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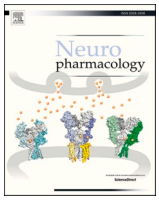

Sex-specific alterations in emotional behavior and neurotransmitter systems in LPA_1 receptor-deficient mice

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ABSTRACT

Lysophosphatidic acid (LPA) and the endocannabinoid system (ECS) are critical lipid signaling pathways involved in emotional regulation and behavior. Despite their interconnected roles and shared metabolic pathways, the specific contributions of LPA signaling through the LPA_1 receptor to stress-related disorders remain poorly understood. This study investigates the effects of LPA_1 receptor deficiency on emotional behavior and neurotransmitter-related gene expression, with a focus on sex-specific differences, using $maLPA_1$-null mice of both sexes. We hypothesized LPA_1 receptor loss disrupts the interplay between LPA and the endocannabinoid 2-arachidonoylglycerol (2-AG) signaling, resulting in distinct behavioral and molecular alterations. $maLPA_1$-null mice exhibited increased anxiety-like behaviors and altered stress-coping responses compared to wild-type counterparts, with more pronounced effects observed in females. Female mice also displayed higher corticosterone levels, though no genotype-related differences were observed. Plasma analyses revealed elevated LPA levels in $maLPA_1$-null mice, suggesting a compensatory mechanism, and reduced 2-AG levels, indicating impaired ECS signaling. Gene expression profiling in the amygdala and medial prefrontal cortex showed significant alterations in the gene expression of key components of LPA and 2-AG signaling pathways, as well as neuropeptide systems such as corticotropin-releasing hormone (CRH) and neuropeptide Y (NPY). Glutamatergic signaling components also exhibited sex-specific variations. These findings suggest that LPA_1 receptor deficiency impacts behavioral response and disrupts sex-specific neurotransmitter signaling, emphasizing the importance of LPA-ECS crosstalk in emotional regulation. This study provides insights into the molecular mechanisms underlying stress-related disorders such as depression and anxiety, which may inform the development of sex-specific therapeutic approaches.

1. Introduction

The lysophosphatidic acid (LPA) and the endocannabinoid system (ECS) are two closely related lipid signaling systems that share several characteristics, including that both systems are expressed at central and peripheral levels, they are not stored in intracellular compartments but are synthesized on demand by cleavage from membrane lipid precursors, these lipids share metabolic pathways, and they are rapidly inactivated by specific enzymes (Orio et al., 2013). Moreover, both lipid signaling systems regulate the function of the central nervous system (CNS) with implications for behavior, playing a key role in the modulation of emotions and mood (Moreno-Fernandez et al., 2018; Serrano

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and Natividad, 2022; Serrano and Parsons, 2011).

LPA is a naturally occurring bioactive lipid that participates in multiple biological processes. LPA is synthetized through different metabolic pathways, with the enzyme autotaxin (ATX) playing a crucial role (Aoki et al., 2008). ATX is an enzyme with lysoPLD activity and acts as the primary enzyme responsible for maintaining LPA concentrations in the blood (Riaz et al., 2016). This lipid exerts its signaling effects by interacting with a complex family of G protein-coupled receptors ($LPA_{1\text{-}6}$) widely distributed throughout the body (Choi and Chun, 2013; Choi et al., 2010), regulating plasticity and behavior (Penalver et al., 2017; Yung et al., 2015). Among LPA receptors, the LPA_1 receptor is the most extensively studied (Choi and Chun, 2013; Choi et al., 2010; Yung et al., 2014, 2015). This receptor plays a role in the regulation of emotions and the stress response, and its inactivation leads to functional alterations in crucial brain regions involved in the regulation of emotional behaviors and the stress response (Moreno-Fernandez et al., 2017, 2018; Pedraza et al., 2014). The majority of studies conducted to examine the function of LPA-LPA_1 receptors signaling have been carried out using mice lacking this receptor (LPA_1-null mice). These mutant mice exhibit certain impairments in both the hippocampus and cortex (Estivill-Torrus et al., 2008; Matas-Rico et al., 2008), which may contribute partially to the behavioral phenotype observed in these mice, including cognitive alterations, increased anxiety-like behaviors, impaired extinction of contextual fear memories, and dysfunctional coping in response to chronic stress (Blanco et al., 2012; Castilla-Ortega et al., 2010; Moreno-Fernandez et al., 2017; Pedraza et al., 2014; Santin et al., 2009).

The ECS is a complex and ubiquitous signaling system that has important regulatory functions throughout the body. It consists of cannabinoid receptors (CB_1 and CB_2), endogenous ligands or endocannabinoids, and the enzymatic machinery involved in their synthesis and inactivation (Lowe et al., 2021). The most well-characterized endocannabinoids to date are anandamide (AEA) and 2-arachidonoylglycerol (2-AG), both are derivatives of arachidonic acid. Structurally, AEA belongs to the family of acylethanolamides, while 2-AG belongs to another family of signaling molecules called monoacylglycerols, which are present in the CNS with even higher levels than acylethanolamides and have modulatory effects at the synaptic levels. The biosynthesis and degradation pathways for monoacylglycerols involve complex enzymatic cascades that are crucial in regulating the endogenous levels of these lipids. The endocannabinoid 2-AG is closely linked to the synthesis of diacylglycerol (DAG) through the breakdown of an inositol phospholipid by a phospholipase C (PLC). This DAG is then transformed into 2-AG by the action of two specific lipases (DAGLα/β). While it can be hydrolyzed by different enzymes, monoacylglycerol lipase (MAGL) is the main enzyme responsible for its degradation (Ueda et al., 2011). The activation of cannabinoid receptors, CB_1 and CB_2, by 2-AG is involved in regulating numerous physiological processes, including neuroinflammation, mood, anxiety, stress response, and addiction (Serrano and Parsons, 2011).

As previously mentioned, there is a relationship between LPA and the ECS, and both signaling systems play relevant roles in the control of emotional behaviors. Moreover, both systems are closely interconnected, and their endogenous ligands can undergo mutual interconversion within a cell (Zhao and Abood, 2013). In fact, 2-AG can be metabolized to LPA by the action of a MAG kinase (Kanoh et al., 1986; Shim et al., 1989), and the opposite reaction is also possible through the action of a phosphatase (Nakane et al., 2002). Nevertheless, although both endogenous ligands share metabolic pathways, direct interaction between CB receptors and LPA is not observed, and similarly, 2-AG does not exhibit affinity for the LPA receptors (Nakane et al., 2002). Furthermore, a reciprocal interaction has been observed between LPA_1 and CB_1 receptors, wherein the absence of one of these receptors results in modulation of the other (Gonzalez de San Roman et al., 2019).

Both LPA and the ECS are involved in the regulation of emotional behaviors, but their effects may differ between males and females due to biological and hormonal factors. Sex hormones such as estrogen and testosterone influence the regulation of LPA and ECS signaling in the brain by modulating their expression and function (Conde et al., 2017; Gonzalez-Arenas et al., 2008; Santoro et al., 2021). In this regard, estrogens have been shown to modulate emotional behavior through the ECS, which may contribute to a greater susceptibility in females to emotional disorders such as depression and anxiety, as well as differing stress responses (Hill et al., 2007). Similarly, recent evidence suggests a sexual dimorphism in LPA signaling, with distinct LPA profiles observed in men and women that are associated with psychiatric comorbidities, such as anxiety and personality disorders, particularly in individuals with cocaine use disorder (Requena-Ocana et al., 2023). Together, these findings highlight the complex interplay between LPA signaling, the ECS, and sex-specific hormonal regulation in shaping emotional behavior and vulnerability to psychiatric conditions.

The main aim of this study was to explore how LPA_1 receptor deficiency influences emotional behavior and gene expression related to neurotransmitter systems in the brain, with a focus on sex-specific differences. We hypothesize that the absence of the LPA_1 receptor will disrupt the interplay between the 2-AG and LPA signaling systems, leading to distinct alterations in emotional behaviors and neurotransmitter-related gene expression in male and female mice. To test this hypothesis, we first evaluated the effects of LPA_1 receptor deficiency on anxiety-like and stress-coping behaviors in both male and female wild-type and LPA_1-null mice. Furthermore, we examined the gene expression of key components of the 2-AG and LPA signaling systems, as well as the corticotropin-releasing hormone (CRH), neuropeptide Y (NPY), and glutamatergic systems, within the amygdala and medial prefrontal cortex (mPFC) of these mice. We focused on these brain regions due to their essential roles in the regulation of emotional and stress-related behaviors. This study aims to elucidate the sex-specific regulation of emotional behavior and neurotransmitter signaling in the absence of the LPA_1 receptor. Understanding the crosstalk between LPA and 2-AG in the regulation of emotional behaviors could reveal new mechanisms underlying emotional disorders and guide the development of sex-specific therapeutic approaches.

2. Material and methods

2.1. Animals and ethical statement

All studies were performed on adult male and randomly cycling female mice. The mice were age-matched littermates from the wild-type and the Malaga variant of the LPA_1-null (maLPA_1-null) mice, which were maintained in a hybrid C57BL/6J × 129 × 1/SvJ background. The generation and characterization of the maLPA_1-null mice have been described previously (Estivill-Torrus et al., 2008). 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*.

Fig. S1 provides a summary of the timeline and experimental procedures.

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). All protocols and procedures were approved by the Ethic and Research Committee of the Universidad de Malaga (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. Behavioral procedures

The elevated-plus maze (EPM) and tail suspension test (TST) were conducted between 09:00 h and 15:00 h during the light phase of the

diurnal cycle in a testing room illuminated at 300 lux. From the same cohort of mice, separate sets of wild-type and maLPA$_1$-null mice were used for each test to ensure that all animals were naïve to the testing procedures. Specifically, one set (n = 11–12) was used for the EPM, and another set (n = 8) was used for the TST. The use of different sets of animals for each test was necessary to ensure that mice did not undergo prior testing, minimizing potential test effects on the behavior of the animals. For each test, individual mice were placed in the designated apparatus, and their behavior was recorded on digital video. The analysis was performed using the software EthoVision XT 12 (Noldus Information Technology, Wageningen, The Netherlands).

2.2.1. Elevated-plus maze (EPM)

Anxiety-like behavior and locomotion were evaluated using the EPM as previously described (Pavon et al., 2021). The apparatus was composed of two oppositely positioned open arms (35 × 5 cm) and two oppositely positioned closed arms of the same size and 15 cm-high walls. The arms were connected by a central area (5 × 5 cm). The entire apparatus was elevated 30 cm above the floor. At the start of the test, each mouse was placed in the center of the maze, facing an open arm, and was allowed to freely explore the maze for 5 min. The total number of entries into the arms, the percent of the time spent in the open arm (time spent in the open arms/total time × 100) and the percent of entries into the open arms (number of entries into the open arms/total entries x 100) were calculated. Additionally, the anxiety index was calculated as previously described (Tabbai et al., 2019). A score approaching 1 suggests a higher anxiety-like behavior. Moreover, head dipping frequency was also evaluated (number of times that the mice protrude the head over the edge of the open arms).

2.2.2. Tail suspension test (TST)

This test was used to assess depression-like behavior or stress-coping behavior as previously described (Pavon et al., 2021). Each mouse was suspended from its tail using adhesive tape on a metal bar located 30 cm above a flat surface for 6 min. Immobility was defined as the absence of any limb or body movements, except those caused by respiration. The apparatus was wiped with a cleaning solution and dried with paper towels between each test. The following variables were scored in the TST: the latency to the first episode of immobility (duration of vigorous movements at the beginning) and the total immobility time (seconds).

2.3. Sample collection for immunoassays and molecular determinations

A third set of wild-type and maLPA$_1$-null mice were euthanized by decapitation, and blood and brain samples were collected. Blood samples were centrifuged (2000×*g* for 15 min) and plasma aliquots were kept for further analysis. The brains were frozen on dry ice and stored at −80 °C until molecular determinations.

2.3.1. Brain dissection

The frozen brains were dissected into 1 mm thick coronal brain slices using a mouse brain slicer matrix (Zivic Instruments, Pittsburgh, PA, US). The mPFC and amygdala were identified (Paxinos and Franklin, 2004) and removed. Samples were stored at −80 °C until they were used for gene analysis.

