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Cocaine-seeking behaviour is differentially expressed in male and female mice exposed to maternal separation and is associated with alterations in AMPA receptors subunits in the medial prefrontal cortex.

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ABSTRACT

According with clinical data, women evolve differently from drug use to drug abuse. Among drugs of abuse, cocaine is the most consumed psychostimulant. Animal studies demonstrated that females show increased motivation to seek cocaine during the self-administration paradigm (SA) than males. Moreover, suffering childhood adversity or major depressive disorder are two factors that could increase the predisposition to suffer cocaine addiction. Maternal separation with early weaning (MSEW) is an animal model that allows examining the impact of early-life stress on cocaine abuse. In this study, we aimed to explore changes in MSEW-induced cocaine-seeking motivation to determine potential associations between despair-like behaviour and cocaine-seeking. We also evaluated possible alterations in the AMPA receptors (AMPArs) composition in the medial prefrontal cortex (mPFC) of these mice. We exposed mice to MSEW and the behavioural tests were performed during adulthood. Moreover, GluA1, GluA2 mRNA and protein expression were evaluated in the mPFC. Results show higher cocaine-seeking in standard nest females, as well as an increase in GluA1 and GluA2 protein expression. Moreover, MSEW induces downregulation of Gria2 and increases the Gria1/Gria2 ratio, only in male mice. In conclusion, female mice show different composition of the AMPA receptor in the mPFC and MSEW alters the glutamatergic system in the mPFC of male mice.

1. Introduction

Epidemiological data show that there are fewer women who use or abuse of drugs (UNODC 2020) leading to think that women are less vulnerable to drug abuse than men. However, several studies show that women have an accelerated course to addiction than men, acquiring faster the criteria for dependence after initial use (Haas and Peters 2000; Lynch 2008).

Among illicit drugs, cocaine is one of the most consumed psychostimulants with more than 18 million users (UNODC 2019). Several animal studies yield that females show increased motivation for cocaine-seeking than males, contributing to the faster acquisition of addicted behaviour (Lynch 2008; Lynch and Taylor 2005). This higher cocaine-seeking motivation is generally related with increased impulsivity and compulsivity behaviours that may enhance the vulnerability to suffering drug addiction (Adams et al. 2019; Butelman et al. 2019; Jupp et al. 2020; Nicholls et al. 2014; Rømer Thomsen et al. 2018). However, the changes that contribute to this higher cocaine-seeking

Abbreviations: AMPArs, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; CSF, Lumbar cerebrospinal fluid; GABA, Gamma-aminobutyric acid; GluA1, AMPA receptor subunit 1; GluA2, AMPA receptor subunit 2; Gria1, Glutamate Ionotropic Receptor AMPA Type Subunit 1 gene; Gria2, Glutamate Ionotropic Receptor AMPA Type Subunit 2 gene; MDD, Major depression disorder; NAc, Nucleus accumbens; mPFC, medial prefrontal cortex; MSEW, Maternal separation with early weaning; PD, Postnatal day; PFC, Prefrontal cortex; RRID, Research Resource Identifier; SA, Self-administration; SN, Standard nest; SUD, Substance use disorder; TST, Tail suspension test; VTA, Ventral tegmental area.

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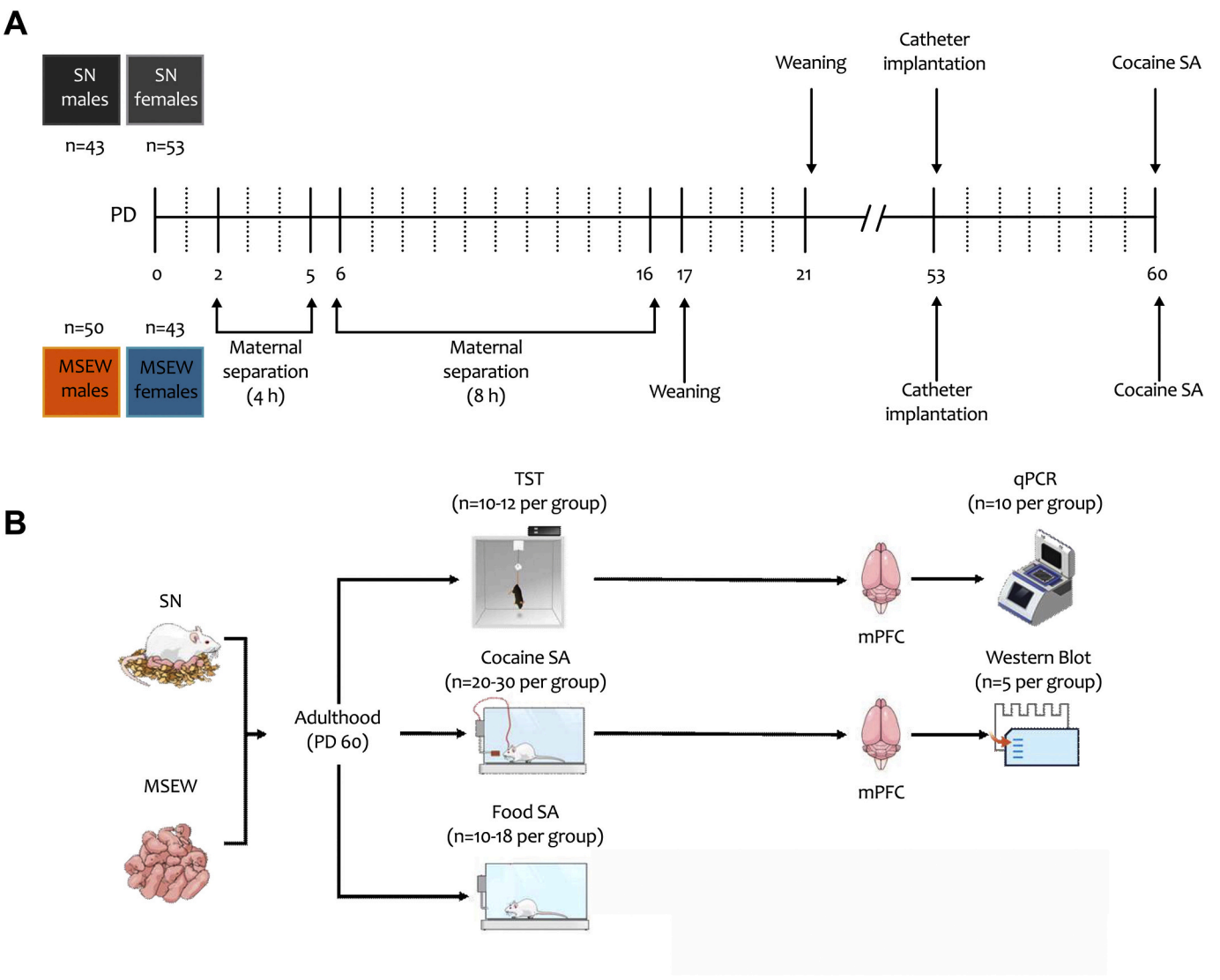

Fig. 1. Schematic representation of the experimental schedule. (A) Schematic representation of the MSEW model and the (B) timeline in which the experiments were performed.

motivation remain unknown.

Another important factor that increases the predisposition to cocaine addiction is the presence of other psychiatric disorders like major depressive disorder (MDD) (Torrens et al. 2015). Depression is the most common psychological disorder affecting almost 300 million people worldwide (GBD 2017 Disease and Injury Incidence and Prevalence Collaborators, 2018). This disorder is characterized by sadness, loss of interest or pleasure, feelings of guilt, low self-worth, disturbed sleep or appetite, tiredness, and poor concentration (WHO, 2017).

