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Prenatal, but not postnatal exposure to chlorpyrifos affects social behavior of mice and the excitatory-inhibitory balance in a sex-dependent manner

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ABSTRACT

The balance between excitatory and inhibitory neurotransmitters is essential for proper brain development. An imbalance between these two systems has been associated with neurodevelopmental disorders. On the other hand, literature also associates the massive use of pesticides with the increase of these disorders, with a particular focus on chlorpyrifos (CPF) a world-wide used organophosphate pesticide. This study was aimed at assessing social autistic-like behaviors on mice pre or postnatally exposed to CPF (0 or 1 mg/kg/day), in both sexes. In prenatal exposure, C57BL/6J pregnant mice were exposed to CPF through the diet, between gestational days (GD) 12 and 18, while a positive control group for some autistic behaviors was exposed to valproic acid (VPA) on GD 12 and 13. To assess postnatal exposure, C57BL/6J mice were orally exposed to the vehicle (corn oil) or CPF, from postnatal days (PND) 10–15. Social behavior and gene expression analysis were assessed on PND 45. Results showed social alterations only in males prenatally treated. GABA system was upregulated in CPF-treated females, whereas an increase in both systems was observed in both treated males. These findings suggest that males are more sensitive to prenatal CPF exposure, favoring the sex bias observed in ASD.

1. Introduction

Pesticides have been used extensively in developed countries for both agriculture and residential uses, with consequential effects on non-target organisms such as humans (Karalliedde et al., 2003; Suratman et al., 2015). Exposure to pesticides can occur through different routes: ingestion (e.g., food contamination), inhalation (e.g., environmental and household pollution) and dermal absorption (e.g., farmers) (Kim et al., 2017).

Organophosphate (OP) pesticides, and specially chlorpyrifos (CPF), are widely used in agriculture to protect crops from insect attacks (Jokanović, 2001). However, there is a great deal of evidence demonstrating adverse health effects on humans leading to regulations in the United State, where agricultural and food uses were banned in 2022 (EPA, 2021). In turn, in 2020, the European Union did not continue renewing the authorization to use CPF (EFSA, 2019). However, several countries still use this pesticide, which will lead to negative effects on human health for years to come.

Bioactivation of CPF is produced by its oxidation to CPF-oxon (active metabolite) in the liver by cytochrome P450. Subsequently, CPF-oxon is hydrolyzed to diethylphosphate and 3,5,6-trichloro-2-pyridinol (TCPy) (Chambers and Chambers, 1989; Sams et al., 2004). The toxic effects of OP are related to the irreversible inhibition of the enzyme cholinesterase (ChE), which causes an overstimulation of the cholinergic system (Casida and Quistad, 2004; Flaskos, 2012). However, other targets have also been identified (Casida, 2017).

There is a growing body of literature that points towards the relationship between CPF exposure and the increase in neurodevelopmental disorders such as autism spectrum disorder (ASD), which is characterized by difficulties with social interaction and communication, as well as

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a high prevalence of repetitive patterns of behavior or stereotypes (Baird, 2003). The increased prevalence in ASD observed in the last few years has triggered many authors to hypothesize a gene-environment interaction involved in autism etiology (Havdahl et al., 2021; Waye and Cheng, 2018). Although existing data are not always consistent regarding CPF exposure and its relation to autism symptomatology, a systematic review recently conducted by our group (Biosca-Brull et al., 2021) informed consistent positive associations between OP exposure and neurodevelopmental disorders, including autism-related traits, in clinical studies. Moreover, in preclinical studies, exposure to low or high doses of CPF, during gestation or the first developmental stages, produce social deficits in the adulthood in a sex-dependent manner (Venerosi et al., 2006, 2015), while effects in earlier stages such as adolescence, are more heterogeneous (Ricceri et al., 2003; Venerosi et al., 2008). Besides, studies that evaluated both prenatal and postnatal exposure also reported short- and long-term impairments in motor and novelty-related response (Laporte et al., 2018). Nonetheless, Lan et al. (2019) reported that autism is a sexually dimorphic disorder, with a male bias inasmuch as for every four males diagnosed, only one female is affected.

