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Fluoxetine modulates both endocannabinoid and lysophosphatidic acid pathways in a region-specific Manner during alcohol withdrawal in male rats

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ABSTRACT

Anxiety is a major symptom associated with alcohol withdrawal and a major factor increasing the risk of relapse. Although fluoxetine, a selective serotonin reuptake inhibitor, is used to alleviate these symptoms, its effects on brain lipid signaling pathways involved in withdrawal-related anxiety remain unclear. This study evaluated, in a preclinical model, the behavioral and molecular effects of chronic alcohol exposure and fluoxetine treatment during early abstinence. Male Wistar rats received oral alcohol (3 g/kg) or saline for 14 days, followed by 7 days without alcohol, during which fluoxetine (10 mg/kg) was administered to designated groups. Anxiety-like behavior was assessed using the elevated plus maze. Circulating plasma levels of corticosterone, 2-arachidonoylglycerol (2-AG), lysophosphatidic acid (LPA), and interleukin-10 (IL-10) were quantified, and gene expression analyses were performed in the amygdala and medial prefrontal cortex (mPFC). Chronic alcohol administration increased anxiety-like behavior and plasma 2-AG, while reducing LPA and IL-10 levels. Fluoxetine induced an anxiolytic effect in controls but was ineffective in alcohol-exposed rats, only normalizing the alcohol-induced increase of plasma 2-AG. At the molecular level, fluoxetine modulated gene expression region-specifically, altering 2-AG-related genes in the amygdala and enhancing LPA signaling in the mPFC. Hierarchical clustering revealed coordinated downregulation of 2-AG pathway genes in the alcohol-fluoxetine group and partial restoration of anti-inflammatory markers. These findings indicate fluoxetine modulates lipid signaling and immune-related genes during alcohol withdrawal, but its anxiolytic efficacy may be limited after alcohol exposure. These findings may contribute to the development of targeted therapeutic strategies for alcohol-related anxiety and relapse prevention.

1. Introduction

Alcohol consumption represents a global public health concern, with high rates of dependence and relapse (Geneva: World Health Organization, 2024; Schellekens et al., 2015). After alcohol cessation, many individuals experience intense anxiety symptoms, which significantly increase the risk of relapse and complicate long-term recovery (Geneva: World Health Organization, 2024; Schellekens et al., 2015).

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These affective disturbances are not merely psychological in nature but reflect deeper neurobiological changes. Thus, in preclinical models, alcohol withdrawal precipitates anxiety-like behaviors, accompanied by alterations in brain regions critical for emotional regulation, such as the amygdala (Gilpin et al., 2015) and the medial prefrontal cortex (mPFC) (George et al., 2012).

To alleviate withdrawal-induced anxiety and prevent relapse, selective serotonin reuptake inhibitors (SSRIs) like fluoxetine are sometimes prescribed in clinical settings. However, there are relevant reports limiting the efficacy of SSRIs as antidepressants in the context of long-term alcohol use disorders, indicating that alcohol disrupts their mechanism of action (Tirado Muñoz et al., 2018; Torrens et al., 2005). Fluoxetine is primarily known for enhancing serotonergic neurotransmission by inhibiting serotonin reuptake (Sohel et al., 2024). However, beyond its classical mechanism of action, increasing evidence suggests that fluoxetine may also modulate neuroimmune and lipid signaling pathways (Serra and Fratta, 2007; Yang et al., 2020). These alternative pathways could be particularly relevant in the context of alcohol withdrawal, where disruptions in lipid-based neuromodulation have been implicated in anxiety-like states.

One such pathway is the endocannabinoid system (ECS), which plays a key role in regulating emotional behaviors and is strongly implicated in the neurobiology of addiction and withdrawal (Serrano and Natividad, 2022; Wolfe et al., 2022). One of its main lipid mediators, 2-arachidonoylglycerol (2-AG), acts predominantly on cannabinoid receptor type 1 (CB_1), and to a lesser extent CB_2 (Gallego-Landin et al., 2021), influencing processes such as mood regulation, anxiety, stress reactivity, neuroinflammation, and reward signaling (Serrano and Parsons, 2011). 2-AG is synthesized from diacylglycerol (DAG) by the enzymes diacylglycerol lipase-α (DAGLα), encoded by *Dagla*, and DAGLβ, encoded by *Daglb*. While DAGLα is mainly expressed in neurons and contributes to synaptic 2-AG signaling (Gao et al., 2010; Katona and Freund, 2012; Schuele et al., 2022), DAGLβ is more abundant in microglia and peripheral immune cells, contributing to tonic 2-AG production under inflammatory conditions (Bainbridge et al., 2022; Shin et al., 2020; Young and Denovan-Wright, 2022). 2-AG is mainly degraded by monoacylglycerol lipase (MAGL), encoded by *Mgll* (Gallego-Landin et al., 2021). In brain areas like the amygdala, this signaling cascade modulates fear, anxiety, and stress-related responses, and may be disrupted during alcohol withdrawal (Serrano et al., 2018).

A second lipid-based signaling system, the lysophosphatidic acid (LPA) pathway, also plays a crucial role in brain function, particularly in neurodevelopment, synaptic plasticity, immune regulation, and emotional behavior (Kasatkina et al., 2021; Yung et al., 2015). LPA is primarily produced from lysophosphatidylcholine (LPC) by the enzyme autotaxin (ATX), encoded by *ENPP2*. Alternatively, LPA can be generated intracellularly via phosphorylation of monoacylglycerols by acylglycerol kinase (AGK), encoded by *Agk* (Pagès et al., 2001). LPA acts through a family of G protein-coupled receptors ($LPA_{1\text{-}6}$), with LPA_1 (encoded by *Lpar1*) being the most studied in the brain (Li and Li, 2024).

Several studies suggest functional cross-talk between the ECS and LPA system, both of which may be dysregulated during alcohol withdrawal and jointly contribute to anxiety-related pathology (González de San Román et al., 2019; Sánchez-Marín et al., 2025; Zhao and Abood, 2013). These systems share overlapping metabolic pathways, and their endogenous ligands can undergo mutual interconversion: 2-AG can be phosphorylated to LPA via monoacylglycerol kinase, while LPA can be dephosphorylated back to 2-AG through phosphatases (Kanoh et al., 1983; Nakane et al., 2002). At the metabolic interface of both systems lies phospholipase C beta 3 (PLCβ3), encoded by *Plcb3*, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce DAG. DAG is a precursor for 2-AG (via DAGLα/β), which can be phosphorylated by AGK to form LPA. This places PLCβ3 as a pivotal upstream regulator at the convergence of the endocannabinoid and LPA signaling networks, integrating lipid-mediated pathways involved in mood regulation and neuroinflammation (Falzone and MacKinnon, 2023). Interestingly, neuroinflammatory processes have been implicated in the pathophysiology of both anxiety disorders and alcohol-related conditions (Flores-Bastías and Karahanian, 2018). In this context, the anti-inflammatory cytokine interleukin-10 (IL-10) is a key immunoregulatory molecule that counteracts pro-inflammatory signaling and promotes the restoration of homeostasis in the central nervous system (Chen et al., 2025; Colton, 2009). IL-10 has been shown to restore aberrant GABAergic transmission associated with alcohol withdrawal (Patel et al., 2021), suggesting it may also contribute to the behavioral effects of fluoxetine during abstinence.

Taken together, these findings underscore the potential involvement of lipid signaling pathways in the neurobiological effects of fluoxetine during alcohol withdrawal. However, it remains unclear whether fluoxetine can modulate anxiety-related behavior and associated molecular pathways during early abstinence, specially taking in consideration the described reduced efficacy as antidepressant in abstinent AUD patients (Torrens et al., 2005). A growing body of evidence suggests that serotonergic, lipid, and immune signaling systems are closely interconnected and together regulate emotional and stress-related processes, particularly during alcohol withdrawal. Anatomical and functional studies have demonstrated a bidirectional crosstalk between serotonergic and endocannabinoid systems (Colangeli et al., 2021). Moreover, serotonergic activity has been also shown to regulate phospholipase C-dependent lipid pathways that converge on both 2-AG and LPA production (Parrish and Nichols, 2006). Serotonergic signaling also influence immune function by modulating cytokine release and neuroinflammatory responses (Saggu et al., 2025). Thus, disruptions in serotonergic transmission during alcohol withdrawal may indirectly impact lipid-mediated neuromodulation and immune balance. In addition, fluoxetine, beyond its role as a serotonin reuptake inhibitor, has been reported to exert anti-inflammatory and lipid-modulating effects, including alteration of endocannabinoid signaling and upregulation of the anti-inflammatory cytokine IL-10 (Gallant et al., 2025; Mato et al., 2010; Suárez et al., 2020). Collectively, these findings support the notion that fluoxetine may restore emotional and molecular homeostasis during alcohol withdrawal not only through serotonergic mechanisms but also by engaging lipid and immune pathways. Accordingly, we hypothesized that fluoxetine might influence both endocannabinoid and LPA signaling cascades during early withdrawal, contributing to the modulation of anxiety-like behavior and neuroinflammatory responses. To address this hypothesis, the present study investigated whether fluoxetine can influence anxiety-like behaviors and key molecular markers during early withdrawal from chronic alcohol exposure. Male rats were administered alcohol via oral gavage for 14 consecutive days, followed by a 7-day alcohol-free period during which they received fluoxetine treatment. At the end of this period, anxiety-like behavior was assessed using the elevated plus maze (EPM). In parallel, we quantified circulating levels of 2-AG, LPA, corticosterone, and IL-10 to evaluate systemic responses. At the central level, we examined the expression of genes involved in 2-AG and LPA synthesis and degradation, as well as *Il10*, in the amygdala and the medial prefrontal cortex (mPFC), two brain regions critically involved in emotional regulation and anxiety, particularly during alcohol withdrawal. Measuring IL-10 levels allows us to assess neuroimmune alterations during early abstinence and to explore whether fluoxetine exerts anxiolytic effects partly through immunomodulatory mechanisms.