The understanding of this comorbidity (cocaine addiction and depression) is critical to improving considerably the treatment, clinical evolution, medication adherence and recovery of these patients (Rappeneau and Bérod 2017). Clinical studies show that cocaine addiction includes poor inhibitory control for goal-directed behaviour in the frontal cortical regions, evidencing an impulse control disorder which induces drug-seeking behaviour (Barrós-Loscertales et al. 2020).

The medial prefrontal cortex (mPFC) is a region that modulates cognitive and executive functions, including inhibitory control (Narayanan and Laubach 2017). Moreover, the mPFC is involved in in the reward response, due to the modulation of the GABAergic function in the NAc and the VTA dopamine neurons. Additionally, it has been reported that the stimulation of D1 receptors in the mPFC plays a key role in cocaine-seeking behaviour (Devoto et al. 2016). Also, post-mortem analysis and neuroimaging evaluations of depressed patients evidenced reductions in grey-matter volume in the prefrontal cortex (PFC) and hippocampus (Krishnan and Nestler 2008).

Molecular analyses demonstrated that cocaine-seeking behaviour in rats alters molecules related to neuronal plasticity in the mPFC like c-Fos and γ-protein kinase C (Mandyam 2013). Moreover, one of the neuronal cells affected by cocaine-seeking behaviour is the pyramidal excitatory glutamatergic neurons (Mandyam 2013).

Several studies have demonstrated that cocaine exposure induces changes in the AMPA glutamatergic receptors (AMPArs) subunit composition (Bowers et al. 2010; Castro-Zavala et al. 2020a, 2020b). AMPArs are made up of four subunit proteins (GluA1-A4) and generally composed of GluA2 in complex with GluA1 or GluA3 (Bowers et al. 2010). Previous studies reported increased AMPArs function after cocaine exposure, because of the induction of long-term potentiation (Kauer and Malenka 2007). This high activity of AMPArs could be explained by the insertion of GluA2-lacking AMPArs, because this kind of receptors are calcium-permeable, have greater channel conductance and trigger calcium-dependent signalling cascades (Bowers et al. 2010).

Recently, the GluA2-lacking AMPArs were suggested to be a common link between depression and cocaine abuse (Castro-Zavala et al. 2020b;

Goffer et al. 2013; Martínez-Rivera et al. 2017). In line with this, some studies report that fluoxetine triggers the phosphorylation of GluA1 in the prefrontal cortex, hippocampus and striatum (Svenningsson et al. 2002); in contrast, serotonin depletion in the PFC decreases the expression of GluA1 but increase GluA2 in the cerebral cortex (Shutoh et al. 2000).

An important observation is that being victim of abuse or negligence in childhood increase the risk of developing psychiatric disorders (Lippard and Nemeroff 2020). For example, history of childhood adversity increases in 28.8% the possibility of depression and 16.5% the risk of illicit drug use (Anda et al. 2006).

Maternal separation with early weaning (MSEW) is an animal model that allows researchers to reproduce the effects of childhood adversity (Bian et al. 2015; George et al. 2010; Vetulani 2013). Moreover, MSEW induces a depression phenotype that permits the examination of the impact of early-life stress on cocaine use or abuse (Castro-Zavala et al. 2020b; Liu et al. 2018; Vannan et al. 2018). We have previously reported, using a model of MSEW, increased despair- and anxiety-like behaviour (Gracia-Rubio et al. 2016; Portero-Tresserra et al. 2018), higher acquisition in the cocaine self-administration (SA) paradigm in males but not in females (Castro-Zavala et al. 2020b) and increased GluA1/GluA2 in the NAc and VTA of males exposed to MSEW and cocaine SA (Castro-Zavala et al. 2020b). Moreover, we also observed an increased acquisition percentage in females, which was independent of the early-life stress (Castro-Zavala et al. 2020b), being in accordance with the telescoping effect observed in women regarding drug use disorders (Haas and Peters 2000).

In this context, this work aimed to explore the changes in cocaine-seeking motivation induced by the MSEW model in male and female CD1 mice. Moreover, we determined a possible association between despair-like behaviour, cocaine-seeking motivation, as well as alterations of AMPArs subunit (mRNA and protein levels). To achieve these goals, we evaluated cocaine-seeking motivation (as a percentage of response efficiency) in the SA paradigm. We also determined GluA1 and GluA2 protein expression and evaluated Gria1 and Gria2 mRNA expression in the mPFC, a brain area involved in inhibitory control of behaviours.

2. Materials and methods

2.1. Animals

Sixteen male and sixteen female CD1 adult mice of 10 weeks of age were used as breeders (Charles River, Barcelona, Spain). Animals were received at our animal facility, UBIOMEX, PRBB. The animals were placed in pairs in standard cages at a temperature- (21 ± 1 °C) and humidity- (55% ± 10%) controlled room and subjected to a 12 h light/dark cycle; with the lights on from 8:00 to 20:00 h and *ad libitum* access to food and water. Ten days later, the males were removed from the cages. The sex distribution estimation of the offspring was 53% for males and 46% for females. Once offspring had been weaned, mice were assigned randomly to the cocaine SA, food SA or to naïve condition. A different group of mice was used to perform the tail suspension test (TST). The total number of animals used for this work was 189 (49% males, 51% females). Experimenters were blinded to the different experimental procedures. The experiments were carried out in accordance with ARRIVE, BJP guidelines for reporting experiments involving animals (Kilkenny et al. 2010; McGrath and Lilley 2015) in accordance with the guidelines of the European Communities Directive 88/609/EEC regulating animal research. The local ethical committee (CEEA-PRBB) approved all procedures, and every effort was made to minimize animal suffering and discomfort as well as the number of animals used.

2.2. Rearing conditions

The rearing conditions were as previously described (Castro-Zavala et al. 2020a, 2020b; Gracia-Rubio et al. 2016; Portero-Tresserra et al. 2018). New-born mice were randomly assigned to the experimental groups: standard nest (SN) and MSEW (Fig. 1A). The day of birth was considered the postnatal day (PD) 0. Animals in the MSEW were separated from their mothers for 4 h per day (9:00 to 13:00 h) from PD2 to PD5, and 8 h per day (9:00 to 17:00 h) from PD6 to PD16. As for the separation, the mother was removed and placed in another cage and room, leaving the pups in their home box. To maintain the body temperature of the pups, home boxes were placed upon electric blankets until the mother was duly returned. Animals in the SN remained with their mother until weaning (PD21), whilst animals in the MSEW were weaned at PD17. In both cases (SN and MSEW), cages were cleaned on PD10. We distributed the pups of each litter between the different experimental groups in order to avoid a litter effect. MSEW procedure does not affect body weight (Gracia-Rubio et al. 2016; Portero-Tresserra et al. 2018), mortality (George et al. 2010), morbidity (George et al. 2010) or the male/female ratio (Koob and Zorrilla 2010).

2.3. Tail suspension test

We used the tail suspension test (TST) for assessing despair-like behaviour (Planchez et al. 2019). At the beginning, there is a period of active behaviour during the animal tries to scape; after this period, animals exhibit immobility, and this immobility is taken as an indication of behavioural despair and is commonly considered to reflect depression-like states (Planchez et al. 2019). Mice underwent the TST on PD60 as previously described (Gracia-Rubio et al. 2016). Briefly, each mouse was suspended 50 cm above a benchtop for 6 min (using adhesive tape attached 1 cm from the tip of the tail). The time (s) that the animal was immobile during this interval was recorded.

2.4. Drugs

Cocaine hydrochloride was purchased from Alcatel (Ministry of Health, Madrid, Spain) and was dissolved in sterile physiological saline (0.9%, NaCl solution). A dose of 1 mg/kg/infusion was used for the acquisition phase of the SA procedure.