The Three-Chamber test described by Crawley (2004) has been largely used to study social behavior and social recognition in rodents (Silverman et al., 2010). Brain regions such as amygdala, anterior cingulate cortex, medial prefrontal cortex and hippocampus are critical for social memory formation and consolidation (Tanimizu et al., 2017). A considerable amount of literature has highlighted the CA2 hippocampal region as the principal area involved in social memory regulation (Garrido-Zinn et al., 2016; Hitti and Siegelbaum, 2014; Montagrin et al., 2018). Furthermore, it is suggested that an imbalance between excitatory and inhibitory neurotransmitters (glutamate and gamma-aminobutyric acid (GABA), respectively) is present in some neurodevelopmental disorders (Lopatina et al., 2018). GABA is synthesized from glutamate by glutamic acid decarboxylase (GAD). Then, vesicular GABA or glutamate transporters release their neurotransmitters to the synapses through exocytosis, leading GABA or glutamate to join their receptors (Rowley et al., 2012). Alterations in neurotransmitter balance, together with neurodevelopmental delays, were observed in the offspring of epileptic mothers treated with valproic acid (VPA) (Godhe-Puranik et al., 2013). Exposure to VPA during the first trimester, when organogenesis occurs, is associated with a high prevalence of ASD in offspring (Arndt et al., 2005). In mice and rats, organogenesis is developing between gestational day (GD) 8 and GD 15 (Ergaz et al., 2016). Prenatal VPA exposure (GD 11.5) showed deficits in GAD levels and a downregulation of the GABAergic system in rats (Win-Shwe et al., 2018), while glutamate expression levels were upregulated (Markram et al., 2008). On the other hand, post-mortem studies of autism showed a reduction in hippocampal $GABA_A$ receptor subunits and GAD (Bozzi et al., 2018). In the same line, developmental exposure to low and high doses of CPF was associated with an increase in the glutamate N-methyl-D-aspartate (NMDA) receptor, particularly, in the GluN2A and GluN2B receptor subunits) (Gómez-Giménez et al., 2018; Gultekin et al., 2007). In contrast, less is known about the effects of CPF in the GABA system. However, Perez-Fernandez et al. (2020a) found an upregulation of GABA-A α2 subunits in rats that were postnatally exposed to low doses of CPF, but no effects were observed in GAD1, GAD2 and GABA-A α1 subunit. Taking together all these results, it could be suggested that both CPF and VPA exposure impair GABAergic neurotransmission and enhance glutamatergic neurotransmission, leading to an altered excitatory/inhibitory (E/I) balance.

Some evidence points towards the contribution of CPF on neurodevelopmental disorders being the developmental period of exposure critical in producing different deficits. For this reason, the aims of this study were to evaluate the effects of prenatal and early postnatal exposure to CPF on social behavior, as well as to determine whether low pesticide exposure alters GABAergic and glutamatergic hippocampal signaling, while looking for a plausible association between CPF exposure and neurodevelopmental disorders.

2. Material and methods

2.1. Animals

Adult male and female C57BL/6J mice were obtained from Charles River Laboratories (Barcelona, Spain). One male and two females were mated for 3 h. When a vaginal plug was detected, this day was designated as GD 0. Animals were housed in plastic cages containing between three and five mice until GD 12, when pregnant females were housed individually. The delivery day was designated as postnatal day (PND) 0. Only litters with at least four live pups were used in this study. Pregnant females were randomly assigned to one of the three treatments (control (CNT), chlorpyrifos (CPF_1) or VPA) for the prenatal exposure experiment or to one of the two treatment groups (vehicle or CPF_2) for the postnatal exposure experiment. Animals were maintained in a 12-h light/dark automatic cycle (light ON between 8 a.m. and 8 p.m.) with controlled temperature (22 ± 2 °C) and humidity (50 ± 10%). Food and water were administered *ad libitum*. The present study was assigned an authorization code (number 10735) by the Government of Catalonia. It was conducted in compliance with Spanish Royal Decree 53/2013 on the protection of animals used in experiments and the European Communities Council Directive (86/609/EEC) and was approved by the Animal Care and Use Committee of the Universitat Rovira i Virgili (Catalonia, Spain).