2.4. Immunoassays and molecular determinations

2.4.1. Corticosterone levels in the plasma

Corticosterone levels were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit [Abcam (ab108821), Cambridge, UK]. A corticosterone-specific antibody was precoated onto 96-well plates and blocked. Standards or test samples were added to the wells, followed by the addition of biotinylated corticosterone. After washing with wash buffer, an avidin-biotin-peroxidase complex was added, and unbound conjugates were removed through additional washing. Tetramethylbenzidine (TMB) substrate was then used to visualize the horseradish peroxidase (HRP) enzymatic reaction. The HRP catalyzed TMB to produce a blue product, which turned yellow upon the addition of an acidic stop solution. The intensity of the yellow coloration was inversely proportional to the amount of corticosterone captured on the plate. Corticotropin was expressed as nanograms per milliliter (ng/mL).

2.4.2. 2-AG and total LPA in the plasma

The levels of 2-AG and total LPA were measured in plasma samples using specific ELISA kits following the respective manufacturer's instructions. The plasma levels of 2-AG were measured with a separate ELISA kit [CLOUD-CLONE CORP. (CEO443Ge), Houston, Texas, USA] and reported as nanograms per milliliter (ng/mL). Total LPA levels were determined using an ELISA kit [MyBioSource (MBS7269921), San Diego, CA, USA] and expressed as micrograms per milliliter (μg/mL). Due to a combination of technical issues during the ELISA procedures and insufficient plasma volume for repeated measurements of total LPA, reliable readings could not be obtained for certain samples. To ensure the reliability and integrity of the data, these samples were excluded from the final dataset, as reflected in the reported ranges of sample sizes for each determination (n = 6–8 for 2-AG; and n = 5 for LPA per experimental group).

2.4.3. RNA isolation and RT-qPCR analysis in the brain

Real-time PCR was employed to quantify the relative mRNA levels of enzymes and receptors associated with the signaling pathways of 2-AG and LPA, including their respective receptors: CB$_1$ (*Cnr1*), CB$_2$ (*Cnr2*), LPA$_1$ (*Lpar1*), and LPA$_2$ (*Lpar2*); and enzymes involved in their synthesis or degradation: DAGLα (*Dagla*), DAGLβ (*Daglb*), MAGL (*Mgll*), ATX (*Enpp2*), PLCβ (*Plcb3*), and AGK (*Agk*). The analysis also included components of the corticotropin-releasing hormone (CRH) and neuropeptide Y (NPY) systems, specifically CRH (*Crh*), CRH receptors CRHR1 (*Crhr1*) and CRHR2 (*Crhr2*); NPY (*Npy*), and its receptors NPY1r (*Npy1r*) and NPY2r (*Npy2r*). Additionally, we evaluated the glutamatergic signaling pathways, focusing on kidney-type glutaminase isoforms (KGA, *Gls*), metabotropic glutamate receptors mGluR3 (*Grm3*) and mGluR5 (*Grm5*), ionotropic N-methyl D-aspartate (NMDA) receptor subunits NR1 (*Grin1*) and NR2A (*Grin2a*), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunits GluR1 (*Gria1*) and GluR2 (*Gria2*), as well as the glutamate transporter EAAC1 (*Slc1a1*). Total RNA was extracted from brain samples using Trizol Reagent (Gibco BRL Life Technologies, Baltimore, MD, USA) and the concentrations were quantified using a spectrophotometer to ensure ratios of absorbance at 260–280 nm of 1.8–2.0. The 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). The absolute values from each sample were normalized relative to the housekeeping gene β-actin (*Actb*), which was found to be stable among the groups in the amygdala and the mPFC. The relative quantification was calculated using the ΔΔCt method and normalized to the control group. Primers for the RT-qPCR were obtained based on the Applied Biosystems genome database of mouse mRNA references (http://bioinfo.appliedbiosystems.com/genome-database/gene-expression.html) (Table S1).

2.5. Statistical analysis

All data for graphs are expressed as the mean ± SEM. The normal distribution of data was evaluated by Kolmogorov-Smirnov normality test. The significance of differences within and between groups was evaluated using two-way analysis of variance (ANOVA) [factors: "genotype" (wild-type/*maLPA$_1$-null*) and "sex" (male/female)] followed by

Sidak's multiple comparisons test.

The mRNA expression of LPA_1 receptors was analyzed using Student's t-test. Test statistic values and degrees of freedom are indicated in the results and a *p*-value less than 0.05 was considered statistically significant. All statistical analyses were performed using the Graph-Pad Prism version 5.04 software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Effects of LPA_1 receptor deficiency on anxiety-like behaviors in male and female mice

First, we assessed the effects of LPA_1 receptor deficiency on anxiety-like behaviors in male and female mice using the EPM.

Two-way ANOVA of the percentage of time spent in open arms revealed significant main effects of "genotype" ($F_{(1,43)} = 6.31$; $p = 0.016$) and "sex" ($F_{(1,43)} = 8.42$; $p = 0.006$), but no significant interaction between both factors (Fig. 1A). As we can see in the figure, maLPA$_1$-null mice spent less time exploring the open arms compared to wild-type mice, and female mice spent less time in the open arms than male mice. We also evaluated the number of entries into the open arms (Fig. 1B). The statistical analysis revealed a main effect of "sex" ($F_{(1,43)} = 9.42$; $p = 0.004$), and a significant interaction between "genotype" and "sex" ($F_{(1,43)} = 5.76$; $p = 0.021$). *Post hoc* tests for multiple comparisons showed a significant decrease in the number of entries into the open arms in all groups compared to male wild-type mice (*$p < 0.05$ and **$p < 0.01$). Additionally, the total number of arm entries was determined for each group: a) male wild-type mice, 16.83 ± 1.64; b) male maLPA$_1$-null mice, 17.75 ± 0.94; c) female wild-type mice, 15.55 ± 0.79; d) female maLPA$_1$-null mice, 17.08 ± 0.83. Statistical analysis revealed no significant effects of "genotype" or "sex" on total entries. Furthermore, we calculated the anxiety index for each group (Fig. 1C), and we found

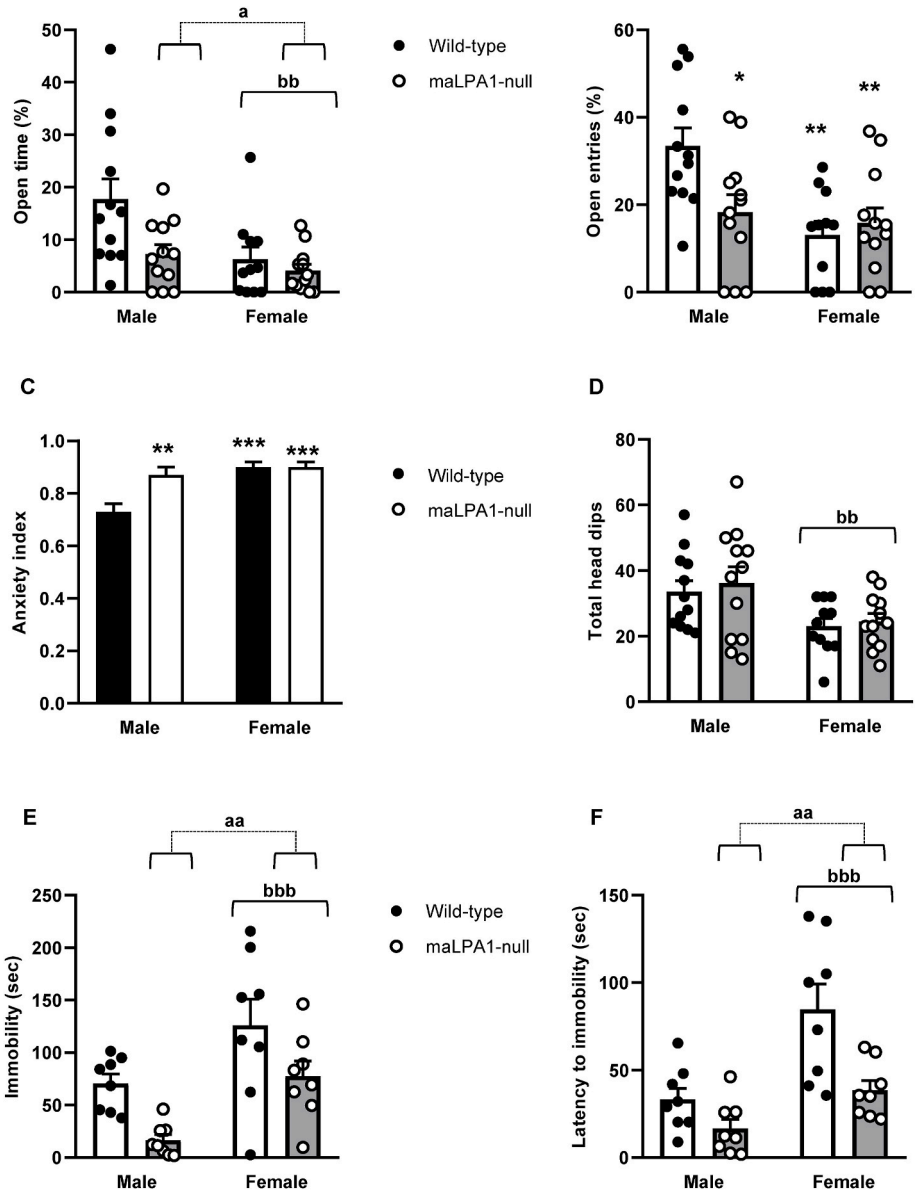

Fig. 1. Anxiety-like and stress-coping behaviors in male and female wild-type and maLPA$_1$-null mice. Percentage of time spent in the open arms (A), percentage of open arm entries (B), anxiety index (C), and total head dips (D) in the EPM; and the total immobility time (E) and the latency to the first episode of immobility (F) in the TST. Bars represent the mean and SEM (8–12 mice/group). Data were analyzed using two-way ANOVA. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ denote significant differences compared to male wild-type mice using *post hoc* test for multiple comparisons when an interaction between factors was found. $^{a}p < 0.05$ and $^{aa}p<0.01$ denote main effect of "genotype"; $^{bb}p<0.01$ and $^{bbb}p<0.001$ denote main effect of "sex".

significant main effects of "genotype" ($F_{(1,43)} = 7.39$; $p = 0.009$) and "sex" ($F_{(1,43)} = 15.09$; $p < 0.001$), and a significant interaction between both factors ($F_{(1,43)} = 7.39$; $p = 0.009$). In fact, a significantly higher anxiety index was observed in male maLPA$_1$-null (**$p < 0.01$) and female wild-type and maLPA$_1$-null mice (***$p < 0.01$) compared to male wild-type mice. Regarding ethological parameters, such as head dipping (Fig. 1D), the statistical analysis only revealed a main effect of "sex" ($F_{(1,43)} = 10.27$; $p = 0.003$), with female mice exhibiting a lower frequency of head dipping than male mice.

3.2. Effects of LPA$_1$ receptor deficiency on stress-coping behavior in male and female mice

Next, we used the TST to assess the effects of LPA$_1$ receptor deficiency on stress-coping behavior in male and female mice. The statistical analysis revealed a significant main effect of "genotype" on the total immobility time ($F_{(1,28)} = 11.10$; $p = 0.002$) and on the latency ($F_{(1,28)} = 12.77$; $p = 0.001$) (Fig. 1E and F). Specifically, maLPA$_1$-null mice showed lower immobility time and latency to the first episode of immobility compared to wild-type mice. There was also a significant main effect of "sex" on the total immobility time ($F_{(1,28)} = 14.34$; $p < 0.001$) and on the latency ($F_{(1,28)} = 17.32$; $p < 0.001$), and female mice showed higher immobility and latency than male mice.

3.3. Effects of LPA$_1$ receptor deficiency on plasma levels of corticosterone, 2-AG, and LPA in male and female mice

We first examined the effects of "genotype" and "sex" on plasma corticosterone levels in wild-type and maLPA$_1$-null mice (Fig. 2A). A two-way ANOVA revealed a significant main effect of "sex" on plasma corticosterone levels ($F_{(1,24)} = 65.31$;$p < 0.001$), with female mice showing higher corticosterone levels than male mice.