2. Material and methods

2.1. Animals and ethical statement

Thirty-two male Wistar rats aged 8 weeks were obtained from Charles River Laboratories (France) and housed in our animal facility. Animals were housed in group of four animals in a humidity- and temperature-controlled room under a 12-h light/dark cycle (lights on at 08:00 h) with water and food provided ad libitum.

All the procedures were conducted in accordance with the ARRIVE guidelines and the European directive 2010/63/EU for the protection of animals used for scientific purposes and the Spanish regulations for the care and use of laboratory animals (Real Decreto 53/2013 and 178/2004, Ley 32/2007 and September 2003 and Decreto 320/2010), and were approved by the research ethics committees of the University of Málaga (CEUMA: 67-2020-A). All efforts were made to reduce the number of animals used and to minimize unnecessary pain and/or distress.

2.2. Drugs

Fluoxetine hydrochloride (Merck Life Science S.L.U., Madrid, Spain) was freshly prepared each day by dissolving it in 0.9 % saline and administered intraperitoneally (i.p.) at a dose of 10 mg/kg in a volume of 2 mL/kg of body weight, in accordance with previously established protocols (Aranda et al., 2021; Suárez et al., 2020).

2.3. Experimental design

A total of 32 male rats were randomly assigned to two initial groups: control (n = 16) and alcohol (n = 16). The alcohol group received daily intra-gastric alcohol administrations via gavage at a dose of 3 g/kg in a volume of 15 mL/kg (25 % alcohol in saline, v/v) for 14 consecutive days (Fig. 1). Although blood alcohol concentrations (BAC) were not measured in the present study to minimize additional stress or procedural interference, this protocol is widely considered a reliable model of binge-like alcohol consumption producing pharmacologically relevant intoxication. Previous studies from our group using the same procedure have reported BAC values exceeding 80 mg/dL, consistent with the National Institute on Alcohol Abuse and Alcoholism (NIAAA) criteria for binge drinking (National Institute on Alcohol Abuse and Alcoholism, 2004; Sánchez-Marín et al., 2022b, 2022a). In those studies, this exposure protocol also produced robust behavioral alterations, including increased anxiety-like behavior and changes at transcriptional level. Given the similar experimental conditions and well-established reproducibility of the protocol, comparable BAC can be expected. It is important to note that the binge-like alcohol exposure model used in this study involves forced administration via intragastric gavage rather than voluntary drinking. This procedure ensures precise control over alcohol dose and timing but also represents a non-voluntary and potentially stressful approach that differs from free-choice paradigms. The administered dose corresponds to a high and pharmacologically relevant level of alcohol exposure that, in some animals, may transiently impair motor coordination or induce a partial loss of the righting reflex (Forbes et al., 2013; Vetreno et al., 2023). Therefore, while this model reliably reproduces binge-like patterns of alcohol intoxication and withdrawal, the forced nature and intensity of exposure may amplify stress-related or neuroimmune responses. The control group received an isovolumetric equivalent of saline following the same schedule and procedure.

After alcohol exposure, each main group was further divided into two subgroups (n = 8 per group), resulting in four experimental conditions: control-vehicle, control-fluoxetine, alcohol-vehicle, and alcohol-fluoxetine. Fluoxetine was administered i.p. at a dose of 10 mg/kg once daily for 7 days during an alcohol-free period. After the completion of fluoxetine treatment, all animals underwent behavioral testing.

2.4. Elevated plus maze

Twenty-four hours after the final fluoxetine administration and 7 days of alcohol withdrawal, animals were tested in the elevated plus maze (EPM) to assess anxiety-like behavior. This time-point of alcohol withdrawal was chosen to evaluate persistent emotional disturbances beyond the acute withdrawal symptoms (Heilig et al., 2010). The test was conducted between 09:00 and 15:00 h during the light phase of the diurnal cycle, as previously described (Sánchez-Marín et al., 2017, 2022a). The apparatus consisted of a plus-shaped maze elevated 75 cm above the floor, with two open arms and two closed arms (each 45 × 10 cm) connected by a central platform (10 × 10 cm). At the beginning of the test, each rat was placed on the central platform facing one of the open arms and allowed to freely explore the maze for 5 min. The following parameters were analyzed: total distance traveled (cm), time spent in the open arms (s), time spent in the closed arms (s), distance traveled in the open arms (cm), distance traveled in the closed arms (cm), percentage of time spent in the open arms [% time = (time in open arms × 100)/total test time], and percentage of distance traveled in the open arms [% distance = (distance in open arms × 100)/total distance traveled]. All parameters were quantified using EthoVision XT 17 software (Noldus, Wageningen, The Netherlands).

2.5. Sample collection and brain dissection

One week after the last fluoxetine treatment, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and blood and brain samples were collected. Samples were collected at this time point to capture stable neurobiological adaptations associated with protracted alcohol withdrawal and fluoxetine exposure. This timing was based on our previous studies using an intermittent binge-like alcohol model, in which molecular analyses were performed two weeks after the last alcohol administration (Sánchez-Marín et al., 2022a, 2022b). Following the same rationale, we maintained a comparable interval between the last alcohol exposure and brain collection in the current continuous-exposure model. This sampling time also takes into account the slow pharmacokinetics of fluoxetine, since both fluoxetine and its active metabolite norfluoxetine display prolonged half-lives in rodents, ensuring substantial serotonergic modulation during the week following treatment cessation (Caccia et al., 1990; Fuller and Perry, 1992). This approach minimizes the influence of acute pharmacological or stress-related effects during behavioral testing and allows the assessment of consolidated transcriptional adaptations induced by both alcohol and fluoxetine.

The blood samples were centrifuged (2000×*g* for 15 min) and plasma aliquots were kept for further analysis. The brains were quickly

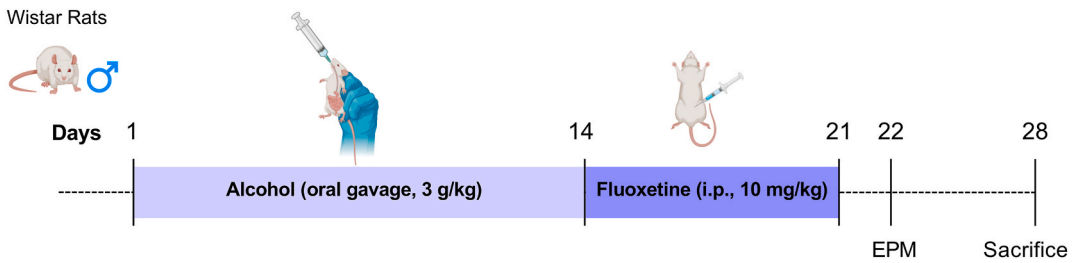

Fig. 1. Experimental workflow. Male rats received alcohol by gavage (via oral, 3 g/kg) for 14 consecutive days. This was followed by a 7-day alcohol-free period during which animals were treated with vehicle or fluoxetine (i.p., 10 mg/kg). Spontaneous motor activity and anxiety-like behaviors were evaluated 24h after the last fluoxetine administration using the EPM. All animals were sacrificed 1 week later, and brain and blood samples were collected for determinations.

removed, immediately frozen on dry ice and stored at −80 °C until mRNA analyses. The frozen brains were placed in acrylic rat brain matrices, and 2 mm thick slices were obtained using brain matrix razor blades. The mPFC and the entire amygdaloid complex (hereafter referred to as the amygdala) were dissected bilaterally. The amygdala was collected without distinction among individual nuclei to ensure sufficient tissue for molecular analyses and to evaluate overall gene expression within this limbic structure. Both brain regions were identified using a rat brain atlas (Paxinos and Watson, 2014).

