarri, M., Bailey, A., Herradón, G., 2016. Pleiotrophin overexpression regulates amphetamine-induced reward and striatal dopaminergic denervation without changing the expression of dopamine D1 and D2 receptors: implications for neuroinflammation. Eur. Neuropsychopharmacol 26 (11), 1794–1805. https://doi.org/10.1016/j.euroneuro.2016.09.002.

Xi, G., Demambro, V.E., D'Costa, S., Xia, S.K., Cox, Z.C., Rosen, C.J., Clemmons, D.R., 2020. Estrogen stimulation of pleiotrophin enhances osteoblast differentiation and maintains bone mass in IGFBP-2 null mice. Endocrinology 161 (4), bqz007. https://doi.org/10.1210/endocr/bqz007.

Yao, H., Shen, H., Yu, H., Wang, C., Ding, R., Lan, X., Tash, D., Wu, X., Wang, X., Zhang, G., 2021. Chronic ethanol exposure induced depressive-like behavior in male C57BL/6 N mice by downregulating GluA1. Physiol. Behav. 234, 113387 https://doi.org/10.1016/j.physbeh.2021.113387.