2.2. Treatment and experimental design

Two experiments were designed in order to assess the effects of low doses of CPF at two different developmental periods: late prenatal and early postnatal (Fig. 1). For the prenatal exposure experiment, pregnant mice were exposed through the diet to a 0 or 1 mg/kg/day dose of CPF (0,0-diethyl O-(3,5,6-trichloropyridin-2-yl) phosphorothioate) 99.5% purity) (Sigma-Aldrich, Madrid, Spain) between GD 12 and GD 18. The positive control group was based on a model of autism-like behaviors taken from previous literature (Sakai et al., 2018). For the autism model mice, pregnant females were treated with a dose of 300 mg/kg/day of VPA (2-propypentanoic acid sodium, purity 98%) (Sigma-Aldrich, Madrid, Spain) administered by subcutaneous injection for two consecutive days (GD 12 and GD 13). For the postnatal exposure experiment, CPF was dissolved in corn oil (vehicle) and adjusted to administer an oral dose of 1 mg/kg in 1 μL/g of body weight, using a micropipette. Treatment was administered to pups from PND 10 to PND 15. The control group received the vehicle during the same period.

In both cases, on PND 28 (weaning day), mice were separated in order to assess social behavior. Animals were housed in groups of two to four animals of the same sex per cage until behavioral test day (PND 45). On PND 46, animals were euthanized by exsanguination under isoflurane anesthesia, where brains were removed and stored at −80 °C until analysis. The number of mice used for each analysis is given in Table 1.

2.3. Behavioral assessment: three-chamber test

Sociability and preference for social novelty were evaluated in adolescent males and females on PND 45, using a nonautomated three-chamber Plexiglas box based on the Three-Chamber test described by Crawley (2004). Briefly, the apparatus consisted of a rectangular box (60 × 30 × 30 cm) with three interconnected chambers (20 × 30 × 30 cm). The two middle walls of the box had doorways allowing the mice to move between chambers. There were also two empty wire cups (7 × 7 cm) at both ends of the box. Before starting the test, the mice were transported to the testing room. Then, the animals were introduced into the space and allowed to explore freely for 10 min. Next, we assessed sociability by placing an inanimate object (i.e., red plastic frog, 2.5 ×

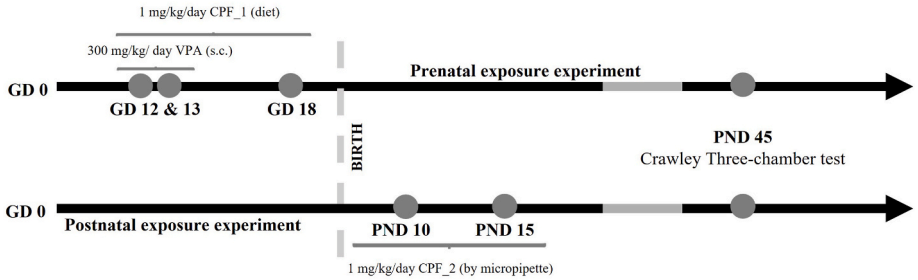

Fig. 1. Experimental design of both exposure periods. In the prenatal exposure experiment, pregnant females were exposed to CPF_1 (1 mg/kg/day) from GD 12 to GD 18, whereas in the positive control of autism, mice were exposed to VPA (300 mg/kg/day) on GD 12 and GD 13. In the postnatal exposure experiment, mice were exposed to the vehicle (corn oil) or CPF_2 (1 mg/kg/day) from PND 10 to PND 15. A social behavioral test was performed on PND 45.

Table 1
Number of animals used in the study.

	Prenatal exposure			Postnatal exposure	
	CNT	CPF	VPA	CNT	CPF
Social behavior					
Males	15	10	15	10	10
Females	15	13	13	10	10
Gene expression					
Males	5	6	6	–	–
Females	6	6	5	–	–
Protein expression					
Males	4	5	6	–	–
Females	5	5	6	–	–

CNT-Control; CPF-Chlorpyrifos; VPA-Valproic acid.