2.6. Determination of plasma of corticosterone, 2-AG, LPA, and IL-10

Commercially available enzyme-linked immunoassay (ELISA) kits were used to measure plasma levels of corticosterone (ab323743, Abcam Ltd., Cambridge, UK), 2-AG (CEO443Ge, Cloud-Clone Corp, Hoston, Tx, USA), LPA (MBS2612504, MyBioSource, San Diego, CA, USA), and IL-10 (ab100764, Abcam Ltd., Cambridge, UK), following the manufacturer's instructions.

2.7. RNA isolation

Total RNA was extracted from brain tissue using Trizol Reagent (Gibco BRL Life Technologies, Baltimore, MD, USA), and RNA concentration and purity were determined by spectrophotometry, ensuring 260/280 absorbance ratios between 1.8 and 2.0. Reverse transcription was performed using the Transcriptor Reverse Transcriptase kit and random hexamer primers (iScript™ Reverse Transcription Supermix, Bio-Rad Laboratories, Hercules City, CA, USA). The RT-qPCR was performed using a CFX Duet Real-Time PCR System (Bio-Rad Laboratories, Hercules City, CA, USA) and the FAM dye label format for the TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). We assessed the expression of genes encoding endocannabinoid and LPA receptors (*Cnr1, Cnr2, Lpar1*), as well as key enzymes involved in the synthesis and degradation of these lipid mediators (*Dagla, Daglb, Enpp2, Plcb3, Agk, Mgll*), along with the gene expression of the anti-inflammatory cytokine IL-10 (*Il10*).

Primer sequences and assay IDs were selected from the Applied Biosystems rat mRNA references databases (http://bioinfo.appliedbiosystems.com/genome-database/gene-expression.html) (Table S1).

2.8. Relative quantification for gene expression

Gene expression levels were normalized to the housekeeping gene *Actb* (β-actin), which showed stable expression across experimental conditions in both the amygdala and the mPFC. Relative quantification was calculated using the $2^{\wedge}-\Delta\Delta Ct$ method, with expression levels normalized to the control group. This method evaluates each gene independently and provides average fold changes for each treatment condition. It is particularly well-suited for detecting statistically significant differences in gene expression at the individual gene level.

2.9. Hierarchical clustering analysis

To explore coordinated transcriptional patterns, in the amygdala and mPFC, hierarchical clustering was performed using the Morpheus web-based platform. For each brain region, ΔCt values normalized to the housekeeping gene (*Actb*) were used as input, and data were further standardized using Z-score transformation per gene, enabling relative comparisons across genes and treatment groups. Both genes (rows) and samples (columns) were clustered using Euclidean distance as the similarity metric and the average linkage method. Heatmaps with dendrograms were generated to visualize expression patterns and to identify potential gene clusters with shared regulatory or functional relationships. Clustering results were subsequently re-plotted in R (v4.5.0) to optimize graphical representation of the figures.

2.10. Gene network and pathway analysis

Gene clusters identified in the hierarchical clustering were further analyzed using GeneMANIA, which integrates multiple datasets including protein and genetic interactions, co-expression, co-localization, pathways, and protein domain similarity. The list of clustered genes was submitted for analysis in *Rattus norvegicus*. Functional enrichment results were retrieved to highlight over-represented pathways and biological processes.

2.11. Statistical analysis

The data were assessed for normality (Kolmogorov-Smirnov's test), sphericity (Mauchly's test) and homoscedasticity (Levene's test). A two-way ANOVA was employed with factor 1 (f1) = *alcohol* (levels: "saline" and "alcohol"); and factor 2 (f2) = *fluoxetine* (levels: "vehicle" and "fluoxetine") as independent factors. The Bonferroni test was used as post-hoc analysis for multiple pairwise comparisons of the subgroups when an interaction (f1 x f2) was revealed by two-way ANOVA. Statistical analyses were performed using Graph-Pad Prism 9.0.2 (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± SEM, and significance was set at $p < 0.05$.

3. Results

3.1. Effects of chronic alcohol exposure and fluoxetine treatment on locomotor activity and anxiety-like behaviors in rats

We evaluated the effects of chronic alcohol exposure and/or repeated fluoxetine treatment on anxiety-like behaviors in rats. Twenty-four hours after the last fluoxetine administration, animals were tested on the EPM to evaluate spontaneous motor activity and anxiety-like behaviors (Fig. 2).

Representative heatmaps of locomotor activity were generated by overlaying the trajectories of all animals within each group (Fig. 2A), illustrating overall spatial exploration patterns for each treatment condition. As a control for locomotor activity, we first measured the total distance traveled. No significant differences were found between groups, indicating that the treatments did not affect general locomotion (Fig. 2B).

Regarding time spent in the open arms (Fig. 2C), a two-way ANOVA revealed a significant main effect of *fluoxetine* (f2: $F_{1,25} = 4.91$; $p < 0.05$), indicating that fluoxetine increased time spent in these arms. Additionally, a significant interaction between *alcohol* and *fluoxetine* was found for time spent in the closed arms (f1 × f2: $F_{1,25} = 6.77$; $p < 0.05$) (Fig. 2D). Post-hoc analysis showed that fluoxetine reduced time spent in the closed arms in control rats ($p < 0.05$), but not in alcohol-exposed rats. For the percentage of time spent in the open arms (Fig. 2E), a significant main effect of *fluoxetine* was also observed (f2: $F_{1,25} = 4.90$; $p < 0.05$), with treated animals spending a higher proportion of time in these arms. Regarding the distance traveled in the open arms (Fig. 2F), there was a significant main effect of *alcohol* (f1: $F_{1,25} = 6.43$; $p < 0.05$), showing that alcohol reduced open-arm exploration. There was also a trend toward a main effect of *fluoxetine* (f2: $F_{1,25} = 4.06$; $p = 0.055$), suggesting a potential increase in open-arm exploration with fluoxetine. Finally, the percentage of distance traveled in the open arms showed a significant main effect of *alcohol* (Fig. 2H) (f1: $F_{1,25} = 4.33$; $p < 0.05$), in which alcohol reduced the percentage of distance traveled in open arms.

3.2. Effects of chronic alcohol exposure and fluoxetine treatment on plasma levels of corticosterone, 2-AG, LPA, and IL-10

We first examined the effects of chronic alcohol exposure and/or fluoxetine treatment on plasma corticosterone levels. As shown in Fig. 3A, a two-way ANOVA revealed a significant interaction between *alcohol* and *fluoxetine* (f1 × f2: $F_{1,25} = 5.19$; $p < 0.05$) (Fig. 3A). Post-hoc