2.5. Apparatus for self-administration experiments

The SA experiments were carried out in mouse operant chambers (Model ENV-307A-CT, Medical Associates, Cibertec S.A., Madrid, Spain) containing two holes; one was defined as active and the other as inactive. Nose-poking into the active hole produced a reinforcer delivery (cocaine infusion or a food pellet) that was paired with two stimulus lights, one of which was placed inside the nose-poke and the other above the active hole. Mice received a maximum of 150 reinforcers, and each reinforcer was followed by a 15 s time-out period, in which no reinforcers were delivered. Nose-poking into the inactive hole had no consequences. The side on which the active/inactive hole was placed was counterbalanced.

At the beginning of each session, the house light was ON for 3 s and OFF for the rest of the experiment. The session started with a food pellet release or a cocaine priming injection and 4 s presentation of the light cue, situated above the active hole.

2.6. Cocaine self-administration

The SA experiments were conducted as described (Castro-Zavala et al. 2020a, 2020b; Ferrer-Pérez et al. 2019). Briefly, when the SN (males $n = 23$, females n = 23) and MSEW (males $n = 30$, females $n = 20$) animals reached PD53, a jugular-vein catheter implantation was performed. The surgery was done following anaesthetization with a mixture of ketamine/xylazine (50 mg/mL, 10 mg/mL, administrated in a volume of 0.15 mL/10 g). Animals were treated with analgesic (Meloxicam 0.5 mg/kg; i.p, administrated in a volume of 0.10 mL/10 g)

and antibiotic solution (Enrofloxacin 7.5 mg/kg, i.p., administrated in a volume of 0.03 mL/10 g). After surgery, animals were housed individually, placed over electric blankets, and allowed to recover. At least 3 days after surgery, animals were trained, on a fixed ratio 1, to self-administer cocaine (1.0 mg/kg per infusion). During 10-day sessions (2 h each), we counted the responses in the *time in* (cocaine infusions) and the responses in the *time in* + *time out* (nosepokes). Mice were considered to have acquired a stable SA behaviour when the following criteria were met on 2 consecutive days: ≥5 responses in the active hole and ≥ 65% of responses in the active hole. All animals accomplished the 10 sessions independently of the day of acquisition.

2.7. Food self-administration

Four days before testing commenced (PD56), mice SN (males $n = 10$, females $n = 18$) and MSEW (males n = 10, females $n = 12$) were food-restricted and for that, mice were fed accordingly to the 95% of their body mass daily. Food restriction lasted the duration of food-maintained operant behaviour. Water was available *ad libitum* during the experimental phase. The animals were trained, on a fixed ratio 1, to nosepoke for food pellets (Grain-Based Rodent #5001, Test Diet, Sawbridgeworth, UK) for 10-day sessions (2 h each). We counted the responses in *time in* (food pellets) and the responses in *time in* + *time out* (nosepokes) during all the experiment.

2.8. Percentage of response efficiency and motivation for cocaine

Due to higher motivation for cocaine is related to higher cocaine seeking (Le et al. 2017), we consider that a reduced response efficiency (more nosepokes than reinforcers) means higher motivation for cocaine.

We calculated the percentage of response efficiency with the data obtained in the self-administration paradigm.

For the cocaine SA, we used the following formula:

$$\%Response\ efficiency = \frac{Number\ of\ cocaine\ infusions}{Number\ of\ nosepokes} \times 100$$

In the case of food SA, we calculated the percentage of response efficiency as follows:

$$\%Response\ efficiency = \frac{Number\ of\ food\ pellets}{Number\ of\ nosepokes} \times 100$$

2.9. Animal sacrifice and sample collection

Animals were sacrificed by cervical dislocation. Brains were immediately removed from the skull and placed in a cold plaque. Samples were dissected at different phases: without any behavioural test (drug-naïve), after cocaine SA (drug-experienced) and after TST (PD60). The drug-experienced animals are exclusively the mice that acquired the cocaine SA behaviour. To dissect mPFC, we used a brain matrix for coronal slicing. mPFC was dissected (bregma +1.2 mm to +0.6 mm) and immediately stored at −80 °C until the biochemical analysis was performed. Samples from naïve- and drug-experienced mice were used for the western blot. For the qPCR, we utilised samples from mice that completed the TST.

2.10. Western Blot for GluA1 and GluA2

We evaluate the expression of GluA1 and GluA2 in whole-cell lysate. Samples were homogenized in cold lysis buffer (NaCl 0.15 M, EDTA 0.001 M, Tris pH 7.4 0.05 M, TX-100 1%, Glycerol 10%), supplemented with a protease inhibitor (Complete ULTRA Tablets Mini EASYpack, Roche, Mannheim, Germany) and a phosphatase inhibitor (PhosSTOP EASYpack, Roche, Mannheim, Germany). Protein samples (20 μg) were mixed with 5× loading buffer (TRIS pH 6.8 0.153 M, SDS 7.5%, Glycerol 40%, EDTA 5 mM, 2-β-mercaptoethanol 0.025%, bromophenol blue 0.025%), loaded and run on SDS-PAGE 10% and transferred to PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked with BSA 5% for 1 h at room temperature and incubated overnight at 4 °C with primary antibodies (Table 1). Primary antibodies were detected with fluorescent secondary antibodies (Table 1), incubated for 1 h at room temperature. Images were acquired on a Licor Odyssey Scanner and quantified using Image Studio Lite software v5.2 (LICOR, USA). The expression of GluA1, GluA2 and β-tubulin were evaluated in the mPFC of the different groups: SN drug-naïve males, SN drug-experienced males, MSEW drug-naïve males, MSEW drug-experienced males, SN drug-naïve females, SN drug-experienced females, MSEW drug-naïve females and MSEW drug-experienced females ($n = 4$–5 per group, run in duplicate). We normalized the GluA1 and GluA2 expression to tubulin protein levels.

Table 1
Antibodies.

Antibody	# Catalogue	RRIDs	Dilution	Company
GluA2	AB1768	AB_2313802	1:1000	Milipore
GluA1	ABN241	AB_2721164	1:1000	Milipore
β-tubulin	556,321	AB_396360	1:5000	BD Biosciences
goat anti-mouse IgG H&L (IRDye 800CW)	ab216772	AB_2857338	1:2500	Abcam
goat anti-rabbit IgG H&L (DyLight 680)	611–144-002	AB_1660962	1:2500	Rockland

Table 2
Primers for qPCR.

	Primer sequence (5'-3')
Gria1 Forward	ACA ACT CAA GCG TCC AGA ATA G
Gria1 Reverse	CAT AGC GGT CAT TGC CTT CA
Gria2 Forward	CCT TTC TTG ATC CTT TAG CCT ATG A
Gria 2 Reverse	CTG CTG ACC AGG AAT AAA ACT ACA CT
36B4 Forward	TCC AGG CTT TGG GCA TCA
36B4 Reverse	CTT TAT CAG CTG CAC ATC ACT CAG A

2.11. RNA isolation and real-time (RT)-PCR

RNA extraction from mPFC samples was performed using trizol as previously described (Cardenas-Perez et al. 2018). RT-PCR was performed by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) using random primers and following standardized protocols.

2.12. Quantitative PCR for Gria1 and Gria2

For the qPCR, we use cDNA (20 ng), Light Cycler SBYR green 480 Master Mix (Roche LifeScience, Product No. 04707516001) and the specific primers (Table 2) for Gria1, Gria2 and 36B4 as housekeeping gene (Integrated DNA Techologies, Inc.). The qPCR was performed in LightCycler ® 480 Instrument II (Roche LifeScience) using next program: 95 °C-10s, 60 °C-20s, 72 °C-10s for 45 cycles.