Next, we analyzed lipid mediators in plasma using a two-way ANOVA with "genotype" and "sex" as factors. The analysis of 2-AG plasma levels (Fig. 2B) demonstrated significant main effects of both "genotype" ($F_{(1,24)} = 36.29$; $p < 0.001$) and "sex" ($F_{(1,24)} = 9.02$; $p = 0.006$). Specifically, maLPA$_1$-null mice displayed lower 2-AG levels compared to wild-type mice, while female mice exhibited lower 2-AG levels than male mice. In contrast, the analysis of total LPA (Fig. 2C) revealed a significant main effect of "genotype" ($F_{(1,16)} = 11.86$; $p = 0.003$) and maLPA$_1$-null mice showed higher LPA levels than wild-type mice.

3.4. Effects of LPA$_1$ receptor deficiency on the mRNA expression of 2AG-signaling and LPA-related genes in the amygdala of male and female mice

Next, we evaluated the impact of LPA$_1$ receptor deficiency on the mRNA expression of genes related to components of the ECS involved in the production, signaling, and degradation of 2-AG, as well as the mRNA expression of genes related to enzymes and receptors of LPA, in the amygdala of both male and female mice.

3.4.1. Cannabinoid receptors

As shown in Fig. 3A, a two-way ANOVA revealed a significant interaction between "genotype" and "sex" ($F_{(1,37)} = 5.51$; $p = 0.024$) on the levels of *Cnr1*. *Post hoc* tests for multiple comparisons showed a significant increase in the mRNA levels of this receptor in male maLPA$_1$-null and female wild-type mice (*$p < 0.05$) compared to male wild-type mice.

Regarding the expression of *Cnr2* (Fig. 3B), the statistical analysis revealed only a significant main effect of "sex" ($F_{(1,37)} = 14.61$; $p < 0.001$), with female mice displaying higher mRNA levels of this receptor

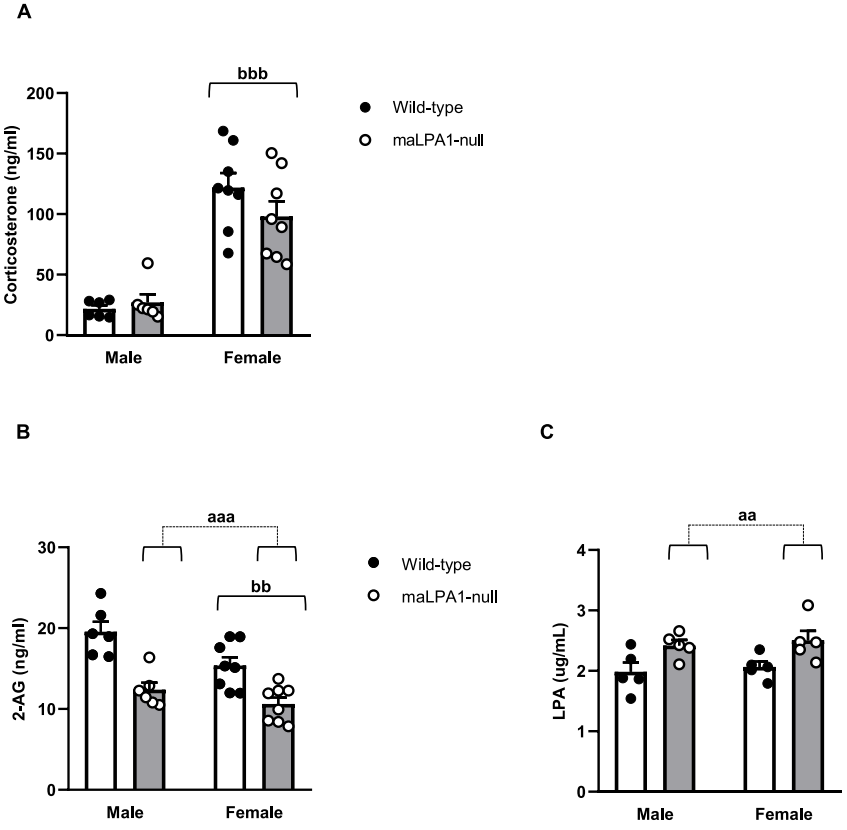

Fig. 2. Plasma levels of corticosterone, 2-AG, and total LPA in male and female wild-type and maLPA$_1$-null mice. Plasma levels of corticosterone (A), 2-AG (B), and total LPA (C). Bars represent the mean and SEM (5–8 mice/group). Data were analyzed using two-way ANOVA. $^{aa}p<0.01$ and $^{aaa}p<0.001$ denote main effect of "genotype"; $^{bb}p<0.01$ and $^{bbb}p<0.001$ denote main effect of "sex".

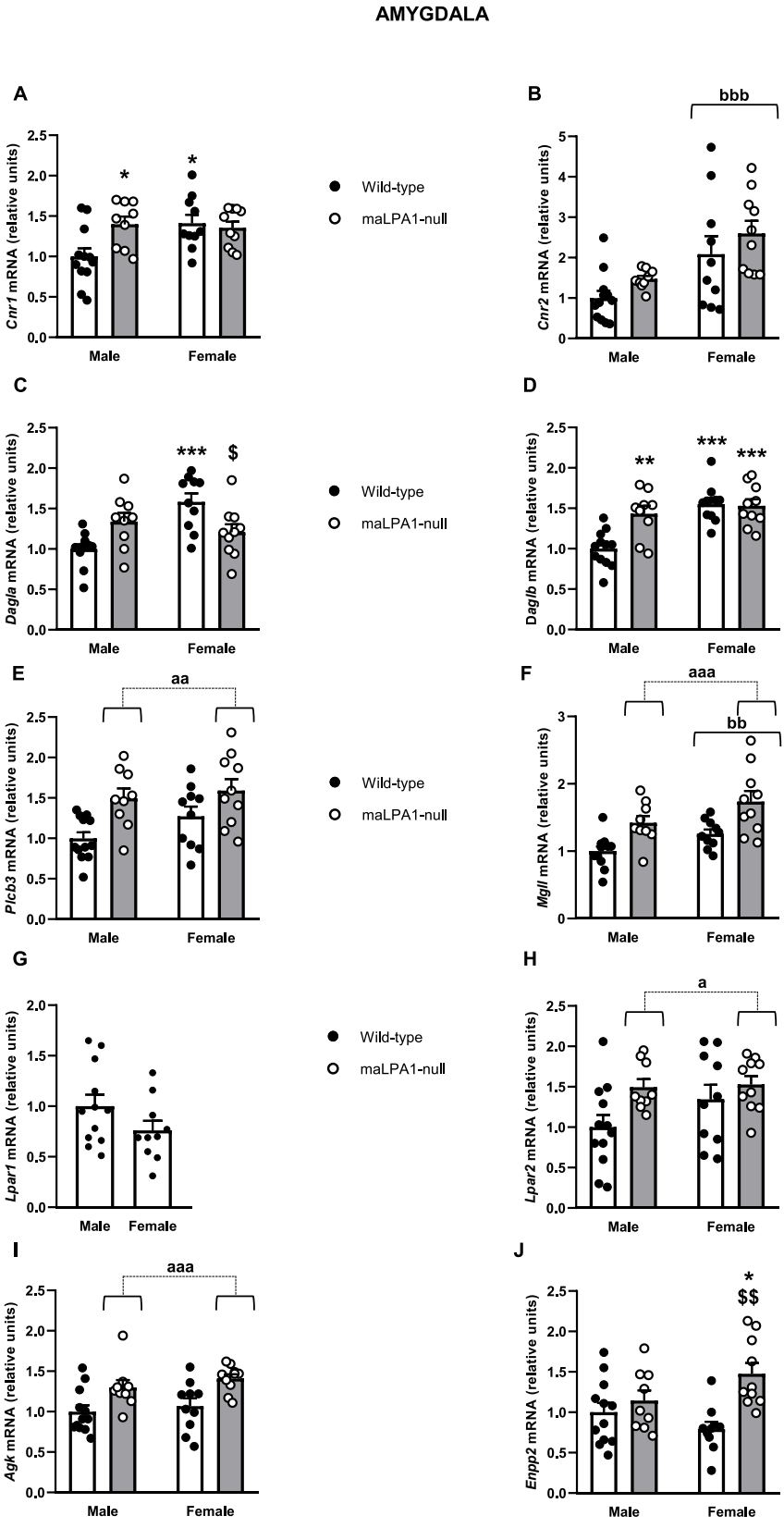

Fig. 3. Relative mRNA expression levels of 2-AG-signaling and LPA-related genes in the amygdala of male and female wild-type and maLPA$_1$-null mice. Relative mRNA expression of *Cnr1* (A), *Cnr2* (B), *Dagla* (C), *Daglb* (D), *Plcb3* (E), *Mgll* (F), *Lpar1* (G), *Lpar2* (H), *Agk* (I), and *Enpp2* (J) in the amygdala of wild-type and maLPA$_1$-null mice of both sexes. Bars represent the mean and SEM (9–12 mice/group). Data were analyzed using two-way ANOVA. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ denote significant differences compared to male wild-type mice; and $^{\$}p < 0.05$ and $^{\$\$}p < 0.01$ denote significant differences compared to female wild-type mice using *post hoc* test for multiple comparisons when an interaction between factors was found. $^{a}p < 0.05$, $^{aa}p<0.01$, and $^{aaa}p<0.001$ denote main effect of "genotype"; $^{bb}p<0.01$ and $^{bbb}p<0.001$ denote main effect of "sex".

than male mice.

3.4.2. Cannabinoid enzymes

A two-way ANOVA revealed a significant main effect of "sex" ($F_{(1,37)} = 6.03$; $p = 0.019$), and a significant interaction between "genotype" and "sex" ($F_{(1,37)} = 15.00$; $p < 0.001$) on the mRNA levels of *Dagla* (Fig. 3C). *Post hoc* tests for multiple comparisons showed a significant increase in the mRNA levels of this enzyme in female wild-type mice compared to male wild-type mice (***$p < 0.001$), and a significant decrease in the female maLPA$_1$-null mice compared to female wild-type ($^{\$}p < 0.05$).

Regarding the expression of *Daglb* (Fig. 3D), we found significant main effects of "genotype" ($F_{(1,37)} = 6.88$; $p = 0.013$) and "sex" ($F_{(1,37)} = 17.10$; $p < 0.001$), and a significant interaction between both factors ($F_{(1,37)} = 8.28$; $p = 0.007$). *Post hoc* tests for multiple comparisons indicated a significant increase in the mRNA levels of this enzyme in all groups compared to male wild-type mice (**$p < 0.01$ and ***$p < 0.001$).

In addition to these enzymes involved directly in the synthesis of 2-AG, we also analyzed the mRNA levels of *Plcb3*, a phospholipase involved in the production of diacylglycerol (DAG), a precursor of 2-AG. The statistical analysis only revealed a significant main effect of "genotype" ($F_{(1,37)} = 12.66$; $p = 0.001$) (Fig. 3E). Specifically, maLPA$_1$-null