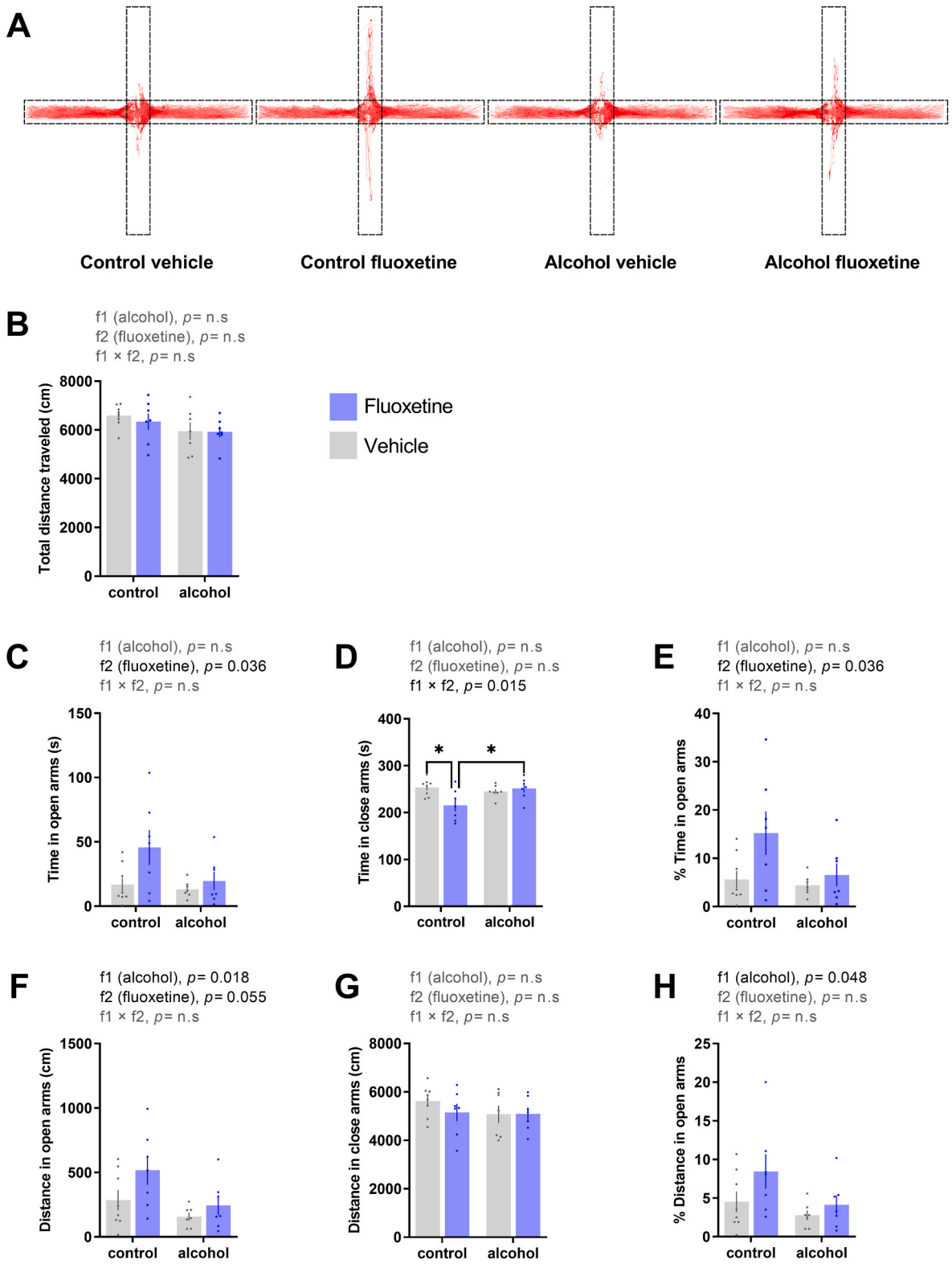

Fig. 2. Effects of chronic alcohol exposure and/or fluoxetine treatment on locomotor activity and anxiety-like behaviors. (A) Representative heatmaps of locomotor activity in the EPM; (B) total distance traveled; (C) time spent in open arms; (D) time spent in close arms; (E) percentage time in open arms; (F) distance in open arms; (G) distance in close arms; and (H) percentage of distance in open arms in the EPM 24h after the last fluoxetine administration. Data are expressed as mean ± SEM (n ≈ 8 rats per group). Data were analyzed by two-way ANOVA. Bold *p*-values indicate significant main effects of factors (f1 and f2) or a significant interaction (f1 × f2). Significant post hoc tests for multiple comparisons are indicated with lines (*$p < 0.05$).

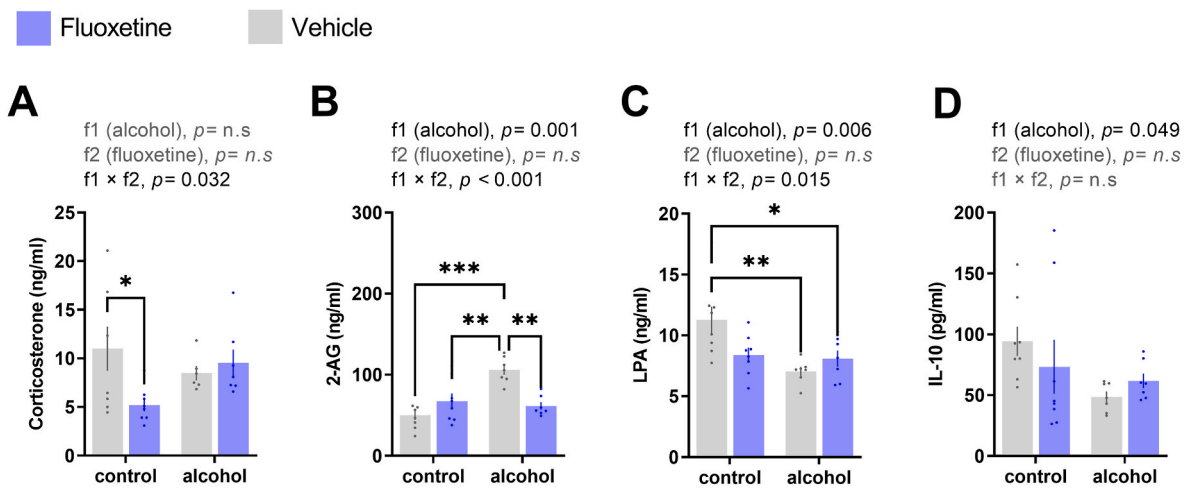

Fig. 3. Plasma levels of corticosterone, lipid mediators, and inflammatory markers. (A) Corticosterone; (B) 2-AG; (C) LPA; and (D) IL-10. Data are expressed as mean ± SEM (n ≈ 8 rats per group). Data were analyzed by two-way ANOVA. Bold *p*-values indicate significant main effects of factors (f1 and f2) or a significant interaction (f1 × f2). Significant post hoc tests for multiple comparisons are indicated with lines (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

tests for multiple comparisons showed that control-fluoxetine group displayed lower corticosterone levels than control-vehicle animals ($p < 0.05$).

Next, we analyzed plasma levels of the lipid mediators 2-AG and LPA. For 2-AG, the two-way ANOVA revealed a significant main effect of *alcohol* (f1: $F_{1,26} = 12.90$; $p < 0.01$) and a significant interaction between *alcohol* and *fluoxetine* (f1 × f2: $F_{1,26} = 19.80$; $p < 0.001$) (Fig. 3B). Post-hoc tests for multiple comparisons showed that rats in the alcohol-vehicle group had significantly higher 2-AG levels than those in the control-vehicle group ($p < 0.001$). In contrast, fluoxetine-treated rats without alcohol exposure (control-fluoxetine group) exhibited significantly lower 2-AG levels compared to the alcohol-vehicle group ($p < 0.01$). Furthermore, fluoxetine administration attenuated the alcohol-induced increase in 2-AG levels, as rats in the alcohol-fluoxetine group showed significantly lower 2-AG concentrations compared to the alcohol-vehicle group ($p < 0.01$). For LPA, a two-way ANOVA revealed a significant main effect of *alcohol* (f1: $F_{1,26} = 9.05$; $p < 0.01$), and a significant interaction between *alcohol* and *fluoxetine* treatments (f1 × f2: $F_{1,26} = 6.75$; $p < 0.05$) (Fig. 3C). Post-hoc tests for multiple comparisons showed that rats in the alcohol-vehicle group had significantly lower LPA levels compared to the control-vehicle group ($p < 0.01$). Similarly, the alcohol-fluoxetine group also exhibited reduced LPA levels compared to control-vehicle group ($p < 0.05$).

Finally, we examined plasma levels of IL-10 in these animals. As shown in Fig. 3D, a two-way ANOVA revealed a significant main effect of *alcohol* (f1: $F_{1,26} = 4.22$; $p < 0.05$), indicating that alcohol exposure decreased IL-10 levels in comparison to control rats.

3.3. Effects of chronic alcohol exposure and fluoxetine treatment on the mRNA expression of 2AG-signaling and LPA-related genes in the amygdala of rats

Next, we evaluated the effects of chronic alcohol exposure and/or fluoxetine treatment on the mRNA expression of genes associated with the synthesis, signaling, and degradation of 2-AG, as well as genes related to LPA-associated enzymes and receptors, in the amygdala of male rats. Although the amygdala is a heterogeneous structure composed of multiple nuclei that play distinct and sometimes opposing roles in anxiety regulation and alcohol-related behaviors (Gilpin et al., 2015), we analyzed the amygdaloid complex as a whole to capture overall molecular adaptations associated with alcohol withdrawal and fluoxetine treatment.

3.3.1. Cannabinoid receptors

As shown in Fig. 4A, a two-way ANOVA showed no significant main effects of *alcohol* or *fluoxetine,* nor a significant interaction both factors on mRNA levels of *Crn1* (Fig. 4A). Regarding the expression of *Cnr2* (Fig. 4B), the statistical analysis revealed a significant main effect of *fluoxetine* (f2: $F_{1,26} = 17.30$; $p < 0.001$), and a significant interaction between *alcohol* and *fluoxetine* (f1 × f2: $F_{1,26} = 8.81$; $p < 0.01$). Post-hoc tests for multiple comparisons showed a significant decrease in the mRNA levels of this receptor in alcohol-fluoxetine rats compared to control-vehicle ($p < 0.05$) and alcohol-vehicle ($p < 0.001$) rats.

3.3.2. Cannabinoid enzymes

We analyzed the mRNA expression of *Plcb3*, a phospholipase involved in the production of DAG, a precursor of 2-AG. However, the statistical analysis showed no significant main effects of *alcohol* or *fluoxetine,* nor a significant interaction between both factors on mRNA levels of this enzyme (Fig. 4C).