2.13. Statistical analysis

Data were analysed for conditions of normality (Kolmogorov-Smirnov's test), sphericity (Mauchly's test) and homoscedasticity (Levene's test). Data from the TST, qPCR, average of response efficiency and western blot results of drug-naïve mice, were analysed using a two-way ANOVA with *rearing* and *sex* as independent factors. Western blot results of drug-naïve and drug-experienced animals were analysed using a three-way ANOVA with *rearing*, *sex* and *treatment* as factors. For the SA results, we used a four-way ANOVA with two variables between-subjects

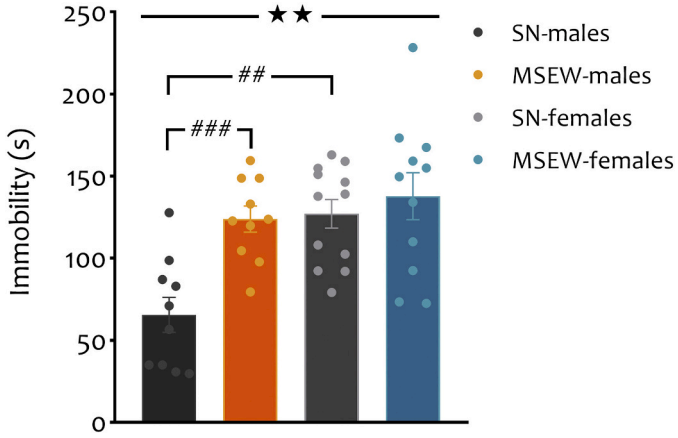

Fig. 2. Effects of MSEW on the tail suspension test. Total immobility time in SN and MSEW adult male and female mice. *Sex* main effect of the ANOVA (★★$p <$ 0.01). Bonferroni post-hoc comparison for the interaction *sex* × *rearing* is indicated with the lines (##$p < 0.01$, ###$p < 0.001$). Data are expressed as mean ± SEM (n = 10–12 per group).

(*sex* and *rearing*) and two variables within-subjects (*days* and *time*). When F achieved $p < 0.05$, the ANOVA was followed by the Bonferroni post-hoc test if a main effect and/or interaction was observed. All possible post-hoc comparisons were evaluated. Statistical analyses were performed using SPSS Statistics v23. Data are expressed as mean ± SEM and a value of $p < 0.05$ was considered significant.

3. Results

A summary of the statistical analysis of the experiments is available in Supplementary Material, showing all significant and non-significant effects and comparisons.

3.1. Maternal separation increases despair-like behaviour only in male mice

The effects observed in the TST were evaluated in female and male mice during adulthood (PD60) in MSEW and control mice (Fig. 2). A two-way ANOVA of immobility time showed a significant effect of *sex* ($F_{1,39} = 12.324$, $p = 0.001$), *rearing* ($F_{1,39} = 10.291$, $p = 0.003$) and the interaction between these factors ($F_{1,39} = 4.908$, $p = 0.033$). Bonferroni post-hoc test for the interaction showed that SN females spent more time immobile than SN males ($p = 0.000$) and also that MSEW males showed a higher immobile time than SN males (p = 0.001).

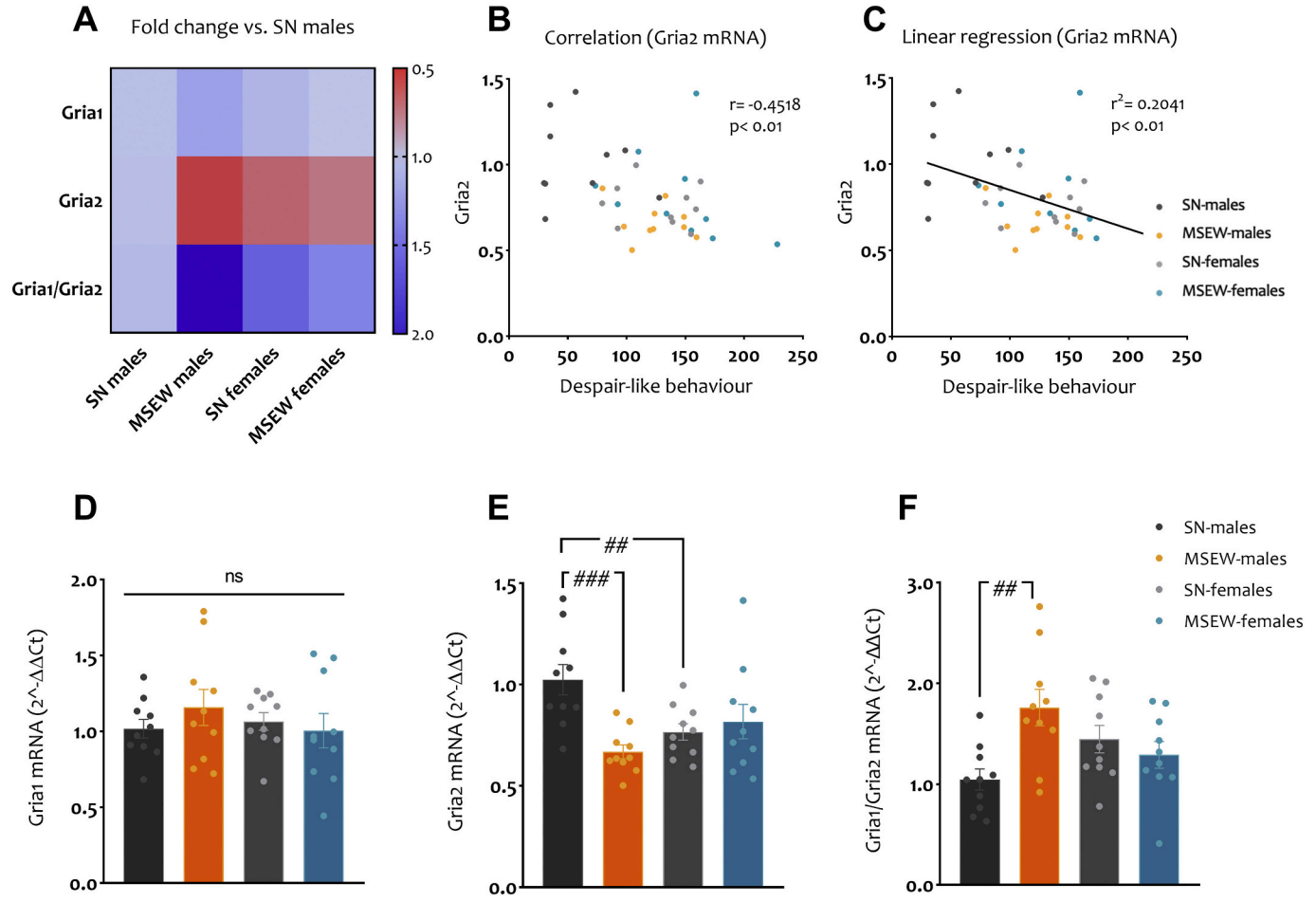

Fig. 3. mRNA expression of two AMPArs subunits in the mPFC of SN and MSEW drug-naïve mice. (A) Heatmap representing mRNA expression of Gria1, Gria2 and Gria1/Gria2 ratio in the mPFC of SN and MSEW mice relative to SN males as determined by qPCR. (B) Scatter blot illustrating the negative correlation between Gria2 and despair-like behaviour in mice. (C) Linear regression representing the relationship between despair-like behaviour and Gria2 expression in mice (Y = −0.0022× + 1.075). Mean fold change relative to SN males of (D) Gria1, (E) Gria2 and (F) Gria1/Gria2 mRNA levels in the mPFC of drug-naïve mice. Bonferroni post-hoc comparison for the interaction *sex* × *rearing* is indicated with the lines (##p < 0.01, ###p < 0.001). Data are expressed as mean ± SEM (n = 10 per group, run in duplicate).