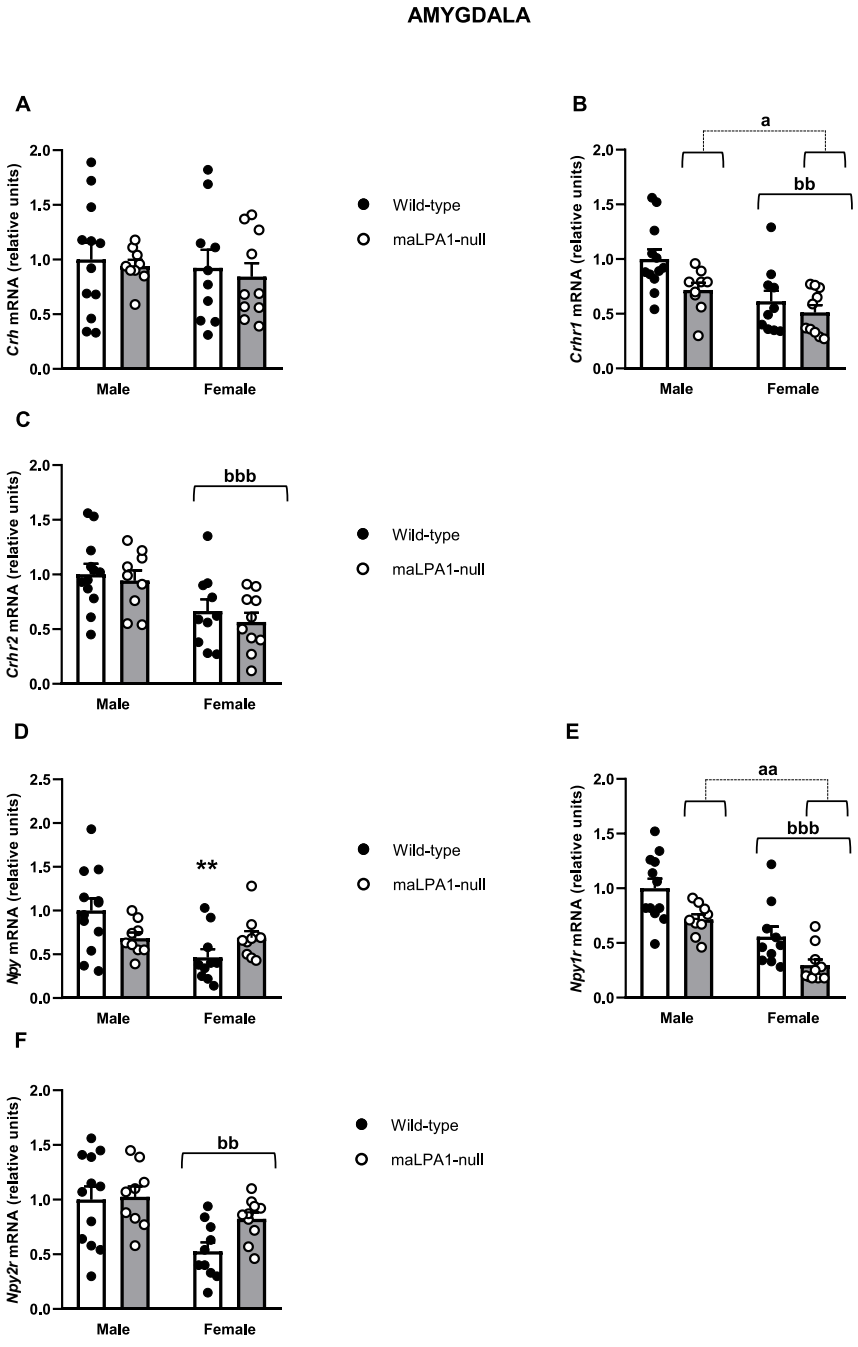

Fig. 4. Relative mRNA expression levels of CRH and NPY systems in the amygdala of male and female wild-type and maLPA$_1$-null mice. Relative mRNA expression of *Crh* (A), *Crhr1* (B), *Crhr2* (C), *Npy* (D), *Npy1r* (E), and *Npy2r* (F) in the amygdala of wild-type and maLPA$_1$-null mice of both sexes. Bars represent the mean and SEM (9–12 mice/group). Data were analyzed using two-way ANOVA. **$p < 0.01$ denotes significant differences compared to male wild-type mice using *post hoc* test for multiple comparisons when an interaction between factors was found. $^{a}p < 0.05$ and $^{aa}p<0.01$ denote main effect of "genotype"; $^{bb}p<0.01$ and $^{bbb}p<0.001$ denote main effect of "sex".

mice displayed higher levels of the mRNA of this enzyme than wild-type mice.

Finally, the statistical analysis revealed a significant main effect of "genotype" ($F_{(1,37)} = 18.95$; $p < 0.001$) and "sex" ($F_{(1,37)} = 7.66$; $p = 0.009$) on the levels of *Mgll* (Fig. 3F), but no significant interaction between both factors. Thus, maLPA$_1$-null mice showed higher mRNA levels compared to the wild-type mice, and female mice showed higher mRNA levels of this enzyme compared to male mice.

3.4.3. LPA receptors

As shown in Fig. 3G, there were no significant differences between male and female mice in the mRNA expression of *Lpar1*. However, the two-way ANOVA revealed a significant main effect of "genotype" ($F_{(1,37)} = 5.75$; $p = 0.022$) on the levels of *Lpar2* (Fig. 3H), and maLPA$_1$-null mice showed higher mRNA levels of this receptor than wild-type mice.

3.4.4. LPA enzymes

A two-way ANOVA revealed a significant main effect of "genotype" ($F_{(1,37)} = 15.52$; $p < 0.001$) on the levels of *Agk* (Fig. 3I). Thus, maLPA$_1$-null mice showed higher mRNA levels of this enzyme compared to wild-type mice.

Finally, we found significant main effects of "genotype" ($F_{(1,37)} = 12.48$; $p = 0.001$) and a significant interaction between "genotype" and "sex" ($F_{(1,37)} = 5.27$; $p = 0.028$) on the mRNA levels of *Enpp2* (Fig. 3J). *Post hoc* tests for multiple comparisons indicated that female maLPA$_1$-null mice had a significant increase in the expression of this enzyme compared to male wild-type ($^{*}p < 0.05$) and female wild-type ($^{\$\$}p < 0.01$) mice.

3.5. Effects of LPA$_1$ receptor deficiency on the mRNA expression of anxiety-related genes in the amygdala of male and female mice

Next, we investigated the impact of LPA$_1$ receptor deficiency on the mRNA expression of many genes related to the CRH and NPY systems in the amygdala of both male and female mice.

3.5.1. CRH

As shown in Fig. 4A, a two-way ANOVA showed no significant main effects of "genotype" and "sex" or interaction between both factors on mRNA levels of *Crh*.

3.5.2. CRH receptors

The statistical analysis revealed a significant main effect of "genotype" ($F_{(1,37)} = 5.39$; $p = 0.026$) and "sex" ($F_{(1,37)} = 12.68$; $p = 0.001$) on the mRNA levels of *Crhr1* (Fig. 4B), but no significant interaction between both factors. Thus, maLPA$_1$-null mice showed lower mRNA levels compared to the wild-type mice, and female mice showed lower mRNA levels of this receptor than male mice. Regarding the levels of *Crhr2* (Fig. 4C), there was a significant main effect of "sex" ($F_{(1,37)} = 13.92$; $p < 0.001$), and female mice displayed lower mRNA levels of this receptor than male mice.

3.5.3. NPY

As shown in Fig. 4D, a two-way ANOVA revealed a main effect of "sex" ($F_{(1,37)} = 6.43$; $p = 0.016$), and a significant interaction between "genotype" and "sex" ($F_{(1,37)} = 6.66$; $p = 0.014$). *Post hoc* tests for multiple comparisons showed that female wild-type mice had a significant decrease in the expression of *Npy* compared to male wild-type mice ($^{**}p < 0.01$).

3.5.4. NPY receptors

The statistical analysis revealed a significant main effect of "genotype" ($F_{(1,37)} = 12.71$; $p = 0.001$) and "sex" ($F_{(1,37)} = 31.44$; $p < 0.001$) on the expression of *Npy1r* (Fig. 4E), but no significant interaction between both factors. Thus, maLPA$_1$-null mice showed lower mRNA levels compared to the wild-type mice, and female mice showed lower mRNA levels of this receptor than male mice. Regarding the levels of *Npy2r* (Fig. 4F), there was a significant main effect of "sex" ($F_{(1,37)} = 12.13$; $p = 0.001$), and female mice displayed lower mRNA levels of this receptor than male mice.

3.6. Effects of LPA$_1$ receptor deficiency on the mRNA expression of glutamatergic-signaling genes in the amygdala of male and female mice

Next, we investigated the impact of LPA$_1$ receptor deficiency on the mRNA expression of the glutamatergic neurotransmission in the amygdala of both male and female mice.

3.6.1. Enzyme of synthesis and transporter of glutamate

As shown in Fig. 5A, a two-way ANOVA showed no significant main effects of "genotype" and "sex" or interaction between both factors on mRNA levels of *Gls*.

Regarding the levels of *Slc1a1* (Fig. 5B), there was a significant main effect of "sex" ($F_{(1,37)} = 40.81$; $p < 0.001$), and female mice displayed lower mRNA levels of the transporter of glutamate than male mice.

3.6.2. NMDA receptors

There were significant main effects of "genotype" and "sex" on the mRNA levels of both subunits, *Grin1* (genotype: $F_{(1,37)} = 4.43$; $p = 0.042$; sex: $F_{(1,37)} = 15.28$; $p < 0.001$) (Fig. 5C) and *Grin2a* (genotype: $F_{(1,37)} = 10.72$; $p = 0.002$; sex: $F_{(1,37)} = 8.44$; $p = 0.006$) (Fig. 5D). Specifically, maLPA$_1$-null mice showed lower mRNA levels compared to the wild-type mice, and female mice showed lower mRNA levels of these NMDA receptor subunits than male mice.

3.6.3. AMPA receptors

There were significant main effects of "genotype" and "sex" on the mRNA levels of the subunit *Gria1* (genotype: $F_{(1,37)} = 14.05$; $p < 0.001$; sex: $F_{(1,37)} = 23.63$; $p < 0.001$) (Fig. 5E). Specifically, maLPA$_1$-null mice showed lower mRNA levels compared to the wild-type mice, and female mice showed lower mRNA levels of this AMPA receptor subunit than male mice. Regarding the levels of *Gria2* (Fig. 5F), there was a significant main effect of "sex" ($F_{(1,37)} = 12.89$; $p = 0.001$), and female mice displayed lower mRNA levels of this subunit than male mice.

3.6.4. Metabotropic receptors

There were significant main effects of "genotype" and "sex" on the mRNA levels of the metabotropic receptor *Grm3* (genotype: $F_{(1,37)} = 8.64$; $p = 0.006$; sex: $F_{(1,37)} = 17.79$; $p < 0.001$) (Fig. 5G). Specifically, maLPA$_1$-null mice showed lower mRNA levels compared to the wild-type mice, and female mice showed lower mRNA levels of this receptor than male mice. In contrast, as shown in Fig. 5H, a two-way ANOVA showed no significant main effects of "genotype" and "sex" or interaction between both factors on mRNA levels of the other metabotropic receptor *Grm5*.

3.7. Effects of LPA$_1$ receptor deficiency on the mRNA expression of 2AG-signaling and LPA-related genes in the mPFC of male and female mice

Next, we evaluated the impact of LPA$_1$ receptor deficiency on the mRNA expression of genes related to components of the ECS involved in the production, signaling, and degradation of 2-AG, as well as the mRNA expression of genes related to enzymes and receptors of LPA, in the mPFC of both male and female mice.

3.7.1. Cannabinoid receptors

As shown in Fig. 6A, a two-way ANOVA revealed significant main effects of "genotype" ($F_{(1,37)} = 121.6$; $p < 0.001$) and "sex" ($F_{(1,37)} = 29.74$; $p < 0.001$), and a significant interaction between "genotype" and "sex" ($F_{(1,37)} = 19.11$; $p < 0.001$) on the levels of *Cnr1*. *Post hoc* tests for multiple comparisons showed a significant increase in the mRNA levels