Next, we examined the mRNA expression of *Dagla* and *Daglb*, two enzymes directly involved in the synthesis of 2- AG. For both genes, a two-way ANOVA revealed a significant main effect of *fluoxetine* (f2: *Dagla*: $F_{1,26} = 6.17$; $p < 0.05$; f2: *Daglb* ($F_{1,26} = 4.31$; $p < 0.05$; Fig. 4D and E, respectively). Specifically, fluoxetine-treated rats displayed higher mRNA levels of both enzymes compared to vehicle-treated animals.

Regarding *Mgll*, the enzyme responsible for 2-AG degradation, a two-way ANOVA showed no significant main effects of *alcohol* or *fluoxetine,* nor a significant interaction between both factors (Fig. 4F).

3.3.3. LPA receptor

As shown in Fig. 4G, there were no significant main effects of *alcohol* or *fluoxetine,* nor a significant interaction between both factors on the mRNA expression of *Lpar1*.

3.3.4. LPA enzymes

Similarly, no significant main effects of *alcohol* or *fluoxetine,* or significant interaction between both factors, were observed on the mRNA expression of *Enpp2* (Fig. 4H) or *Agk* (Fig. 4I).

3.3.5. Hierarchical clustering

To further explore coordinated changes in gene expression, we performed hierarchical clustering on standardized mRNA levels of genes involved in 2-AG and LPA signaling pathways in the amygdala. As shown in Fig. 4J, clustering of both genes and samples revealed distinct treatment-associated expression profiles, particularly highlighting

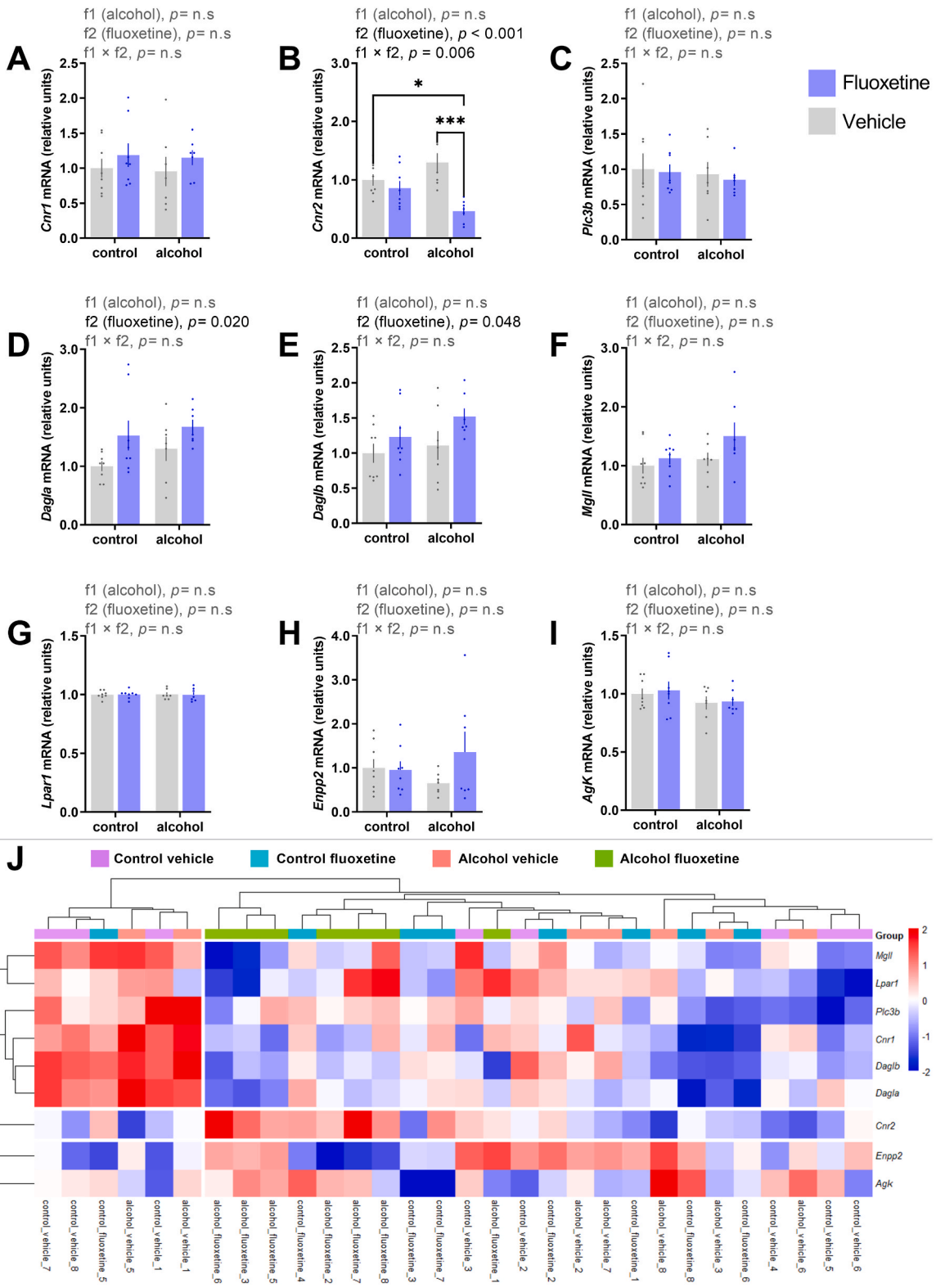

Fig. 4. Relative mRNA expression levels of genes related to 2-AG and LPA signaling in the amygdala. Relative gene expression was calculated using the $2^{\wedge}-\Delta\Delta Ct$ method for the following targets: (A) *Cnr1*, (B) *Cnr2*, (C) *Plc3B*, (D) *Dagla*, (E) *Daglb*, (F) *Mgll*, (G) *Lpar1*, (H) *Enpp2*, and (I) *Agk*. Data are expressed as mean ± SEM (n ≈ 8 rats per group). Data were analyzed by two-way ANOVA. Bold *p*-values indicate significant main effects of factors (f1 and f2) or a significant interaction (f1 × f2). Significant post hoc tests for multiple comparisons are indicated with lines (*$p < 0.05$, ***$p < 0.001$). (J) A heatmap showing hierarchical clustering, with genes represented in rows and individual samples in columns. Red indicates significant upregulation; blue indicates downregulation.

fluoxetine-induced transcriptional changes. This analysis emphasizes the concerted regulation of ECS and LPA pathway genes in response to fluoxetine, supporting the individual findings described above.

The analysis identified three major gene clusters. The first cluster included *Plcb3*, *Mgll*, *Cnr1*, *Daglb*, *Dagla*, and *Lpar1*, which showed relatively lower expression in fluoxetine-treated animals, particularly in the alcohol-fluoxetine group, compared to other treatment conditions. Although this did not necessarily indicate absolute downregulation, it suggested that these genes are less prominent within the overall transcriptional profile under combined treatment.

The second cluster, consisting of *Enpp2* and *Agk*, exhibited more heterogeneous expression across samples. A subset of fluoxetine-treated animals showed relatively elevated expression of these genes, potentially reflecting partial activation of the LPA signaling axis.

The third cluster was defined solely by *Cnr2*, which displayed a unique expression profile marked by relatively higher levels in some alcohol-fluoxetine animals.

Moreover, clustering of the 30 samples yielded two main experimental clusters. One cluster predominantly grouped control and control-fluoxetine samples, showing relatively stable gene expression. The other cluster was enriched in alcohol-fluoxetine samples, characterized by a distinct transcriptional profile, marked by reduced expression of several 2-AG–related genes (*Mgll*, *Dagla*, *Cnr1*) and strong upregulation of *Cnr2*. Together, these findings suggest that fluoxetine, particularly in the context of alcohol exposure or withdrawal, induces a coordinated shift in amygdalar gene expression. This shift primarily affects components of the endocannabinoid and LPA signaling systems in the amygdala, indicating region-specific molecular adaptations that may underline the altered behavioral and neurochemical responses observed in this treatment condition.

3.4. Effects of chronic alcohol exposure and fluoxetine treatment on the mRNA expression of 2AG-signaling and LPA-related genes in the mPFC of rats

We also assessed the impact of chronic alcohol exposure and/or fluoxetine treatment on the mRNA expression of genes involved in 2-AG signaling and the LPA system in the mPFC of male rats. To determine whether alcohol and/or fluoxetine treatments affect the brain's endogenous anti-inflammatory mechanisms, we also assessed the gene expression of *Il-10*, a crucial anti-inflammatory cytokine involved in regulating immune responses and maintaining immune homeostasis in the CNS.

3.4.1. Cannabinoid receptors

A two-way ANOVA revealed no significant main effects of *alcohol* or *fluoxetine*, nor a significant interaction between the two factors on the mRNA expression of *Cnr1* and *Cnr2* (Fig. 5A and B, respectively).

3.4.2. Cannabinoid enzymes

As shown in Fig. 5C, no significant main effects or interaction were found for *Plcb3* mRNA expression.