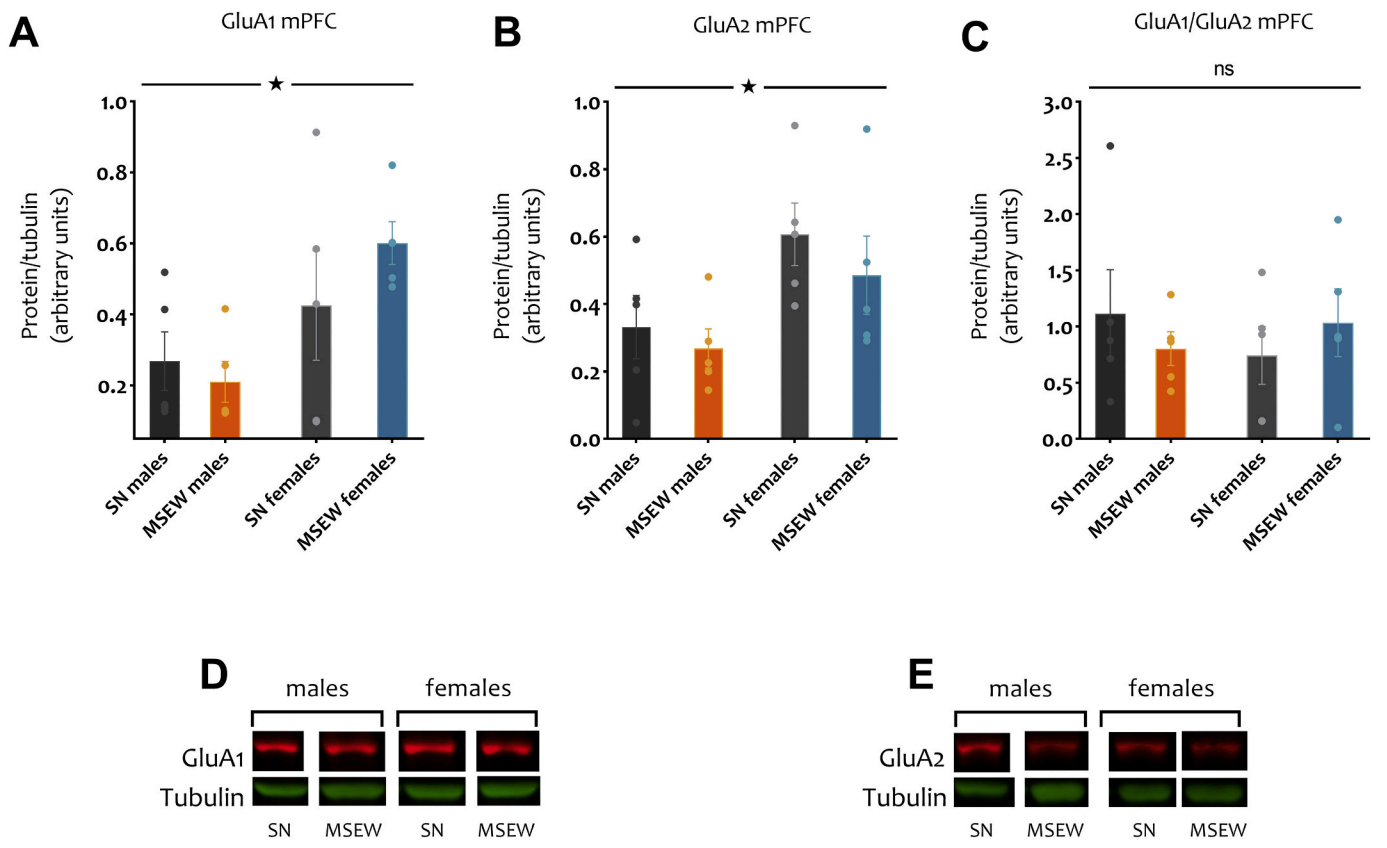

Fig. 4. Protein levels of two AMPArs subunits in the mPFC of SN and MSEW drug-naïve mice. Protein expression of (A) GluA1, (B) GluA2 and (C) GluA1/GluA2 ratio in the mPFC of drug-naïve mice. Representative western blot showing protein levels of (D) GluA1 and (E) GluA2 in the mPFC. The protein of interest in red and tubulin in green. *Sex* main effect of the ANOVA (★$p < 0.05$). Data are expressed as mean ± SEM ($n = 5$ per group, run in duplicate). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. MSEW downregulates Gria2 mRNA and increases the Gria1/Gria2 ratio in the mPFC of male mice

We sought to identify the effect of MSEW on the gene expression of Gria1 and Gria2 and the possible relation between despair-like behaviour and the levels of these genes (Fig. 3A). For this purpose, we performed qPCR in the mPFC of males and females after the TST. For Gria1, we did not find significative differences (Fig. 3D) (see supplementary information, Table S1). Results for the Gria2 showed a significative main effect of *rearing* ($F_{1,36} = 5.939$, $p = 0.020$) and the interaction *sex* × *rearing* ($F_{1,36} = 10.577$, $p = 0.002$) (Fig. 3E) (see supplementary information, Table S2). The interaction *sex* × *rearing* showed a decreased Gria2 mRNA level in SN females in comparison with SN males ($p = 0.006$). Moreover, the interaction also showed that maternally separated males had downregulation of Gria2 mRNA level in comparison with the SN males ($p = 0.000$). For the Gria1/Gria2 ratio the statistical analysis showed a significative difference for the interaction *sex* × *rearing* ($F_{1,36} = 9.516$, $p = 0.004$) (Fig. 3F) (see supplementary information, Table S3). Bonferroni post-hoc test revealed a significant increased Gria1/Gria2 ratio in MSEW males compared to the SN males ($p = 0.024$).

3.3. Despair-like behaviour correlates with Gria2 mRNA levels in the mPFC of mice

We evaluated the relationship between Gria2 mRNA level and the immobility time during the TST in female and male mice. For this purpose, we calculated the Pearson correlation coefficient. There was a significant correlation ($r = -0.4518$, $p = 0.003$, $n = 40$) between Gria2 and the despair-like behaviour among the subjects (Fig. 3B). Linear regression suggests that Gria2 mRNA level could be predicted from the despair-like behaviour ($F_{1,38} = 9.744$, $p = 0.003$) ($y = -0.002244\times + 1.075$) (Fig. 3C).

3.4. Increased GluA1 and GluA2 protein expression in female compared to male mice

Because of the differences observed in the Gria1 and Gria2 mRNA of mice, we decided to evaluate whether these changes were also manifested in the functional form of these genes. Hence, we detected the protein expression of GluA1 (Fig. 4A), GluA2 (Fig. 4B) and the GluA1/GluA2 ratio (Fig. 4C), using western blotting in male and female mPFC of drug-naïve mice (without any behavioural test). The two-way ANOVA showed a *sex* main effect for GluA1 ($F_{1,20} = 8.005$, $p = 0.012$) and GluA2 ($F_{1,20} = 7.087$, $p = 0.017$). We did not find significative differences for *rearing* or the *sex* × *rearing* interaction (See supplementary information, Table S4 and S5). In both cases, the *sex* effect evidenced that females showed higher baseline protein expression of these AMPA receptor subunits compared to males. An interesting observation was that MSEW did not modify the levels of GluA1 or GluA2 in the mPFC neither in females nor in males. No changes were found for the GluA1/GluA2 ratio (see supplementary information, Table S6).