2.5 cm) in one of the two cups (non-social chamber) and an unfamiliar mouse (same sex and age) in the other wire cup (social chamber). Finally, we evaluated the preference for social novelty by replacing the inanimate object with a novel unfamiliar mouse of same sex and age (novel chamber), while the first mouse was kept inside the same wire cup as the familiar mouse (non-novel chamber). In both, the social and novel phases, mice were also allowed to freely explore the three compartments for 6 min. Upon test completion, ethanol 70% was used to clean and eliminate olfactory cues. The time that the subject mouse spent in each of the three compartments was recorded using a video camera (Sony CCD-IRIS), and then computerized using a video-tracking program (Etho-Vision©, Noldus Information Technologies, Wageningen, The Netherlands) to evaluate the innate preference for the novel stimulus (i.e., the preference to explore a new mouse over a familiar one or an inanimate object) (Crawley, 2007). We also analyzed two social variables, which were represented by sociability ratio ((time spent in the social chamber-time in the non-social chamber)/total time exploring), and novelty ratio ((time spent in the novel chamber-time in the non-novel chamber)/total time exploring). "Total time exploring" refers to the sum of time the mice spent in the two lateral compartments.

2.4. Gene expression analysis

Hippocampal RNA was extracted using the SPEEDTOOLS Total RNA Extraction Kit (Biotools, Madrid, Spain). RNA concentration and purity were measured with a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was synthetized from 0.21 μg of RNA samples using a Maxima First Strand cDNA Kit for real time polymerase chain reaction (RT-qPCR) (ThermoFisher Scientific, Waltham, MA, USA). Duplicates of each RNA sample were included in the RT-qPCR, which was performed with a Maxima SYBR Green/ROX qPCR Master Mix (2X) kit (ThermoFisher Scientifics, Waltham, MA, USA) and the Rotor-Gene Q Real-time Q cycler (Qiagen Inc., Hilden, Germany). A quantitative PCR analysis was performed to study the expression of glutamate- and GABA-related genes such as glutamate decarboxylase 1 and 2 (*Gad1* and *Gad2*), vesicular GABA transporter (*Slc32a1*), glutamate ionotropic receptor NMDA type, subunit 2A (*Grin2a*) and 2B (*Grin2b*), $GABA_A$ receptor subunit alpha 1 (*Gabra1*), alpha 2 (*Gabra2*), alpha 5 (*Gabra5*) and beta 3 (*Gabrb3*), solute carrier family 12-member 5 (*Slc12a5*) and 2 (*Slc12a2*), parvalbumin (*Pvalb*) and matrix metallopeptidase 9 (*Mmp9*). Primer sequences are described in detail in Table 2. To calculate the cycle threshold (Ct) we used Rotor-Gene Q Real-Time PCR 2.0 software (Qiagen Inc., Hilden, Germany). Each sample was normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ΔCt) and then standardized to the average of each male control group (ΔΔCt) to assess the relative gene expression levels in accordance with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.5. Western blot analysis

PVALB protein levels were analyzed by western blotting. Hippocampal samples containing 25 μg of protein/sample were mixed with RIPA buffer (Merck, Darmstadt, Germany). Samples were shaken for 40 min and then centrifuged for 15 min (4 °C, 20.000 g). The proteins of the supernatant were separated by electrophoresis on 15% acrylamide gels. Then, proteins were transferred to Immobilon-P PVDF sheets (Millipore Corp., Bedford, MA, USA) using a transblot apparatus (BioRad, Madrid, Spain). Membranes were blocked for 1 h with 5% non-fat milk dissolved in TBS-T buffer (50 mM Tris, 1.5% NaCl, 0.05% Tween 20 at pH 7.5). Primary monoclonal antibody (Cell Signaling, Danvers, MA, USA) against PVALB (molecular weight [MW]: 12 kDa) and GAPDH ([MW]: 37 KDa) was incubated overnight. Blots were washed thoroughly in TBS-T buffer and then incubated with peroxidase-conjugated IgG antibody for 1 h. An ImmunStar Chemiluminescence Kit (BioRad, Madrid, Spain) was used to visualize immunoreactive proteins. Digital images were obtained using the VersaDoc system (BioRad, Madrid, Spain) to perform semi-quantification of the band intensity (Image Lab, Bio-Rad, Madrid, Spain). The protein load was periodically monitored via the immune detection of GAPDH.