Regarding *Dagla* expression (Fig. 5D), the analysis showed significant main effects of both *alcohol* (f1: $F_{1,26} = 10.10$; $p < 0.01$) and *fluoxetine* (f2: $F_{1,26} = 6.41$; $p < 0.05$), revealing that both treatments reduced *Dagla* mRNA levels. In contrast, for *Daglb* (Fig. 5E), the two-way ANOVA did not reveal any significant main effects or interaction between the factors.

Similarly, *Mgll* mRNA levels (Fig. 5F) showed no significant main effects of alcohol or fluoxetine, nor a significant interaction between the two treatments.

3.4.3. LPA receptor

As shown in Fig. 5G, a two-way ANOVA revealed no significant main effects or interaction for *Lpar1* mRNA expression in the mPFC.

3.4.4. LPA enzymes

Regarding *Enpp2* expression (Fig. 5H), a two-way ANOVA revealed a significant main effect of *fluoxetine* (f2: $F_{1,26} = 6.09$; $p < 0.05$), indicating that fluoxetine treatment increased *Enpp2* mRNA levels in the mPFC. For *Agk* mRNA expression (Fig. 5I), the analysis showed significant main effects of both alcohol (f1: $F_{1,26} = 30.2$, $p<0.001$) and fluoxetine (f2: $F_{1,26} = 7.11$, $p < 0.05$), suggesting that the two treatments independently reduced *Agk* expression in the mPFC.

3.4.5. Anti-inflammatory cytokine IL-10

As shown in Fig. 5J, a two-way ANOVA revealed a significant main effect of *alcohol* (f1: $F_{1,26} = 8.02$; $p < 0.01$), indicating that rats exposed to chronic alcohol showed reduced *Il*-10 mRNA expression in comparison with control rats.

3.4.6. Hierarchical clustering

Hierarchical clustering of gene expression in the mPFC revealed treatment-specific patterns across 2-AG and LPA signaling genes (Fig. 5K). Clustering was performed on both genes and samples based on standardized expression values across the dataset, allowing comparison of expression patterns rather than absolute levels.

The analysis identified three major gene clusters. The first cluster included *Lpar1* and *Enpp2*, genes related to LPA signaling, which showed relatively higher expression in fluoxetine-treated groups, suggesting activation of LPA axis. A second cluster, including *Plcb3*, *Cnr1*, *Cnr2*, and *Il-10* showed moderate variation across conditions without a clearly consistent directional pattern, potentially reflecting gene-specific regulatory responses to treatment. The third cluster, including *Mgll*, *Daglb*, *Dagla*, and *Agk*, genes involved in 2-AG metabolism exhibited consistent downregulation, with the most pronounced downregulation observed in the alcohol-fluoxetine group. This suggests a treatment-driven suppression of genes involved in 2-AG metabolism.

Samples were grouped into four main clusters. Notably, alcohol-fluoxetine animals clustered together, displaying a coordinated transcriptional profile marked by relative suppression of several 2-AG–related genes. This suggests that the combined treatment alters the overall gene expression profile in the mPFC, characterized by lower expression of endocannabinoid-related genes and a moderate increase in LPA pathway markers.

To facilitate the integration of the different behavioral, biochemical, and molecular outcomes, Table 1 summarizes the main findings of this study.

3.5. Gene network and pathway analysis of clustered genes

To further investigate whether the clustered genes identified in the amygdala and mPFC participate in common biological pathways, we analyzed them using GeneMANIA, with particular attention to possible associations with serotonergic signaling given its central role in anxiety-related processes. Functional enrichment results confirmed a significant over-representation of lipid catabolic processes (*glycerolipid catabolic process, cellular lipid catabolic process, lipase activity; all FDR* <0.05), consistent with the role of genes such as *Mgll, Enpp2, and Dagla*. In addition, enrichment was observed in retrograde and lipid-mediated trans-synaptic signaling, supporting a role of these clusters in neuroactive lipid signaling. Importantly, enriched terms also included cAMP-mediated signaling and G protein-coupled receptor (GPCR) signaling pathways coupled to cyclic nucleotide second messengers, which are canonical mechanisms underlying serotonergic receptor signaling (5-HT receptors).

Although serotonergic signaling did not appear explicitly as an enriched term, these results indicate that the clustered genes converge on intracellular signaling mechanisms that overlap with serotonergic pathways. This analysis provides functional validation of the transcriptional clusters identified in the amygdala and mPFC, and suggests potential interplay between lipid metabolism, cannabinoid-related

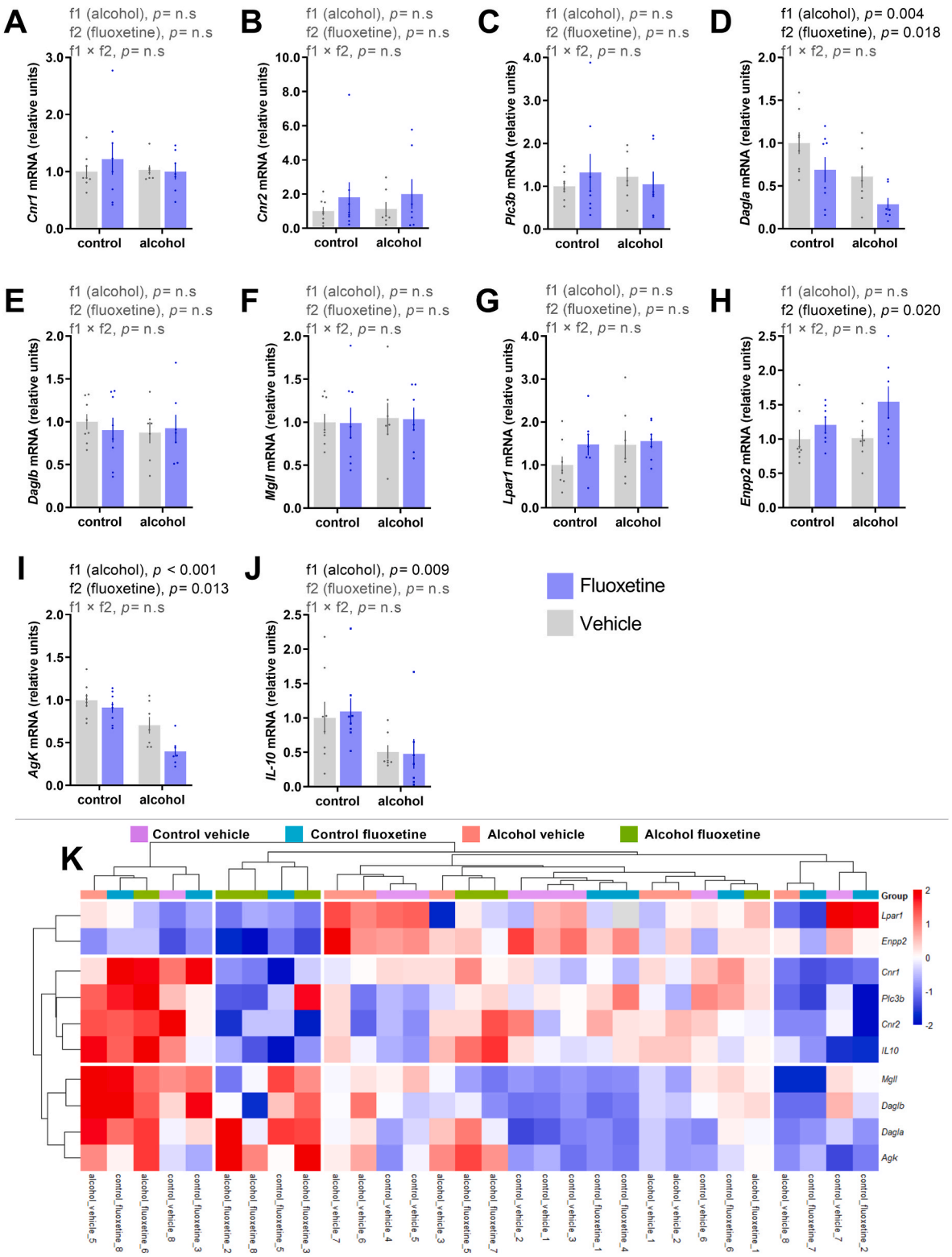

Fig. 5. Relative mRNA expression levels of genes related to 2-AG and LPA signaling, and IL-10 as a marker of inflammation in the mPFC. Relative gene expression was calculated using the 2^−ΔΔCt method for the following targets: (A) *Cnr1*, (B) *Cnr2*, (C) *Plc3b*, (D) *Dagla*, (E) *Daglb*, (F) *Mgll*, (G) *Lpar1*, (H) *Enpp2*, (I) *Agk*, and (J) *Il10*. Data are expressed as mean ± SEM (n ≈ 8 rats per group). Data were analyzed by two-way ANOVA. Bold *p*-values indicate significant main effects of factors (f1 and f2). (K) A heatmap showing hierarchical clustering, with genes represented in rows and individual samples in columns. Red indicates significant upregulation; blue indicates downregulation.