3.5. Females showed higher cocaine-seeking behaviour in standard rearing conditions while MSEW increased cocaine-seeking behaviour in male but no in female mice

The statistical analysis for the cocaine SA (Fig. 5A) showed a main effect of *days* ($F_{9,828} = 19.627$, $p = 0.000$), *time* ($F_{1,92} = 118.436$, $p = 0.000$) and *sex* ($F_{1,92} = 5.442$, $p = 0.022$). Additionally, results yield double interaction between *days* × *rearing* ($F_{9,828} = 2.258$, $p = 0.017$),

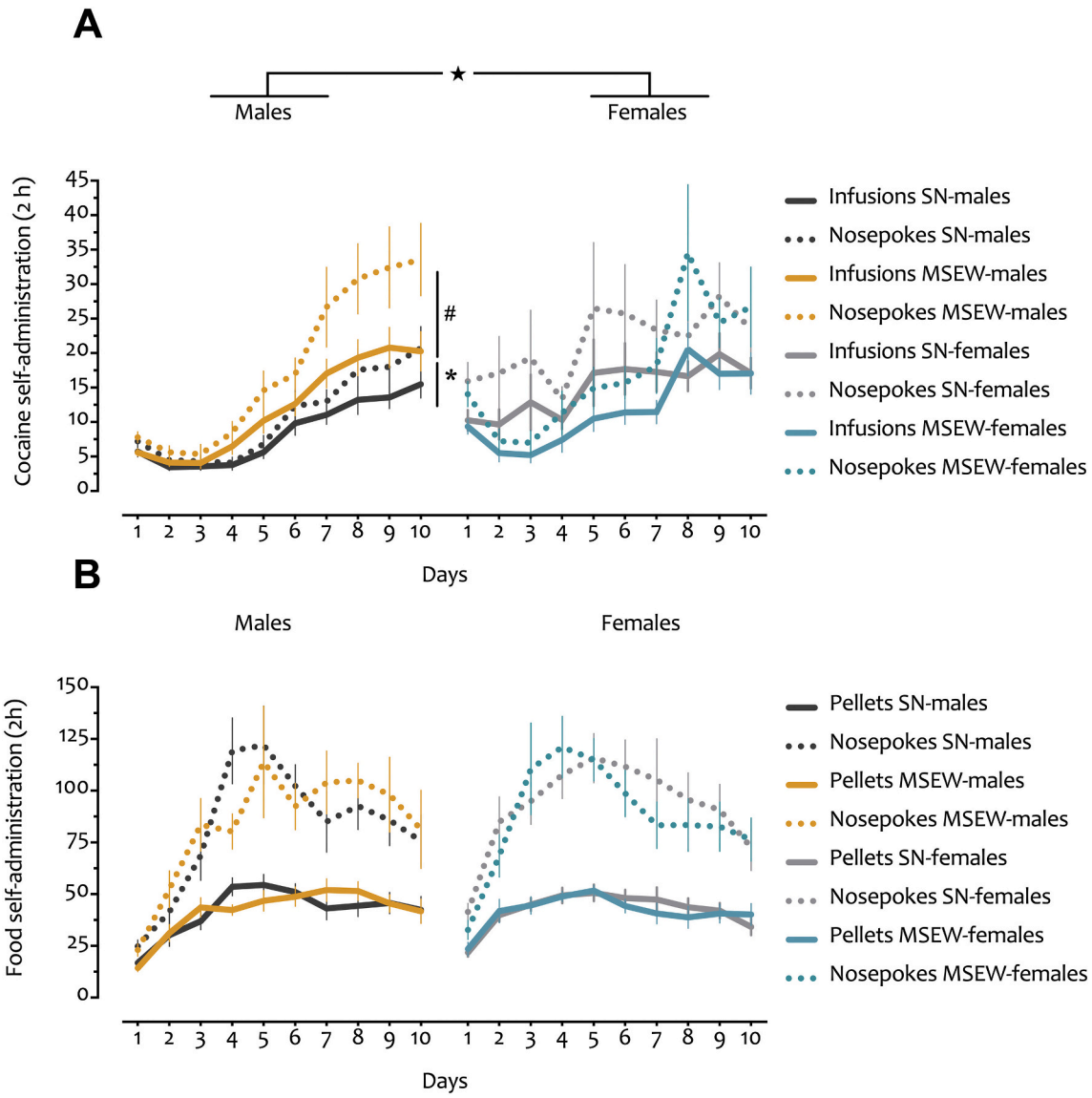

Fig. 5. Results from the self-administration paradigm. (A) Mean of infusions and nosepokes during the cocaine self-administration acquisition phase (n = 20–30 per group). (B) Mean of pellets and nosepokes during the food self-administration acquisition phase (n = 10–18 per group). *Sex* main effect of the ANOVA (★p < 0.05). Bonferroni post-hoc comparison for the interaction *response* × *sex* × *rearing* is indicated with the lines: higher number of infusions (*p < 0.05) and nosepokes (#p < 0.05) performed by MSEW males *vs.* SN males.

time × *sex* ($F_{1,92}$ = 4.345, p = 0.040), *days* × *time* ($F_{9,828}$ = 5.906, p = 0.000) and *sex* × *rearing* ($F_{1,92}$ = 7.444, p = 0.008) (see supplementary information, Table S8). Finally, the analysis also showed triple interaction among *time* × *sex* × *rearing* ($F_{1,92}$ = 5.761, p = 0.018) and *days* × *time* × *rearing* ($F_{9,828}$ = 2.802, p = 0.003). Post-hoc analysis for *time* × *sex* × *rearing* revealed that SN females performed higher number of nosepokes (p = 0.001) and infusions (p = 0.001) than SN males. Additionally, this interaction also showed that MSEW males performed more infusions (p = 0.039) and nosepokes (p = 0.012) than the SN males. The Bonferroni test for the *days* × *time* × *rearing* revealed that MSEW mice achieved more nosepokes in day 8 than the SN animals (p = 0.029).

3.6. MSEW did not modify food seeking during the self-administration paradigm

The four-way ANOVA for the food SA (Fig. 5B) showed a main effect of *days* ($F_{9,414}$ = 26.271, p = 0.000) and *time* ($F_{1,46}$ = 160.009, p = 0.000) (see supplementary information, Table S7). Also, statistical analysis revealed interactions between *days* × *sex* ($F_{9,414}$ = 2.029, p = 0.035) and *days* × *time* ($F_{9,414}$ = 14.095, p = 0.000). The Bonferroni post-hoc test for the interaction *days* × *sex* yield that female mice search more food than males during the first two days (p < 0.05 in both cases), however, in the next days, the food consumption was similar. The post-hoc for *days* × *time* interaction yield that in all the days, animals performed a higher number of nosepokes than the number of pellets obtained (p < 0.001 in all the cases).

3.7. Females showed resilience for the increased cocaine-seeking motivation induced by MSEW

In order to determine if depressed animals exposed to MSEW showed different motivation for cocaine, we performed the cocaine SA procedure and calculated the average of the percentage of response efficiency along the acquisition phase (Fig. 6A). The two-way ANOVA for the average of the response efficiency showed a main effect of *sex* ($F_{1,92}$ = 8.016, p = 0.006) and the interaction *sex* × *rearing* ($F_{1,92}$ = 5.011, p = 0.028). The *rearing* effect did not reach statistical significance ($F_{1,92}$ = 3.455, p = 0.066). The post-hoc test for the interaction *sex* × *rearing*