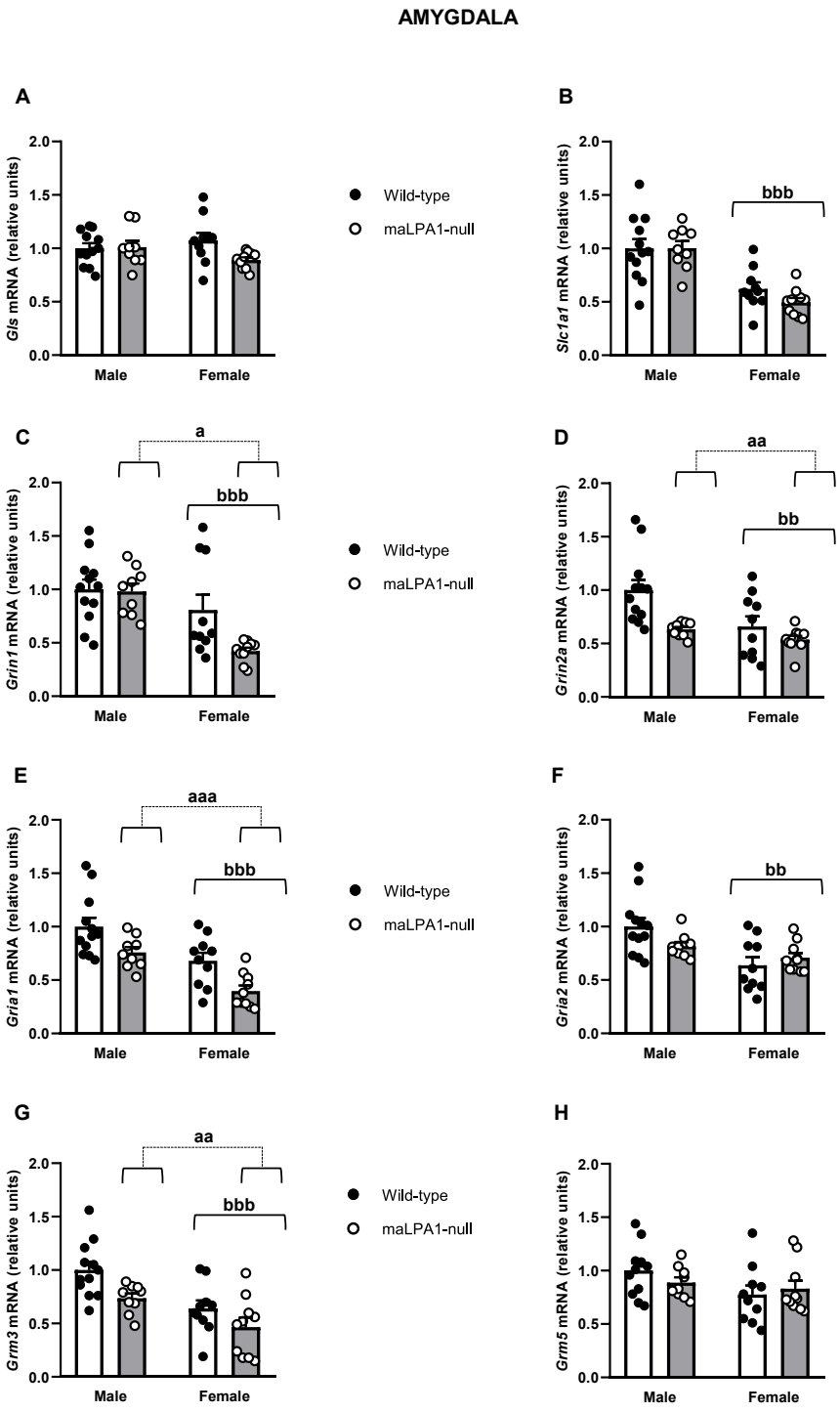

Fig. 5. Relative mRNA expression levels of glutamatergic system in the amygdala of male and female wild-type and maLPA$_1$-null mice. Relative mRNA expression of *Gls* (A), *Slc1a1* (B), *Grin1* (C), *Grin2a* (D), *Gria1* (E), *Gria2* (F), *Grm3* (G), and *Grm5* (H) in the amygdala of wild-type and maLPA$_1$-null mice of both sexes. Bars represent the mean and SEM (9–12 mice/group). Data were analyzed using two-way ANOVA. $^{a}p < 0.05$, $^{aa}p<0.01$, and $^{aaa}p<0.001$ denote main effect of "genotype"; $^{bb}p<0.01$ and $^{bbb}p<0.001$ denote main effect of "sex".

of this receptor in male maLPA$_1$-null ($^{***}p < 0.001$) compared to male wild-type animals. Moreover, the female maLPA$_1$-null mice had a significant increase in the expression of *Cnr1* compared to male wild-type ($^{***}p < 0.001$) and maLPA$_1$-null mice ($^{\#\#\#}p < 0.001$), and female wild-type mice ($^{\$\$\$}p < 0.001$). Regarding the expression of *Cnr2* (Fig. 6B), we found significant main effects of "genotype" ($F_{(1,37)} = 73.42$; $p < 0.001$) and "sex" ($F_{(1,37)} = 16.59$; $p < 0.001$). Thus, maLPA$_1$-null mice showed higher mRNA levels compared to the wild-type mice, and female mice showed higher mRNA levels of this receptor than male mice.

3.7.2. Cannabinoid enzymes

We found significant main effects of "genotype" ($F_{(1,37)} = 9.09$; $p = 0.005$) and "sex" ($F_{(1,37)} = 35.97$; $p < 0.001$), and a significant interaction between both factors ($F_{(1,37)} = 14.74$; $p < 0.001$) on the mRNA levels of *Dagla* (Fig. 6C). *Post hoc* tests for multiple comparisons

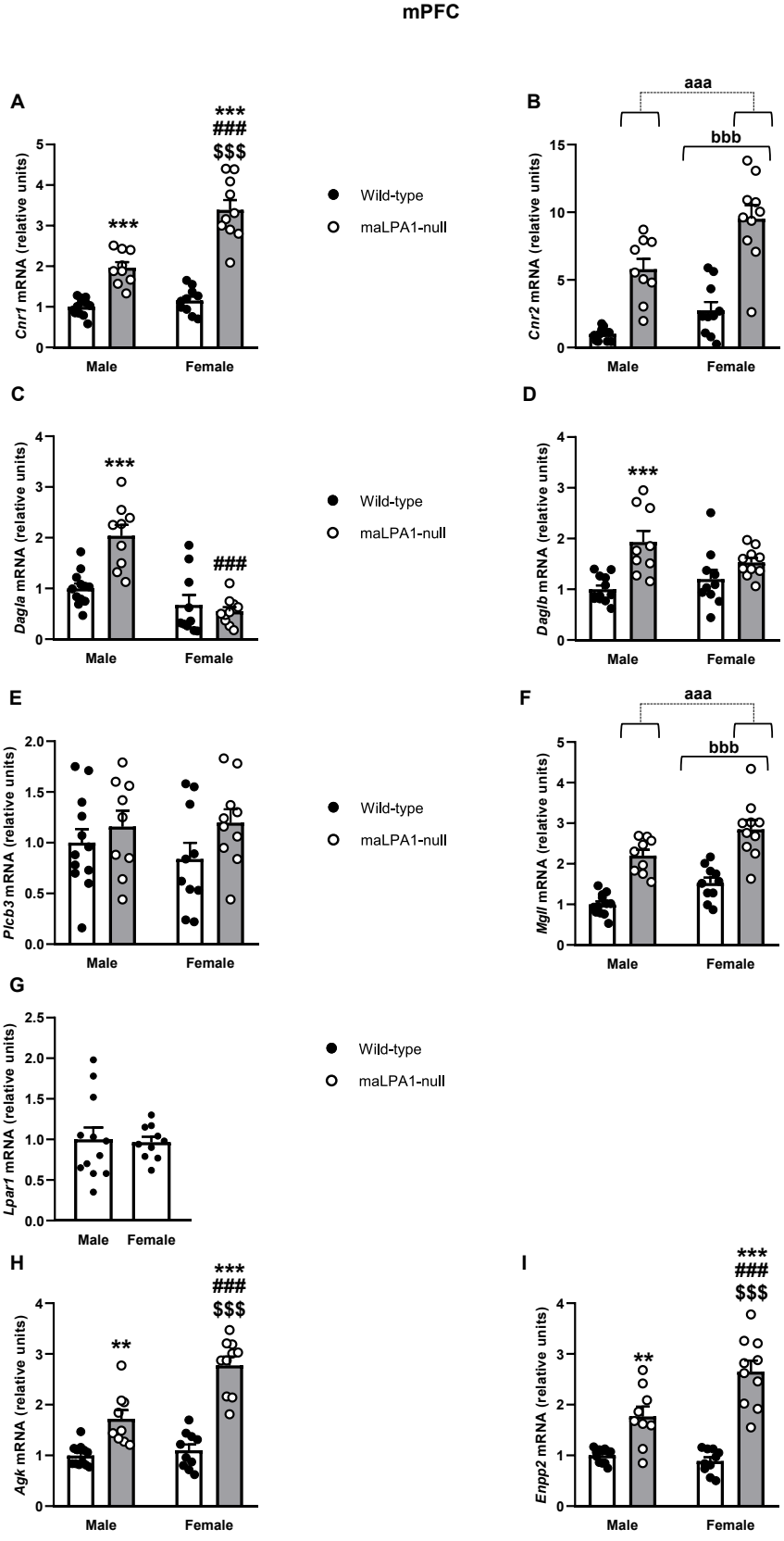

Fig. 6. Relative mRNA expression levels of 2-AG-signaling and LPA-related genes in the mPFC of male and female wild-type and maLPA$_1$-null mice. Relative mRNA expression of *Cnr1* (A), *Cnr2* (B), *Dagla* (C), *Daglb* (D), *Plcb3* (E), *Mgll* (F), *Lpar1* (G), *Agk* (H), and *Enpp2* (I) in the mPFC of wild-type and maLPA$_1$-null mice of both sexes. Bars represent the mean and SEM (9–12 mice/group). Data were analyzed using two-way ANOVA. $^{**}p < 0.01$ and $^{***}p < 0.001$ denote significant differences compared to male wild-type mice; $^{###}p < 0.001$ denotes significant differences compared to male maLPA$_1$-null mice; and $^{\$\$\$}p < 0.001$ denotes significant

differences compared to female wild-type mice using *post hoc* test for multiple comparisons when an interaction between factors was found. $^{aaa}p<0.001$ denotes main effect of "genotype"; $^{bbb}p<0.001$ denotes main effect of "sex".

indicated a significant increase in the mRNA levels of this enzyme in male maLPA$_1$-null mice compared to male wild-type mice ($^{***}p < 0.001$) and female maLPA$_1$-null mice ($^{\#\#\#}p < 0.001$). Regarding *Daglb* (Fig. 6D), a two-way ANOVA revealed a significant main effect of "genotype" ($F_{(1,37)} = 18.82$; $p < 0.001$) and a significant interaction between "genotype" and "sex" ($F_{(1,37)} = 4.35$; $p = 0.045$). *Post hoc* tests for multiple comparisons showed a significant increase in the mRNA levels of this enzyme in male maLPA$_1$-null mice compared to male wild-type mice ($^{***}p < 0.001$).

As shown in Fig. 6E, a two-way ANOVA showed no significant main effects of "genotype" and "sex" or interaction between both factors on mRNA levels of *Plcb3*. Finally, the statistical analysis revealed a significant main effect of "genotype" ($F_{(1,37)} = 70.10$; $p < 0.001$) and "sex" ($F_{(1,37)} = 15.21$; $p < 0.001$) on the levels of *Mgll* (Fig. 6F), but no significant interaction between both factors. Thus, maLPA$_1$-null mice showed higher mRNA levels compared to the wild-type mice, and female mice showed higher mRNA levels of this enzyme than male mice.

3.7.3. LPA receptors

As shown in Fig. 6G, there were no significant differences between male and female mice in the mRNA expression of *Lpar1*. Regarding *Lpar2,* we did not detect its expression in the mPFC in any of our experimental groups (data not shown).

3.7.4. LPA enzymes

There were significant main effects of "genotype" and "sex", and a significant interaction between factors on the mRNA levels of both enzymes, *Agk* (genotype: $F_{(1,37)} = 83.84$; $p < 0.001$; sex: $F_{(1,37)} = 19.58$; $p < 0.001$; genotype × sex: $F_{(1,37)} = 13.30$; $p < 0.001$) (Fig. 6H) and *Enpp2* (genotype: $F_{(1,37)} = 78.89$; $p < 0.001$; sex: $F_{(1,37)} = 7.34$; $p = 0.010$; genotype × sex: $F_{(1,37)} = 12.16$; $p = 0.001$) (Fig. 6I). *Post hoc* tests for multiple comparisons showed a significant increase in the mRNA levels