Table 1
Summary of main behavioral, biochemical, and molecular findings.

Experimental domain	Variable/marker	Effect of alcohol	Effect of fluoxetine	Interaction (alcohol x fluoxetine)	Summary interpretation
Behavior (EPM)	Locomotor activity	–	–	×	No change in general locomotion
	Time/distance in open arms	↓ (anxiogenic)	↑ (anxiolytic)	×	Fluoxetine effective only in controls; no effect after alcohol
	Time in closed arms	–	–	✔	Fluoxetine reduces anxiety only in alcohol-naïve rats
Plasma markers	Corticosterone	–	–	✔	Fluoxetine lowers corticosterone only without alcohol
	2-AG	↑	↓	✔	Alcohol increases 2-AG; fluoxetine normalizes it
	LPA	↓	–	✔	Alcohol reduces LPA; fluoxetine does not restore it
	IL-10	↓	–	×	Alcohol reduces IL-10; fluoxetine no effect
Amygdala (mRNA)	*Cnr1/Plc3b/Mgll*	–	–	×	No significant effects
	Cnr2	–	↓	✔	Fluoxetine downregulates *Cnr2* in alcohol-exposed rats
	Dagla/Daglb	–	↑	×	Fluoxetine upregulates 2-AG synthesis enzymes
	Lpar1/Enpp2/Agk	–	–	×	No significant effects
mPFC (mRNA)	*Cnr1/Cnr2/Plc3b/Daglb/Mgll*	–	–	×	No significant effects
	Dagla	↓	↓	×	Both alcohol and fluoxetine reduce 2-AG synthesis
	Lpar1	–	–	×	No significant effects
	Enpp2	–	↑	×	Fluoxetine enhances LPA synthesis pathway
	Agk	↓	↓	×	Both alcohol and fluoxetine reduce *Agk* expression
	Il-10	↓	–	×	Alcohol reduces anti-inflammatory signaling

↑/↓ = increase/decrease; — = no significant effect; ✔ = significant interaction; × = no interaction.

signaling, and serotonergic neurotransmission. A complete list of enriched functions with their FDR values and coverage is provided in Table S2, together with the network diagrams generated by GeneMANIA (Fig. S1).

4. Discussion

Chronic alcohol consumption and the use of antidepressant medications, such as SSRIs, frequently co-occur in clinical populations, yet their neurobiological interaction remains unclear. The present study aimed to evaluate behavioral and molecular effects of chronic alcohol exposure in combination with repeated fluoxetine treatment in adult male rats. Our results showed that chronic alcohol exposure induced anxiety-like behavior and that fluoxetine produced anxiolytic effects only in alcohol-naïve rats, paralleling the limited SSRI efficacy reported in alcohol use disorders. At the molecular level, alcohol and fluoxetine induced region-specific transcriptional changes in the amygdala and mPFC, particularly in genes related to 2-AG and LPA signaling pathways. Hierarchical clustering analyses revealed coordinated suppression of 2-AG pathway genes, selective regulation of immune-related markers such as *Cnr2* in the amygdala, and fluoxetine-induced upregulation of LPA-related genes in the mPFC. Peripherally, fluoxetine decreased corticosterone only in controls, whereas alcohol increased plasma 2-AG and reduced both LPA and IL-10; fluoxetine partially normalized these effects during withdrawal.

Our behavioral findings demonstrate that chronic alcohol exposure and repeated fluoxetine treatment exert distinct and interacting effects on anxiety-like behavior, without impacting general locomotor activity. Alcohol withdrawal produced an anxiogenic-like phenotype without affecting locomotion, consistent with prior evidence (Kliethermes, 2005). In this study, we assessed anxiety-like behaviors 7 days after the last alcohol exposure to capture persistent emotional alterations that extend beyond the acute withdrawal period, which is often characterized by strong somatic symptoms that can interfere with behavioral evaluation (Heilig et al., 2010). In addition to determining the appropriate timing for alcohol withdrawal assessment, fluoxetine pharmacokinetics must also be considered. Fluoxetine has one of the longest elimination half-lives among SSRIs, making withdrawal syndromes relatively uncommon and delayed compared with other antidepressants (Altamura et al., 1994; Hiemke et al., 2018). In humans, its half-life ranges from 1 to 4 days and that of its active metabolite norfluoxetine from 7 to 15 days (Altamura et al., 1994; Hiemke et al., 2018). Given that full systemic clearance requires several half-lives, norfluoxetine can persist at clinically relevant levels for weeks after discontinuation. In rodents, metabolism is faster but still prolonged, with fluoxetine half-lives of about 16 h and norfluoxetine exceeding 50 h depending on strain and dose (Caccia et al., 1990; Fuller and Perry, 1992). As a result, serotonin transporter occupancy and synaptic 5-HT modulation remain substantial for at least a week after treatment cessation, supporting the biological relevance of sampling 7 days post-fluoxetine. The present results replicate and extend previous results from our group, in which a binge-like alcohol protocol administered during adolescence also increased anxiety-like behavior in male Wistar rats 14 days into abstinence (Sánchez-Marín et al., 2022a, 2022b), reflecting a stable affective state relevant to relapse vulnerability. In the current study, the anxiolytic-like effect of fluoxetine is consistent with its established anxiolytic effects in the EPM (Rogóz and Skuza, 2011; Silva et al., 1999). However, the lack of anxiolytic response in alcohol-exposed animals suggests that chronic alcohol intake may blunt or interfere with the behavioral efficacy of fluoxetine. These results highlight the complex behavioral interaction between chronic alcohol use and antidepressant treatment, supporting the need for searching of alternative therapies for depression associated with an alcohol use disorder, instead of insisting in using SSRIs as a primary therapeutic option.

Next, we examined plasma corticosterone levels as a peripheral marker of hypothalamic-pituitary-adrenal (HPA) axis activity. We found that fluoxetine significantly reduced corticosterone levels in control animals, but this effect was absent in alcohol-exposed animals, suggesting that chronic alcohol intake may impair fluoxetine's capacity to regulate stress hormone levels. This blunted response could be partially explained by adaptive changes in serotonergic and stress pathways, including altered 5-HT_1A receptor signaling, impaired reuptake, and reduced postsynaptic responsiveness (Belmer et al., 2015; Breese et al., 2004; Khom et al., 2020; Vlkolinsky et al., 2024). Moreover, alcohol-induced stress sensitization may also produce a ceiling effect, limiting fluoxetine efficacy even after one week of treatment (Breese et al., 2004; Uzbay, 2008; Uzbay et al., 2004).

Alcohol exposure significantly increased plasma 2-AG, consistent with previous evidence linking the ECS to alcohol-induced neuroadaptations (Montesinos et al., 2020; Serrano et al., 2018; Serrano and Natividad, 2022). Interestingly, fluoxetine attenuated this elevation, suggesting modulation of peripheral endocannabinoid tone, possibly

through its anti-inflammatory properties (Blatteau et al., 2015; Caiaffo et al., 2016; Gallant et al., 2025). In contrast, chronic alcohol reduced circulating LPA levels, an effect not reversed by fluoxetine treatment. This reduction may result from alcohol-induced disruptions in lipid metabolism, including altered ATX activity or impaired hepatic synthesis of LPA precursors (Flores-López et al., 2021). Importantly, clinical evidence shows that patients with alcohol use disorder display altered lipidomic profiles, including lower plasma LPA levels and compensatory increases in ATX, suggesting a shift in the balance of LPA signaling (Flores-López et al., 2021). Reduced LPA may contribute to anxiety and neuroimmune imbalance, as LPA is essential for neuroplasticity and emotional regulation (Yung et al., 2015). Notably, our data suggest that fluoxetine is insufficient to restore this LPA deficit at the peripheral level, potentially reflecting a limited capacity of SSRIs to modulate LPA signaling directly. Finally, IL-10 levels were also diminished after alcohol exposure, confirming a pro-inflammatory state (Melkumyan et al., 2025; Sanchez-Alavez et al., 2019; Schrank et al., 2025). Taken together, these results indicate that fluoxetine mitigated some but not all alcohol-induced molecular alterations, indicating selective efficacy across neuroimmune and lipid pathways.