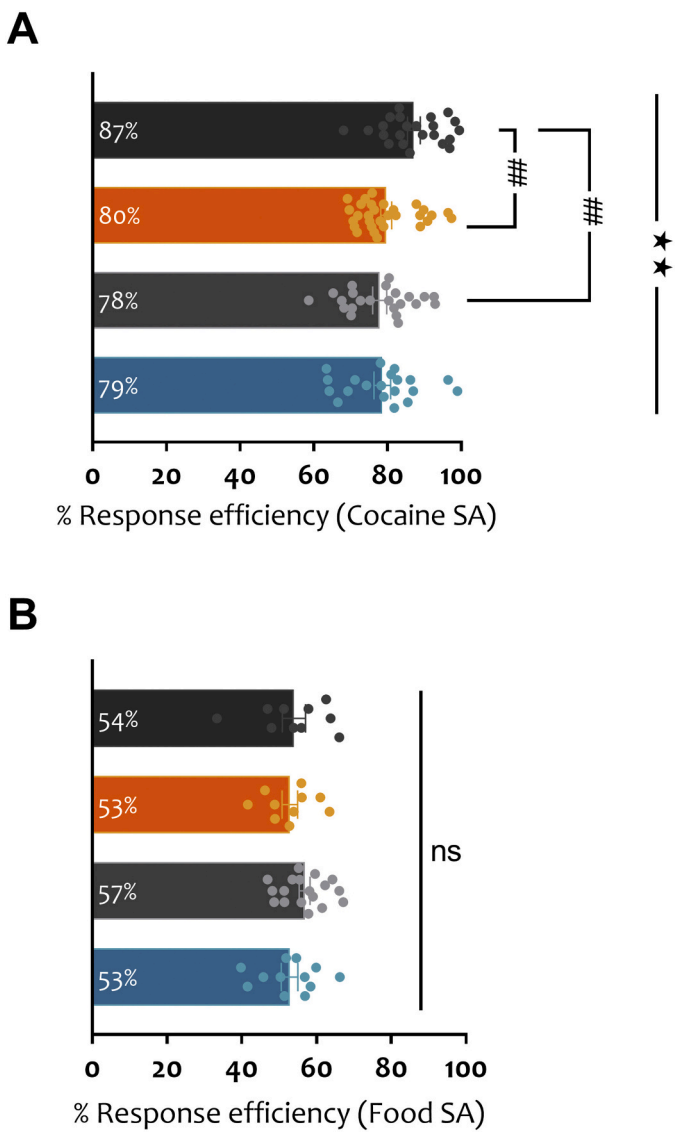

Fig. 6. Percentage of response efficiency in the SA paradigm of SN and MSEW mice. Percentage of response efficiency as an indirect measure of seeking motivation in the (A) cocaine SA (n = 20–30 per group) or the (B) food SA procedure (n = 10–18 per group). *Sex* main effect of the ANOVA (★★$p < 0.01$). Bonferroni post-hoc comparison for the interaction *sex* × *rearing* is indicated with the lines (##p < 0.01). High response efficiency scores mean less seeking motivation whereas low response efficiency signifies more seeking motivation. Data are expressed as mean ± SEM.

revealed that maternally separated male mice had lower response efficiency than SN males (p = 0.003), suggesting that MSEW increases motivation for cocaine-seeking but only in males. This interaction also evidenced that SN females showed higher cocaine-seeking motivation (decreased response efficiency) than SN males (p = 0.001), behaving in the same way than the MSEW males and the MSEW females.

3.8. Cocaine but not natural reward SA is affected by early-life stress in male mice

To evaluate if the MSEW-induced increased motivation was specific for cocaine, we performed a food SA in another set of animals. Once again, we calculated the average of the percentage of response efficiency along the acquisition phase (Fig. 6B). The two-way ANOVA did not show *sex* effect ($F_{1,46} = 0.417$, $p = 0.522$), *rearing* effect ($F_{1,46} = 1.397$, $p = 0.243$) or the interaction between these two factors ($F_{1,46} = 0.455$, $p = 0.503$), suggesting that changes in motivation induced by MSEW is specific for cocaine and no for natural reward.

3.9. Chronic cocaine exposure does not modify the expression of GluA1, GluA2 or the GluA1/GluA2 ratio in the mPFC of mice

In order to evaluate changes in the expression of GluA1 (Fig. 7A), GluA2 (Fig. 7B) or the GluA1/GluA2 ratio (Fig. 7C) due to cocaine exposure, we determined the levels of these proteins in the mPFC of mice that accomplish the cocaine SA. We also compared the values of these animals with the values of the drug-naïve mice. Three-way ANOVA revealed a *sex* effect for GluA1 ($F_{1,32} = 30.803$, $p = 0.000$) and GluA2 ($F_{1,32} = 6.906$, $p = 0.013$). In both cases, this main effect showed that females had higher protein expression than males. We did not find significative differences for *rearing*, *treatment* or the *interactions* (see supplementary information, Table S9 and S10). For the GluA1/GluA2 ratio we did not find any significative differences (see supplementary information, Table S11).

4. Discussion

The present study shows that early-life stress induces a depressive-like behaviour in adult male mice whilst MSEW-exposed females seem to be resilient to this type of stress. Moreover, AMPA glutamate receptor in the mPFC was differently affected due to *rearing* or *sex*. Gria2 levels in the mPFC of MSEW male mice were different than the SN males while MSEW females did not suffer alterations, showing that MSEW affected both sexes differently. However, in the protein expression of GluA2, were not different between the SN males than to those exposed to early-life stress (MSEW). As measured in the cocaine SA, SN female mice showed increased cocaine-seeking motivation than the SN males. Moreover, males were significantly affected by the MSEW due to evidencing a higher cocaine-seeking motivation than SN males. Additionally, we observed that the MSEW-increased cocaine-seeking in males was specific for cocaine, because of the results for the response efficiency in the food SA. Results from western blot in drug-naïve animals showed that female mice expressed higher basal levels of GluA1 and GluA2 in the mPFC, which could explain why they are more vulnerable to cocaine-seeking but resilient to early-life stress. In sum, our results show a sex-dependent effect for the cocaine-seeking motivation.

In previous work, we have also reported that only males were negatively affected by the MSEW in the acquisition of cocaine SA (Castro-Zavala et al. 2020a), showing increased excitability in the nucleus accumbens (increased GluA1/GluA2 ratio), while MSEW-exposed females did not express changes. In the present study, we do confirm that females show resilience to this type of stress.

The mPFC is a region that modulates cognitive and executive functions, including inhibitory control (Narayanan and Laubach 2017). Recent studies show that maternal separation in mice induces depression-like behaviours as well as a reduction of serotonin and dopamine levels in the frontal cortex (Récamier-Carballo et al. 2017). Additionally, animal models of depression suggest a reduced glutamate level in the PFC of depressed mice (Belin et al. 2008), as well as reduced glutamate and glutamate/glutamine levels in depressed rats (Li et al. 2008). Clinical evidence reported increased Gria2 mRNA levels in PFC in patients with major depression of both sexes, and no changes in Gria1 expression (Kleinman et al. 2015). Moreover, it was reported reduced glutamate/glutamine and GABA levels in the PFC of depressed patients (Hasler et al. 2007). These previous studies are in accordance with the fact that the glutamatergic system, especially in the frontal cortex, plays a key role in the modulation of the depressive phenotype.

Our present results showed a negative correlation between Gria2 in the mPFC and despair-like behaviour, meaning that a decreased Gria2 correlates with increased immobility time in the TST. Our biochemical analysis also showed a significant reduction in the Gria2 mRNA levels of male mice exposed to MSEW, which evidences a significant mood