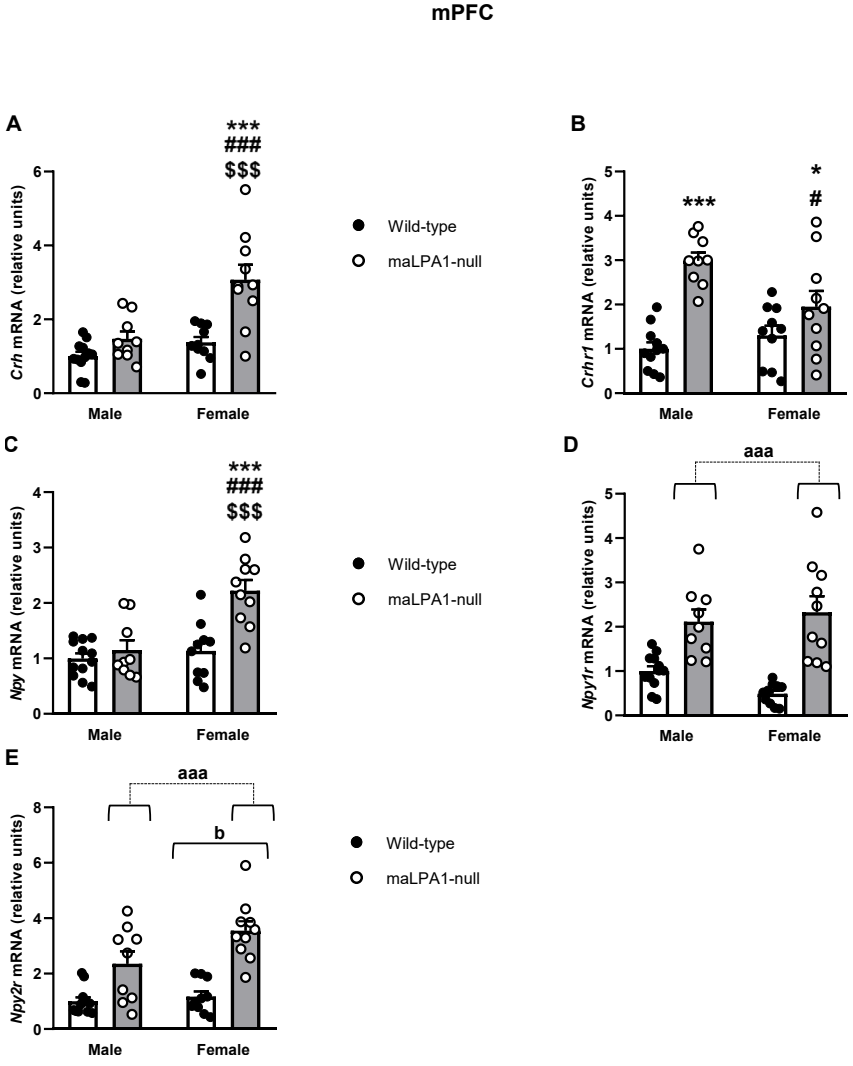

Fig. 7. Relative mRNA expression levels of CRH and NPY systems in the mPFC of male and female wild-type and maLPA$_1$-null mice. Relative mRNA expression of *Crh* (A), *Crhr1* (B), *Npy* (C), *Npy1r* (D), and *Npy2r* (E) in the mPFC of wild-type and maLPA$_1$-null mice of both sexes. Bars represent the mean and SEM (9–12 mice/group). Data were analyzed using two-way ANOVA. $^{*}p < 0.05$ and $^{***}p < 0.001$ denote significant differences compared to male wild-type mice; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ denote significant differences compared to male maLPA$_1$-null mice; and $^{\$\$\$}p < 0.001$ denotes significant differences compared to female wild-type mice using *post hoc* test for multiple comparisons when an interaction between factors was found. $^{aaa}p<0.001$ denotes main effect of "genotype"; $^{b}p < 0.05$ denotes main effect of "sex".

of both enzymes in male maLPA$_1$-null mice ($^{**}p < 0.01$) compared to male wild-type mice. Moreover, the female maLPA$_1$-null mice had a significant increase in the expression of *Agk* and *Enpp2* compared to male wild-type ($^{***}p < 0.001$) and maLPA$_1$-null mice ($^{\#\#\#}p < 0.001$), and female wild-type mice ($^{\$\$\$}p < 0.001$).

3.8. Effects of LPA$_1$ receptor deficiency on the mRNA expression of anxiety-related genes in the mPFC of male and female mice

Next, we investigated the impact of LPA$_1$ receptor deficiency on the mRNA expression of many genes related to the CRH and NPY systems in the mPFC of both male and female mice.

3.8.1. CRH

As shown in Fig. 7A, a two-way ANOVA revealed significant main effects of "genotype" ($F_{(1,37)} = 20.71$; $p < 0.001$) and "sex" ($F_{(1,37)} = 17.16$; $p < 0.001$), and a significant interaction between "genotype" and "sex" ($F_{(1,37)} = 6.61$; $p = 0.014$) on the levels of *Crh*. *Post hoc* tests for multiple comparisons showed that female maLPA$_1$-null mice had a significant increase in the expression of *Crh* compared to male wild-type

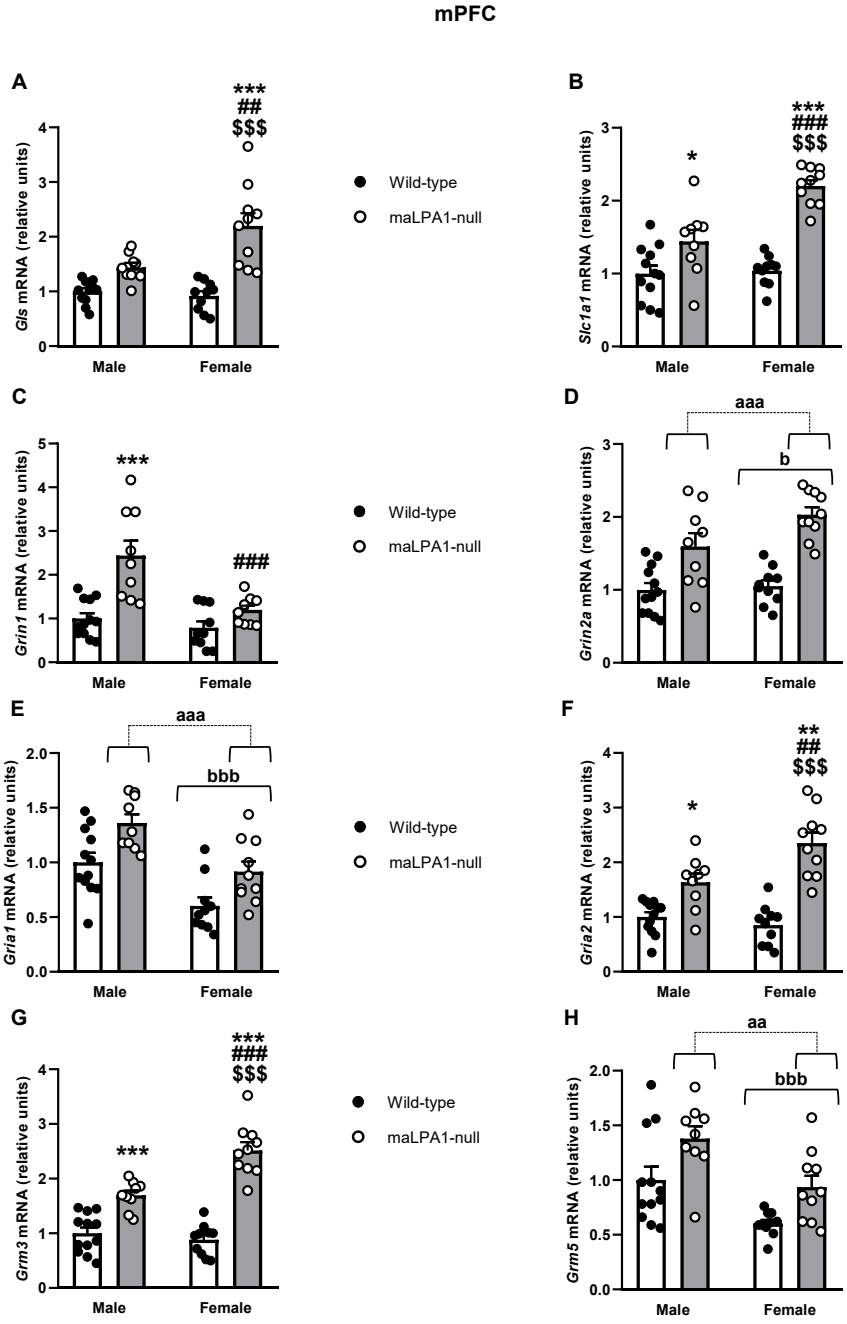

Fig. 8. Relative mRNA expression levels of glutamatergic system in the mPFC of male and female wild-type and maLPA$_1$-null mice. Relative mRNA expression of *Gls* (A), *Slc1a1* (B), *Grin1* (C), *Grin2a* (D), *Gria1* (E), *Gria2* (F), *Grm3* (G), and *Grm5* (H) in the mPFC of wild-type and maLPA$_1$-null mice of both sexes. Bars represent the mean and SEM (9–12 mice/group). Data were analyzed using two-way ANOVA. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ denote significant differences compared to male wild-type mice; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ denote significant differences compared to male maLPA$_1$-null mice; and $^{\$\$\$}p < 0.001$ denotes significant differences compared to female wild-type mice using *post hoc* test for multiple comparisons when an interaction between factors was found. $^{aa}p<0.01$ and $^{aaa}p<0.001$ denote main effect of "genotype"; $^{b}p < 0.05$ and $^{bbb}p<0.001$ denote main effect of "sex".

(***$p < 0.001$) and maLPA$_1$-null mice ($^{\#\#\#}p < 0.001$), and female wild-type mice ($^{\$\$\$}p < 0.001$).

3.8.2. CRH receptors

The statistical analysis revealed a significant main effect of "genotype" ($F_{(1,36)} = 30.25$; $p < 0.001$) and a significant interaction between "genotype" and "sex" ($F_{(1,36)} = 7.85$; $p = 0.008$) on the mRNA levels of *Crhr1* (Fig. 7B). *Post hoc* tests for multiple comparisons showed that male maLPA$_1$-null mice had a significant increase in the expression of this receptor compared to male wild-type (***$p < 0.001$) and female maLPA$_1$-null mice ($^{\#}p < 0.05$), and female maLPA$_1$-null mice had a significant increase compared to male wild-type mice (*$p < 0.05$). Regarding the levels of *Crhr2,* we did not detect its expression in the mPFC in any of our experimental groups (data not shown).

3.8.3. NPY

As shown in Fig. 7C, a two-way ANOVA revealed significant main effects of "genotype" ($F_{(1,37)} = 15.94$; $p < 0.001$) and "sex" ($F_{(1,37)} = 15.02$; $p < 0.001$), and a significant interaction between "genotype" and "sex" ($F_{(1,37)} = 9.05$; $p = 0.005$) on the levels of *Npy*. *Post hoc* tests for multiple comparisons showed that female maLPA$_1$-null mice had a significant increase in the expression of this neuropeptide compared to male wild-type (***$p < 0.001$) and maLPA$_1$-null mice ($^{\#\#\#}p < 0.001$), and female wild-type mice ($^{\$\$\$}p < 0.001$).

3.8.4. NPY receptors

The statistical analysis revealed a significant main effect of "genotype" ($F_{(1,37)} = 42.23$; $p < 0.001$) on the expression of *Npy1r* (Fig. 7D), and maLPA$_1$-null mice showed higher mRNA levels of this receptor than the wild-type mice. Regarding the levels of *Npy2r* (Fig. 7E), there were significant main effects of "genotype" ($F_{(1,37)} = 41.74$; $p < 0.001$) and "sex" ($F_{(1,37)} = 5.61$; $p = 0.023$), but no significant interaction between both factors. Thus, maLPA$_1$-null mice showed higher mRNA levels compared to the wild-type mice, and female mice showed higher mRNA levels of this receptor than male mice.

3.9. Effects of LPA$_1$ receptor deficiency on the mRNA expression of glutamatergic-signaling genes in the mPFC of male and female mice

Finally, we investigated the impact of LPA$_1$ receptor deficiency on the mRNA expression of the glutamatergic neurotransmission in the mPFC of both male and female mice.

3.9.1. Enzyme of synthesis and transporter of glutamate

As shown in Fig. 8A, a two-way ANOVA showed significant main effects of "genotype" ($F_{(1,37)} = 41.76$; $p < 0.001$) and "sex" ($F_{(1,37)} = 6.57$; $p = 0.015$), and a significant interaction between "genotype" and "sex" ($F_{(1,37)} = 9.99$; $p = 0.003$) on the levels of *Gls*. *Post hoc* tests for multiple comparisons showed that female maLPA$_1$-null mice had a significant increase in the expression of this enzyme compared to male wild-type (***$p < 0.001$) and maLPA$_1$-null mice ($^{\#\#}p < 0.01$), and female wild-type mice ($^{\$\$\$}p < 0.001$). Regarding the levels of *Slc1a1* (Fig. 8B), there were significant main effects of "genotype" ($F_{(1,37)} = 55.67$; $p < 0.001$) and "sex" ($F_{(1,37)} = 13.73$; $p < 0.001$), and a significant interaction between "genotype" and "sex" ($F_{(1,37)} = 11.19$; $p = 0.002$) on the levels of *Slc1a1*. *Post hoc* tests for multiple comparisons showed a significant increase in the mRNA levels of this transporter in male maLPA$_1$-null mice (*$p < 0.05$) compared to male wild-type mice. Moreover, the female maLPA$_1$-null mice had a significant increase in the expression of *Slc1a1* compared to male wild-type (***$p < 0.001$) and maLPA$_1$-null mice ($^{\#\#\#}p < 0.001$), and female wild-type mice ($^{\$\$\$}p < 0.001$).