To further explore the molecular mechanisms underlying the behavioral and peripheral effects, we examined the expression of genes in the amygdala's 2-AG and LPA pathways, a region central to emotional regulation and stress processing. In our previous adolescent-exposure study, chronic alcohol reduced *Cnr2*, *Daglb*, and *Mgll* (Sánchez-Marín et al., 2022a), whereas no such changes appeared here in adults. This difference likely reflects developmental sensitivity, since adolescence is marked by heightened neuroplasticity and vulnerability to alcohol (Guerri and Pascual, 2010; Spear, 2018), when alcohol can induce lasting ECS alterations (Pavón et al., 2016; Sánchez-Marín et al., 2017). In adulthood, reduced plasticity (Arellano et al., 2024; Vozella et al., 2023) may limit long-term effects, producing milder transcriptional responses. Fluoxetine, particularly after alcohol exposure, induced a coordinated transcriptional shift in the amygdala that may underlie its reduced anxiolytic efficacy. We found that *Cnr1* mRNA levels were unaffected, while *Cnr2* expression was significantly downregulated by fluoxetine in alcohol-exposed rats, suggesting state-dependent modulation possibly linked to neuroimmune adaptation. Because chronic alcohol provokes amygdalar inflammation and anxiety-like behavior (Melkumyan et al., 2025; Schrank et al., 2025) and the CB2 receptor regulates immune resolution (Turcotte et al., 2016), its downregulation indicates disrupted ECS-immune balance. Interestingly, while qPCR showed *Cnr2* suppression, clustering identified it as a marker of the alcohol–fluoxetine group, implying variable compensatory upregulation in some animals. Fluoxetine increased expression of both *Dagla* and *Daglb*, enzymes driving 2-AG synthesis, regardless of alcohol history, suggesting enhanced biosynthetic capacity and potential anxiolytic effects through elevated endocannabinoid tone. This aligns with evidence that 2-AG modulates amygdalar activity and reduces anxiety (Sciolino et al., 2011; Sumislawski et al., 2011). Previous studies have demonstrated that increasing 2-AG levels reduces anxiety-like behavior (Bedse et al., 2020; Vozella et al., 2023), while impaired signaling heightens it (Marcus et al., 2020). Interestingly, these findings contrast with reduced peripheral 2-AG after fluoxetine, pointing to a possible divergence between central and peripheral responses. It is also notable that *Mgll*, the main degradative enzyme, was unchanged, supporting that fluoxetine primarily boosts synthesis. No LPA-related genes showed individual treatment effects, but hierarchical clustering revealed a broader ECS downregulation in the alcohol-fluoxetine group, even with *Dagla* and *Daglb* upregulated. Thus, hierarchical clustering reveals broader pathway-level changes that are not always evident in single-gene analyses. Overall, the amygdala exhibited opposing regulatory patterns that may explain the altered behavioral and neuroimmune responses to combined alcohol and fluoxetine. Because tissue included the entire amygdaloid complex, results likely reflect integrated activity across its nuclei. Future nucleus-specific analyses are needed to clarify the contributions of individual amygdalar subregions.

To complement our findings in the amygdala, we examined gene expression changes in the mPFC, a region crucial for emotional regulation and antidepressant response. Chronic alcohol and subsequent withdrawal are known to disrupt mPFC function, contributing to anxiety-like behavior (George et al., 2012). In particular, reduced 2-AG tone in this region has been associated with increased anxiety and emotional dysregulation (Marcus et al., 2020; Rosas-Vidal et al., 2025). In our previous adolescent-exposure study (Sánchez-Marín et al., 2022a), alcohol increased expression of *Cnr1*, *Dagla*, *Daglb*, and *Mgll*, whereas here, adult exposure decreased *Dagla*. This developmental divergence supports age-dependent transcriptional responses and highlights the importance of consumption history (Guerri and Pascual, 2010; Spear, 2018). Unlike the amygdala, the mPFC displayed reduced *Dagla* expression after both alcohol and fluoxetine, suggesting a diminished capacity for 2-AG synthesis. Such reduction may enhance serotonergic neurotransmission by decreasing CB_1-mediated inhibition on serotonergic neurons (Haj-Dahmane and Shen, 2011), consistent with fluoxetine's antidepressant mechanism.

Additionally, we observed alcohol-induced downregulation of *Agk*, a gene involved in LPA biosynthesis from monoacylglycerols like 2-AG. This may either reflect a compensatory adaptation to reduced *Dagla* expression or a broader suppression of lipid signaling in the mPFC induced by alcohol. Concomitantly, chronic alcohol exposure significantly reduced *Il10* mRNA levels, indicating a pro-inflammatory shift in the mPFC. This local reduction mirrored the drop in plasma IL-10 levels, suggesting a systemic inflammatory state during alcohol withdrawal that may impair brain function and antidepressant efficacy. Fluoxetine increased *Enpp2*, suggesting activation of the LPA–LPA_1 axis, whereas *Lpar1* itself was unchanged in individual analyses but grouped with *Enpp2* in clustering, indicating coordinated upregulation. These results align with previous findings that deficient LPA_1 signaling promotes alcohol intake and anxiety-like behavior (Castilla-Ortega et al., 2016; Moreno-Fernández et al., 2017; Sánchez-Marín et al., 2025). Although acute LPA elevation can be anxiogenic, sustained activation reduces anxiety (Li and Li, 2024), suggesting that fluoxetine's stimulation of the LPA pathway in the mPFC could counteract withdrawal-related anxiety. Nevertheless, alcohol's suppression of *Dagla, Agk,* and *Il10* points to persistent impairments in endocannabinoid and immune regulation. In summary, the mPFC exhibited a transcriptional profile opposite to that of the amygdala: fluoxetine enhanced LPA-related genes but suppressed 2-AG synthesis, whereas alcohol disrupted both systems. These region-specific molecular interactions between ECS, LPA, and immune pathways likely underlie fluoxetine's limited efficacy following chronic alcohol exposure and emphasize the need for combined therapies targeting lipid and neuroimmune signaling.

5. Conclusions

This study demonstrates that chronic alcohol exposure and fluoxetine treatment exert distinct, region-specific effects on anxiety-like behavior, gene expression, and peripheral biomarkers. While fluoxetine partially reversed alcohol-induced alterations in endocannabinoid and inflammatory pathways, it failed to normalize behavior in alcohol-exposed rats. Hierarchical clustering revealed coordinated regulation of 2-AG and LPA genes, emphasizing pathway-level interactions not evident in single-gene analyses. Fluoxetine enhanced 2-AG synthesis in the amygdala and LPA signaling in the mPFC, whereas alcohol disrupted both lipid and IL-10–mediated anti-inflammatory responses. These molecular imbalances may underlie fluoxetine's reduced anxiolytic efficacy after alcohol exposure.

Overall, fluoxetine engaged compensatory molecular mechanisms that proved insufficient under alcohol-induced neuroadaptations. These findings highlight the need for therapeutic strategies that jointly target serotonergic, lipid, and immune pathways to restore emotional balance during alcohol withdrawal. Integrating quantitative and pattern-based

transcriptomic analyses provides a more comprehensive view of the neurobiological effects of pharmacological interventions in alcohol-related affective disorders.

6. Limitations

This study has several limitations that should be acknowledged. First, we assessed gene expression at the mRNA level only, without measuring corresponding protein levels. While mRNA data provide valuable information about gene regulation, both at the level of individual genes and through broader patterns revealed by clustering, it is important to note that mRNA does not always reflect protein expression or functional activity. Post-transcriptional modifications, protein turnover, and translational control can all affect the final protein output. Therefore, our conclusions based on gene expression should be interpreted with caution until confirmed by protein-level analysis. Second, behavioral and molecular measurements were obtained at slightly different time points. This design was intentionally chosen to evaluate more persistent molecular adaptations associated with early protracted withdrawal and fluoxetine exposure. However, this temporal separation may limit the possibility of establishing direct one-to-one correlations between behavioral and transcriptional outcomes. Additionally, anxiety-like behavior was evaluated after 7 days of withdrawal to focus on the early protracted abstinence phase rather than the acute withdrawal period. While this choice minimizes the confounding effects of somatic withdrawal symptoms, future studies should also include earlier time points as well as complementary behavioral paradigms to capture the full temporal profile of withdrawal-induced anxiety and its molecular correlates. Another limitation is that we included only male rats in this study. It is increasingly recognized that there are sex differences in behavioral responses to stress, substance use, and neuroimmune and lipid signaling pathways. Including female subjects in future research would allow for a more comprehensive understanding of the neurobiological mechanisms underlying alcohol–fluoxetine interactions. Finally, although fluoxetine was administered during a one-week abstinence period, this relatively short duration may not be enough to capture its full therapeutic potential, especially considering the long-term neuroadaptive effects of chronic alcohol exposure. Extending the treatment period or including time-course analyses in future studies would help clarify how these effects evolve over time.

CRediT authorship contribution statement

Adriana Castro-Zavala: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kirstin Boonen:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Laura Sánchez-Marín:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Marisa Roberto:** Writing – review & editing. **Francisco J. Pavón-Morón:** Writing – review & editing, Funding acquisition, Conceptualization. **Fernando Rodríguez de Fonseca:** Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Antonia Serrano:** Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2025.110773.

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