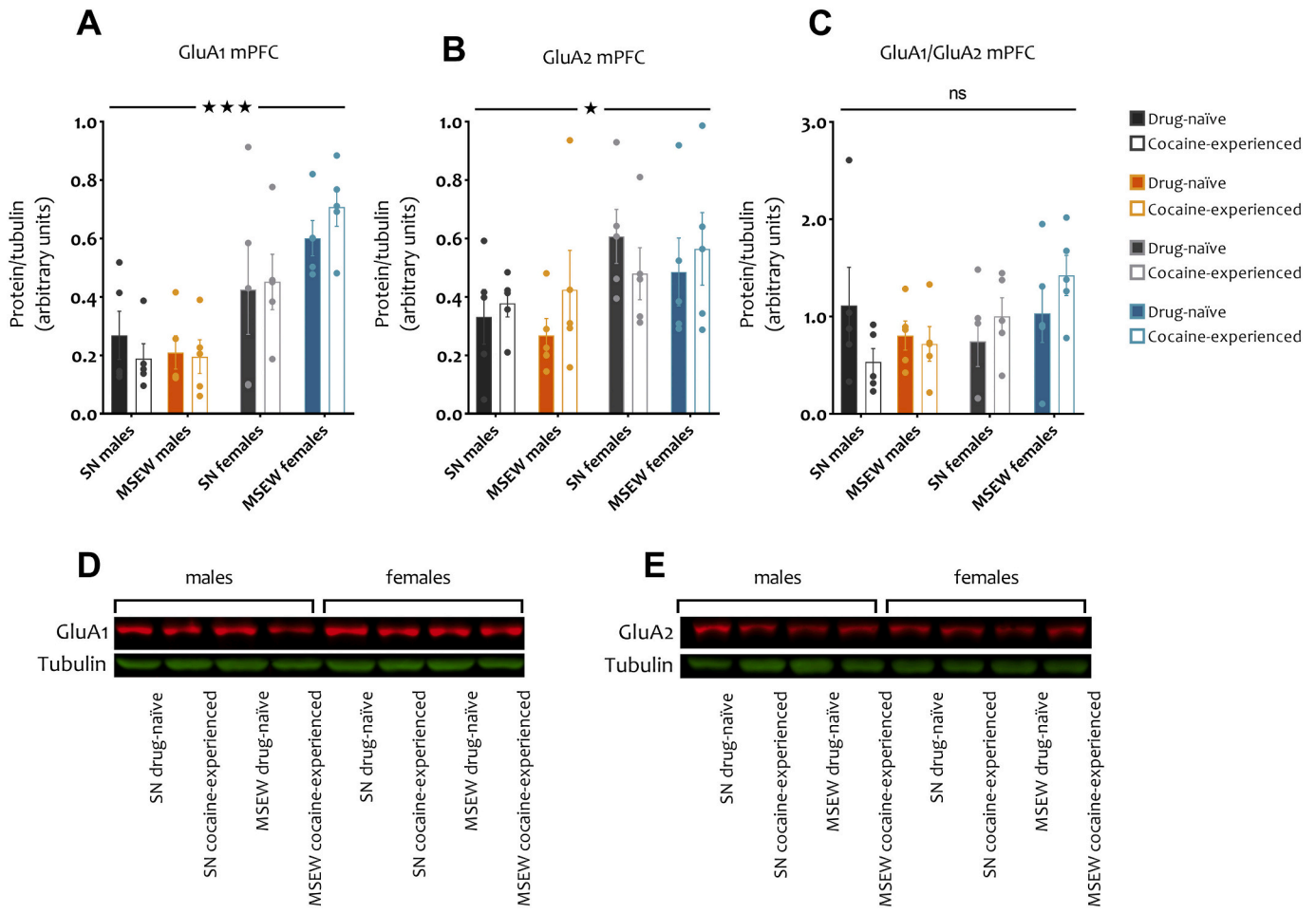

Fig. 7. Protein levels of GluA1, GluA2 and GluA1/GluA2 ratio in the mPFC of SN and MSEW mice after the self-administration procedure. Protein expression of (A) GluA1, (B) GluA2 and (C) GluA1/GluA2 levels in the mPFC. Representative western blot showing protein levels of (D) GluA1 and (E) GluA2 in the mPFC. The western blot images of the drug-naïve animals are the same of the ones presented in Fig. 4. The protein of interest in red and tubulin in green. *Sex* main effect of the ANOVA (★$p < 0.05$, ★★★$p < 0.001$). Data are expressed as mean ± SEM (n = 5 per group, run in duplicate). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

alteration. However, we did not observe significant differences in GluA1 or GluA2 protein level in MSEW exposed mice. Our results are in accordance with Ganguly et al. (2019) who reported that males maternally separated showed decreased Gria2 expression in the mPFC compared to control males. Likewise, they observed that females in general, showed higher GluA2 protein level than male mice, similar to our findings.

In accordance with our results, a study using a model of depression in rats evidenced a dysregulation in glutamate neurotransmission but without changes in the levels of GluA1 or GluA2 in the mPFC of depressed rats (Treccani et al. 2016). Additionally, other authors did not find alterations in Gria1 mRNA level in the mPFC of depressed mice (Belin et al. 2008), which is in agreement with our observations.

Epidemiologic studies show that childhood adversity increases the risk of depression by 28.4% but also to illicit drug use by 16.5% (Anda et al. 2006). Animal studies exploring how stressful situations influence addiction-like behaviours described that acute and chronic stress alters the phosphorylation of GluA2, affecting the function of the AMPA receptor but no the GluA1/GluA2 ratio in the nucleus accumbens and hippocampus (Caudal et al. 2016, 2010; Ellis et al. 2017). This evidence could explain why we were not able to find differences in the GluA1/GluA2 ratio. It is possible that males exposed to MSEW showed decreased-induced phosphorylation of GluA2, which does not modify the GluA2 levels, but alters indirectly the excitability of the AMPA receptor function.

Caffino et al. (2015) reported that a single cocaine exposure in adolescent rats can reduce the number of dendritic spines without any changes of GluA1 or GluA2 in the total mPFC homogenate. In addition, clinical studies indicate that cocaine-dependent patients showed a negative correlation between the activation of the frontoparietal network (network related with the inhibition control) and the dependence severity (Barrós-Loscertales et al. 2020). Moreover, Dhonnchadha et al. (2013) reported that GluA1 expression and the phosphorylation of this subunit, are associated to cellular processes related to cocaine learning and memories, evidencing the behavioural significance of GluA1 alteration in this brain area (Dhonnchadha et al., 2013).

In the present study, we observed that female mice, the group with higher cocaine-seeking motivation had higher GluA1 protein expression at basal level (Fig. 4A) and also after cocaine SA (Fig. 7A). This fact could explain why females showed higher motivation to seek cocaine. Following this idea, it could be expected that MSEW males exposed to cocaine, show an increase in the levels of GluA1, however, we did not find changes in this group of mice.

Other studies using the cocaine SA paradigm reported that disruption of the GluA2 phosphorylation potentiated the acquisition of cocaine SA (Ellis et al. 2017). Therefore, there is a possibility that the differences in the cocaine-seeking motivation during the cocaine SA between the SN males and the MSEW male mice can be explained by changes in the phosphorylation of GluA2. This hypothesis is in line with recent findings in which mutant mice lacking GRIP1 in the mPFC, a scaffolding protein

that stabilizes GluA2 at the surface, showed increased GluA2-containing AMPArs in the cell membrane, and increased cocaine intake during the SA paradigm (Wickens et al. 2019). Moreover, they observed that these effects were cocaine specific and GRIP1 does not influence natural reward-seeking, as we observed in the current work.

In the case of the MSEW females, our hypothesis is that the increased basal level of GluA1 could be compensating the hyperphosphorylation of GluA2, avoiding further increases on motivation to seek cocaine due to MSEW.

5. Conclusion

Taken together, our results propose that female mice exhibit increased expression of GluA1 and GluA2 in the mPFC, which could be explain the difference in the evolution during the cocaine SA paradigm. Additionally, we suggest that MSEW could be affecting several molecular mechanisms to avoid the ability to stabilize GluA2 in the synaptic surface.

In addition, the present study provides novel evidence regarding the molecular mechanisms altered in the glutamatergic system in the prefrontal cortex of mice exposed to early-life stress during the first postnatal days. These alterations could underlie the higher cocaine-seeking motivation due to modifications in the inhibition control.

Ethical statement

Mice were allowed to acclimatize to the new environmental conditions

for at least one week before starting the experiments. Every effort was made to minimize animal suffering and reduce the number of animals used. All procedures were conducted in accordance with national (BOE-2013-1337) and EU (Directive 2010-63EU) guidelines regulating animal research and were approved by the local ethics committee (CEEA-PRBB).

Author contributions

A.C-Z. and O.V. were responsible for the study concept and design. A. C-Z., A.M-S. and L.M-M. carried out the experimental studies. A.C-Z and O.V. drafted the manuscript and A.C-Z, O.V and A.C-M participated in the interpretation of findings. All authors critically reviewed the content and approved the final version for publication.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2021.110262.

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