3.9.2. NMDA receptors

There were significant main effects of "genotype" and "sex" on the mRNA levels of both subunits, *Grin1* (genotype: $F_{(1,37)} = 23.94$; $p < 0.001$; sex: $F_{(1,37)} = 14.86$; $p < 0.001$) (Fig. 8C) and *Grin2a* (genotype: $F_{(1,37)} = 44.82$; $p < 0.001$; sex: $F_{(1,37)} = 4.37$; $p = 0.043$) (Fig. 8D). Additionally, we also found a significant interaction between "genotype" and "sex" ($F_{(1,37)} = 7.46$; $p = 0.010$) on the levels of *Grin1*. *Post hoc* tests for multiple comparisons indicated a significant increase in the mRNA levels of this subunit in male maLPA$_1$-null mice compared to male wild-type (***$p < 0.001$) and female maLPA$_1$-null mice ($^{\#\#\#}p < 0.001$).

3.9.3. AMPA receptors

There were significant main effects of "genotype" and "sex" on the mRNA levels of the subunit *Gria1* (genotype: $F_{(1,37)} = 15.25$; $p < 0.001$; sex: $F_{(1,37)} = 23.93$; $p < 0.001$) (Fig. 8E). Specifically, maLPA$_1$-null mice showed lower mRNA levels compared to the wild-type mice, and female mice showed lower mRNA levels of this AMPA receptor subunit than male mice. Regarding the levels of *Gria2* (Fig. 8F), a two-way ANOVA revealed a significant main effect of "genotype" ($F_{(1,37)} = 56.70$; $p < 0.001$), and a significant interaction between "genotype" and "sex" ($F_{(1,37)} = 9.31$; $p = 0.004$). *Post hoc* tests for multiple comparisons showed a significant increase in the mRNA levels of this AMPA receptor subunit in male maLPA$_1$-null mice (*$p < 0.05$) compared to male wild-type mice. Moreover, the female maLPA$_1$-null mice had a significant increase in the expression of *Gria2* compared to male wild-type (**$p < 0.01$) and maLPA$_1$-null mice ($^{\#\#}p < 0.01$), and female wild-type mice ($^{\$\$\$}p < 0.001$).

3.9.4. Metabotropic receptors

As shown in Fig. 8G, a two-way ANOVA showed significant main effects of "genotype" ($F_{(1,37)} = 106.4$; $p < 0.001$) and "sex" ($F_{(1,37)} = 9.79$; $p = 0.003$), and a significant interaction between "genotype" and "sex" ($F_{(1,37)} = 17.35$; $p < 0.001$) on the levels of *Grm3*. *Post hoc* tests for multiple comparisons showed a significant increase in the mRNA levels of this receptor in male maLPA$_1$-null (***$p < 0.001$) compared to male wild-type animals. Moreover, the female maLPA$_1$-null mice had a significant increase in the expression of *Grm3* compared to male wild-type (***$p < 0.001$) and maLPA$_1$-null mice ($^{\#\#\#}p < 0.001$), and female wild-type mice ($^{\$\$\$}p < 0.001$). Regarding the expression of *Grm5* (Fig. 8H), we found significant main effects of "genotype" ($F_{(1,37)} = 12.19$; $p = 0.001$) and "sex" ($F_{(1,37)} = 16.95$; $p < 0.001$). Thus, maLPA$_1$-null mice showed higher mRNA levels compared to the wild-type mice, and female mice showed lower mRNA levels of this receptor than male mice.

4. Discussion

The LPA$_1$ receptor is a key component of the LPA signaling pathway, which has been implicated in various neural processes, including neurodevelopment, synaptic plasticity, and stress response (Yung et al., 2015). Understanding how LPA$_1$ receptor deficiency influences these processes, especially in a sex-dependent manner, could shed light on the molecular mechanisms underlying emotional disorders and potentially guide the development of sex-specific therapeutic strategies. In this study, we explored the impact of LPA$_1$ receptor deficiency on emotional behavior and neurotransmitter-related gene expression, with a particular focus on sex-specific differences.

Previous studies have reported that male maLPA$_1$-null mice exhibit high anxiety levels (Castilla-Ortega et al., 2010, 2016; Santin et al., 2009). In agreement with that, we have found that LPA$_1$ receptor deficiency leads to an increase in anxiety-like behaviors in both male and female mice. Notably, no significant differences in the total number of arm entries were observed between genotypes, suggesting that the changes in anxiety-like behaviors were not attributable to alterations in general locomotor activity. Interestingly, this effect was stronger in females, suggesting a possible sex-specific role of the LPA$_1$ receptor in modulating anxiety-related responses. Our results also showed that corticosterone levels were significantly higher in females than in males, independent of genotype. These findings suggest that sex-related

differences in corticosterone response to stress might underlie the observed behavioral differences. While the LPA_1 receptor deficiency influences anxiety-like behaviors, the lack of genotype effects on corticosterone levels indicates that these hormonal differences might be regulated by mechanisms that are not directly linked to the LPA_1 receptor. This observation aligns with evidence suggesting that sex hormones, such as estrogen, influence the HPA axis response to stress (Kudielka and Kirschbaum, 2005), which could contribute to the heightened anxiety-like responses in female maLPA$_1$-null mice. Moreover, the interplay between sex hormones and the LPA receptor system could further explain these differences. For example, it has been reported that estrogen may modulate LPA_1-mediated signaling, particularly in hormone-regulated tissues such as the pituitary gland (Moriyama and Fukushima, 2021). In addition, sex hormones like estrogen are known to influence the expression and function of the LPA receptor system in the brain, which could explain the more pronounced anxiety-like behaviors observed in female mice (Gonzalez-Arenas et al., 2008). These findings align with the growing body of evidence indicating that neurochemical and neurobiological responses to stress and anxiety differ between males and females, potentially due to differences in hormone levels, receptor expression, and signaling pathways (Bangasser and Cuarenta, 2021; Marques et al., 2016). The present study further investigated the effects of *Lpar1* deficiency on stress-coping behaviors by employing the TST in both male and female mice. Specifically, maLPA$_1$-null mice exhibited reduced immobility time and shorter latency to immobility compared to their wild-type counterparts. These results are in agreement with previous reports that have described that LPA_1 receptor deficiency in male mice is associated with a depression-related phenotype (Moreno-Fernandez et al., 2017, 2018). In addition to the genotype effect, a significant main effect of sex was observed, with female mice displaying greater immobility and longer latency compared to male mice. These results suggest a more passive response to stress in female, which is often interpreted as a lower ability to cope with stressful situations. This sex difference may be linked to various biological factors, particularly hormonal regulation. Estrogen and other sex hormones are known to influence stress responses and emotional regulation, potentially contributing to the observed differences between male and female (Halbreich and Kahn, 2001).

Next, we analyzed plasma levels of 2-AG and LPA to assess how LPA_1 receptor deficiency impacts lipid signaling pathways and contributes to emotional alterations. Our results showed that maLPA$_1$-null mice had lower 2-AG levels compared to wild-type mice, and female mice had lower levels than males. This reduction in 2-AG in maLPA$_1$-null mice may point to an important interaction between LPA_1 receptor signaling and the ECS. Given that 2-AG is a major endocannabinoid involved in regulating stress, mood, and reward processing (Morena et al., 2016), lower 2-AG levels in these mice may indicate impaired endocannabinoid signaling, potentially contributing to the emotional and behavioral changes observed. The sex differences, where females displaying lower 2-AG levels than males, could be linked to hormonal variations, as estrogen has been shown to modulate endocannabinoid levels (Balint et al., 2016). In fact, numerous evidence points to a bidirectional interaction between the ECS and estrogen [for review see (Forner-Piquer et al., 2024; Santoro et al., 2021)]. This interaction suggests that hormonal fluctuations, particularly estrogen, may shape ECS signaling, contributing to sex differences in emotional processing and susceptibility to psychiatric disorders. The ECS is sexually dimorphic (Ney et al., 2018), and in human, men have significantly higher plasma 2-AG levels than women (Fanelli et al., 2012), which may underlie some of the gender differences observed in stress response and mood disorders. Regarding the analysis of plasma LPA levels, maLPA$_1$-null mice had elevated LPA levels compared to wild-type mice. This increase in total LPA concentrations may suggest a compensatory mechanism in response to the loss of LPA_1 receptor signaling, potentially contributing to some of the neurobehavioral alterations observed in these mice. Supporting these findings, we have previously reported an increase in plasma LPA levels after treatment with Ki16425, a $LPA_{1/3}$ receptor antagonist (Sanchez-Marin et al., 2018).

The present study further explored the effects of LPA_1 receptor deficiency on the expression of genes related to 2-AG, LPA signaling, anxiety-related pathways, and glutamatergic neurotransmission in the amygdala and mPFC of male and female mice. The amygdala was selected for this analysis due to its crucial role in emotional regulation and stress response (Simic et al., 2021), making it an ideal region to investigate how disruptions in lipid signaling pathways may impact emotional behavior. The mPFC was chosen because it cooperates with the amygdala in the regulation of emotional behaviors (Kenwood et al., 2022). Our findings reveal complex interactions between genotype and sex in modulating the mRNA levels of key receptors and enzymes, emphasizing the role of sex-specific mechanisms in response to LPA_1 receptor deficiency.

4.1. 2-AG- and LPA-signaling systems in the amygdala

The observed increase in *Cnr1* mRNA levels in male maLPA$_1$-null mice suggests a compensatory mechanism in response to LPA_1 receptor signaling loss. This elevation could enhance the sensitivity of the ECS to endocannabinoids, which may be crucial for modulating emotional and stress responses. However, a previous study has shown no significant alterations in CB_1 receptor activity in the amygdala of maLPA$_1$-null mice, while demonstrating an increase in LPA_1 receptor activity in CB_1 knockout mice, suggesting a compensatory mechanism between the two receptors in this brain region (Gonzalez de San Roman et al., 2019). Additionally, we observed significant sex differences in the expression of cannabinoid receptors, with mRNA levels of both *Cnr1* and *Cnr2* being higher in females compared to males. These findings underscore the potential sex-specific roles for these receptors in the amygdala, consistent with previous reports suggesting a sex-dependent regulation of the cannabinoid receptors (Liu et al., 2020). Moreover, evidence shows that estrogen regulates cannabinoid receptor expression and activity in the brain (Riebe et al., 2010), highlighting the influence of sex hormones on the ECS and their critical role in mediating sex differences in stress responses and emotional behavior. Enzymes involved in 2-AG metabolism also exhibited significant effects related to genotype and sex. These findings suggest a potential sex-specific modulation of 2-AG metabolism (Bradshaw et al., 2006) and a dysregulation of 2-AG signaling in the amygdala in the context of LPA_1 receptor deficiency. The observed changes in the expression of *Dagla*, *Daglb*, *Plcb3,* and *Mgll* indicated a potential modulation of 2-AG production and degradation, which may be linked to the homeostatic role of the ECS in response to emotional disturbances associated with LPA_1 receptor deficiency (Petrie et al., 2021). Supporting this notion, a preliminary correlation analysis performed between the group means revealed a significant positive association between the anxiety index in the EPM and the expression levels of *Cnr1* ($r = +0.971$; $p = 0.029$) and *Daglb* ($r = +0.997$; $p = 0.001$) in the amygdala. This suggests that the upregulation of CB_1 receptors and DAGLβ may play a functional role in modulating anxiety. However, given the limited sample size and the fact that these analyses were conducted using group averages, these results should be interpreted cautiously and require further validation in larger groups. Furthermore, elevated *Plcb3* mRNA levels in maLPA$_1$-null mice highlight a potential compensatory mechanism to maintain DAG availability, the precursor for 2-AG synthesis, despite the deficiency in LPA_1 receptor signaling. Additionally, an increase in PC levels has been reported in LPA_1 knockout mice, which serves a substrate for *Plcb3* (Gonzalez de San Roman et al., 2019).

Regarding LPA-related genes, we observed an increase in mRNA levels of *Lpar2, Agk,* and *Enpp2* in maLPA$_1$-null mice, suggesting potential compensatory mechanisms within the LPA signaling pathway. The upregulation of *Lpar2*, a receptor known to mediate various physiological responses, may indicate an adaptive response to the absence of LPA_1-mediated signaling (Choi et al., 2010). Moreover, the increased

expression of *Agk*, which converts 2-AG into LPA, presents a compelling connection to the altered 2-AG signaling observed in these mice.

4.2. CRH and NPY systems in the amygdala

Since both the CRH and NPY signaling systems play a role in regulating stress responses, we also examined their mRNA expression in the amygdala of these mice. Although *Crh* expression remained unchanged due to LPA_1 receptor deficiency, *Crhr1* mRNA levels were lower in maLPA_1-null mice, and both receptors were downregulated in females. This may indicate a compensatory strategy to maintain stable CRH production despite diminished receptor availability, leading to altered sensitivity to stress. Furthermore, the downregulation of these receptors could reflect a dysregulation of stress response pathways, particularly in females, highlighting potential sex-specific vulnerabilities in emotional regulation and stress processing. Recently, we have reported similar sex effects in the CRH system in the amygdala of rats (Gobbi et al., 2024).

Regarding the expression of the NPY system, we found a reduced expression of *Npy1r* in maLPA_1-null mice. Since NPY exerts anti-stress and anxiolytic effects primarily through its interaction with NPY1 and NPY2 receptors in the brain (Reichmann and Holzer, 2016), these findings suggest that LPA_1 deficiency may attenuate the protective effects of NPY. Furthermore, the downregulation of the levels of *Npy* and its receptors in females suggests a potential sex-specific dysregulation of the NPY system (Gobbi et al., 2024).

4.3. Glutamatergic signaling system in the amygdala

Significant alterations were also observed in glutamatergic signaling. Lower expression of *Grin1* and *Grin2a* NMDA receptor subunits in maLPA_1-null mice and females indicated a potential disruption in glutamate receptor function, which may affect synaptic plasticity and emotional regulation (Wang et al., 2022). Similarly, the reduction in *Gria1* and *Grm3* expressions points to altered AMPA receptor- and metabotropic-mediated signaling, respectively. These changes may contribute to the behavioral phenotypes associated with LPA_1 receptor deficiency, such as increased anxiety-like behaviors (Bergink et al., 2004). The sex-specific downregulation of glutamatergic signaling genes aligns with previous studies that have shown differential regulation of these pathways in males and females (Kniffin and Briand, 2024).

4.4. 2-AG- and LPA-signaling systems in the mPFC

Similar to our observations in the amygdala, we also noted an increase in mRNA levels for many components of the 2-AG signaling pathway in male maLPA_1-null mice within the mPFC. This suggests a compensatory mechanism in response to the loss of LPA_1 receptor signaling, potentially affecting emotional processing and anxiety-related behaviors (Gonzalez de San Roman et al., 2019). Although we previously reported no significant changes in mRNA expression for various components of the ECS in the PFC of male maLPA_1-null mice, with the exception of *Mgll* (Castilla-Ortega et al., 2016), this apparent discrepancy may be attributed to the specific subregion examined (PFC *vs*. mPFC). The results also revealed sex-specific differences. Thus, the upregulation of *Cnr1* and *Cnr2* mRNA in maLPA_1-null mice, particularly in females, suggests a strong compensatory response to the loss of LPA_1 signaling, indicating a potential interaction between the LPA system, the ECS, and sex hormones (Eymery et al., 2024; Gonzalez de San Roman et al., 2019; Santoro et al., 2021). Moreover, the combination of decreased synthesis and increased degradation likely results in lower levels of 2-AG in female maLPA_1-null mice, potentially leading to reduced ECS signaling. This overall reduction in 2-AG signaling may be associated with an altered stress response in these animals (Bedse et al., 2020).

Previous studies have reported that LPA_2 receptor is expressed in the nervous system (Yung et al., 2015), and *Lpar2* mRNA has been detected in several areas of the developing mouse brain, including the hippocampus, neocortex, cerebellum, and olfactory bulb (Suckau et al., 2019). However, *Lpar2* mRNA expression was not detected in the mPFC of our mice. This does not necessarily mean it is completely absent from this region, and it is possible that very low levels of expression might be present but below the detection threshold of the method employed. Further research would be needed to make a definitive statement about its presence or absence in this brain area. Regarding the enzymes involved in the LPA synthesis, we observed an upregulation of the mRNA of *Agk* and *Enpp2* in maLPA_1-null mice, especially in females, suggesting a potential compensatory mechanism in response to the absence of LPA_1 receptor signaling.

4.5. CRH and NPY systems in the mPFC

The contrasting effects of CRH and NPY signaling in the mPFC and amygdala of these mice highlight the complex and region-specific nature of stress response regulation. The upregulation of the CRH system in the mPFC of maLPA_1-null mice provides a mechanistic insight into the anxiogenic-like behaviors observed in these animals. This finding is particularly significant given the established role of CRH receptor activity in the mPFC in modulating both HPA axis function and anxiety-related behaviors (Jaferi and Bhatnagar, 2007). Thus, the increased CRH signaling in this region likely contributes to an enhanced stress response and heightened anxiety states in these knockout mice. Similarly, the upregulation of the NPY system is interesting, as NPY is generally considered to have anxiolytic effects (Reichmann and Holzer, 2016). The concurrent increase in both CRH and NPY systems might represent a balanced response to maintain emotional stability in the absence of LPA_1 signaling (Thorsell, 2010).

4.6. Glutamatergic signaling system in the mPFC

Region-dependent effects were also observed in glutamatergic signaling in maLPA_1-null mice. In general, these mice exhibited an increase in mRNA expression for most components of the glutamatergic system analyzed in the mPFC. This upregulation was more pronounced in female maLPA_1-null mice, suggesting a sex-specific effect. Interestingly, these findings contrast with previous studies that reported a downregulation of glutamatergic signaling in male maLPA_1-null mice (Castilla-Ortega et al., 2016). These opposite results may be associated with the specific subregion examined (PFC *vs*. mPFC). Moreover, a preliminary correlation analysis performed between the group means revealed a significant positive association between the immobility time in the TST and the expression levels of *Gria1* ($r = +0.998$; $p = 0.001$) and *Grm5* ($r = +0.940$; $p = 0.031$) in the mPFC. These findings suggest that the upregulation of these glutamatergic receptors may be involved in the regulation of behavioral responses related to immobility and stress. However, as with the previous correlation, these results should be interpreted with caution.

In conclusion, our study underscores the significant role of the LPA_1 receptor in the modulation of emotional behavior and neurotransmitter-related gene expression, with a notable emphasis on sex-specific differences. The observed increase in anxiety-like behaviors in both male and female *maLPA$_1$-null* mice, particularly pronounced in females, suggests a distinct involvement of the LPA_1 receptor in stress response mechanisms. Furthermore, the dysregulation of lipid signaling pathways, characterized by altered levels of LPA and 2-AG, points to an intricate interplay between the LPA signaling system and the ECS that influences emotional regulation. The observed sex differences in gene expression related to anxiety and glutamatergic signaling highlight the necessity of considering biological sex in future research and therapeutic approaches. Building upon these findings, it is clear that the LPA_1 receptor could serve as a valuable therapeutic target for anxiety disorders. The observed dysregulation in lipid signaling and its association with altered gene expression provides a strong rationale for developing

interventions aimed at modulating the LPA signaling pathway. Notably, the pronounced sex differences in both behavioral and molecular outcomes suggest that future therapies should be tailored to account for biological sex, potentially leading to more effective treatments with fewer side effects. Moreover, the disruption of glutamatergic signaling observed in our study suggests that a dual-target approach, combining modulation of lipid signaling and regulation of excitatory neurotransmission, could enhance therapeutic outcomes. Overall, our findings contribute to a deeper understanding of the molecular mechanisms of emotional disorders, offering new avenues for the development of more personalized and effective therapeutic strategies.

5. Limitations

We are aware that this study has a number of limitations that future research should take into account. First, we have only analyzed mRNA expression and not protein expression. While gene expression data offer insights into potential regulatory changes at the transcriptional level, they do not directly reflect the actual protein levels or the functional activity of the proteins involved. Protein measurement would be essential to confirm whether the changes observed in gene expression are mirrored by changes in protein synthesis and function. Moreover, protein levels could reveal post-transcriptional regulatory mechanisms, such as translation efficiency and protein stability, which might influence how LPA_1 receptor deficiency manifests behaviorally. Without this protein-level validation, the conclusions drawn from mRNA data should be considered preliminary, and further studies incorporating protein analysis will be needed to offer a more comprehensive view of the underlying biological processes. Another limitation of this study is the lack of control for hormonal fluctuations, particularly variations in the estrous cycle, which may have contributed to observed sex differences in behavioral and molecular outcomes. Hormonal cycles are known to influence emotional behavior and receptor expression, and accounting for these fluctuations in future studies will be essential for clarifying their role in modulating LPA_1 receptor-related pathways. Additionally, the sample size used in the correlation analyses was relatively small, as behavioral and PCR experiments were conducted in separate sets of animals from the same cohort. As a result, the correlations were performed using group averages, which limits the statistical power of some findings. While we observed significant trends, these results require replication in larger samples to confirm their robustness. A larger sample size would allow for more precise estimates and stronger statistical power to validate the observed trends. Finally, although our results show that corticosterone levels were higher in females than in males under basal conditions, measuring corticosterone levels under stress conditions would provide a more comprehensive physiological context to our behavioral and molecular findings. Indeed, future studies will aim to incorporate stress paradigms, including acute and chronic stress models, to examine the corticosterone response in LPA_1-null mice. These studies would expand on our current findings and provide a more comprehensive understanding of how LPA_1 receptor deficiency affects stress reactivity and its downstream effects on behavior and gene expression.

CRediT authorship contribution statement

Laura Sánchez-Marín: Writing – review & editing, Methodology, Investigation. **Violeta Jiménez-Castilla:** Writing – review & editing, Methodology, Investigation. **María Flores-López:** Writing – review & editing, Methodology, Investigation. **Juan A. Navarro:** Writing – review & editing, Methodology, Investigation. **Ana Gavito:** Writing – review & editing, Methodology, Investigation. **Eduardo Blanco-Calvo:** Writing – review & editing, Formal analysis. **Luis J. Santín:** Writing – review & editing, Formal analysis. **Francisco J. Pavón-Morón:** Writing – review & editing, Funding acquisition, Conceptualization. **Fernando Rodríguez de Fonseca:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Antonia Serrano:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Conceptualization.

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Declaration of competing interest

I have nothing to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2025.110325.

Data availability

Data will be made available on request.

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Glossary

2-AG: 2-arachidonoylglycerol
Actb: β-actin gene
Agk: AGK gene
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA: Analysis of variance
ATX: autotaxin
$CB_{1/2}$: cannabinoid receptors type 1 or 2
Cnr1: CB_1 gene
Cnr2: CB_2 gene
CNS: central nervous system
CRH: corticotropin-releasing hormone
Crh: CRH gene
Crhr1: CRH receptor1 gene
Crhr2: CRH receptor 2 gene
DAGLα/β: diacylglycerol lipase α/β
Dagla: DAGLα gene
Daglb: DAGL β gene
ECS: endocannabinoid system

Enpp2: ATX gene
EPM: elevated-plus maze
Gls: kidney-type glutaminase isoform (KGA) gene
Gria1: AMPA receptor subunit GluR1 gene
Gria2: AMPA receptor subunit GluR2 gene
Grin1: NMDA receptor subunits NR1 gene
Grin2a: NMDA receptor subunits NR2A gene
Grm3: metabotropic mGluR3 receptor gene
Grm5: metabotropic mGluR5 receptor gene
LPA: lysophosphatidic acid
Lpar1: LPA_1 receptor gene
Lpar2: LPA_2 receptor gene
MAGL: monoacylglycerol lipase
maLPA$_1$-null: Malaga variant of the LPA_1-null mice
Mgll: MAGL gene
mPFC: medial prefrontal cortex
NMDA: ionotropic N-methyl D-aspartate receptors
NPY: neuropeptide Y
Npy: NPY gene
Npy1r: NPY receptor 1 gene
Npy2r: NPY receptor 2 gene
Plcb3: phospholipase C beta gene
Slc1a1: glutamate transporter EAAC1 gene
TST: tail suspension test