2.6. Statistical analysis

The data were analyzed using SPSS 27.0 software (IBM Corp. Chicago, IL, USA). General univariate and multivariate (body weight, sociability/novelty ratio and gene/protein expression) analyses of variance (ANOVA) were performed to screen general differences of sex, treatment or sex x treatment interaction. A one-way ANOVA followed by the *post-hoc* DMC test was used to analyze the differences between treatments or sex x treatment interactions when it was appropriate. Both social behaviors were evaluated with a paired samples *t*-test in order to find differences in the time spent in social/novel versus non-social/non-novel chambers. The one sample *t*-test was performed to analyze the sociability and novelty ratio. A principal component analysis (PCA) of ΔCt was carried out in gene expression as a general screening. Levene test was performed to assess the homogeneity of variance. Kruskal-

Table 2
Sequences of primers used for the RT-qPCR analysis.

Mus musculus gene	Article Symbology	Forward primer	Reverse primers	Source
Gad1	GAD1	ATGATACTTGGTGTGGCGTAG	GACTCTTCTCTTCCAGGCTATTG	Lee et al. (2017)
Gad2	GAD2	CTCCGGCTTTTGGTCCTTCG	ATGCCGCCCGTGAACTTTTG	Lee et al. (2017)
Slc32a1	VGAT	TCATCGAGCTGGTGATGACG	CTTGGACACGGCCTTGAGAT	Oka et al. (2015)
Grin2a	GluN2A	CCATCAGCAGAGGTATCTAC	CAGTCTGAATGCGTGAAGCT	Chen et al. (2016)
Grin2b	GluN2B	TCCAGGAGTAATGGCACTGTTTC	CGAACATCATCACCCAGACAG	Tsang et al. (2015)
Gabra1	GABA-A α1	CACCATGAGGTTGACCGTGA	CTACAACCACTGAACGGGCT	Mitchell et al. (2018)
Gabra2	GABA-A α2	TTACAGTCCAAGCCGAATGTCCC	ACTTCTGAGGTTGTGTAAGCGTAGC	Tan et al. (2011)
Gabra5	GABA-A α5	CCCTCCTTGTCTTCTGTATTTCC	TGATGTTGTCATTGGTCTCGTCT	Tan et al. (2011)
Gabrb3	GABA-A β3	GAGGTCTTCACAAGCTCAAAATC	AGGCAGGGTAATATTTCACTCAG	Provenzano et al. (2020)
Slc12a5	KCC2	CTCAACAACCTGACGGACTG	GCACAACACCATTGGTT GCG	Aguado et al. (2003)
Slc12a2	NKCC1	AACCGCTTCGTGGTTACATC	TTGCAAGTGATGCATGGAAT	Liu et al. (2014)
Pvalb	PVALB	TGTCGATGACAGACGTGCTC	TTCTTCAACCCCAATCTTGC	Huo et al. (2018)
Mmp9	MMP-9	ACCAAGGGTACAGCCTGTTCCT	GGTAGCTATACAGCGGGTACATGA	Kizaki et al. (2006)

Wallis or Mann-Whitney *U* test were performed when variances were not homogeneous. All data are presented as the mean values ± S.E.M. Statistical significance was set at a threshold of $p < 0.05$.

3. Results

3.1. Body weight

Body weight was measured in all animals during developmental period, from PND 2 to PND 28. Differences of treatment were observed in both exposure periods (prenatal exposure [$F_{7,70} = 2.602$, $p \leq 0.002$] and postnatal exposure [$F_{9,16} = 3.061$, $p = 0.025$]), being in the prenatal exposure the CNT group the ones that had higher body weight, whereas in postnatal exposure higher weight was observed in the CPF-treated group (data not shown). During development treated groups reach the value of controls and by PND 45, both pre or postnatal exposure had no effect on body weight. We observed that sex had a general effect on both periods of exposure (prenatal exposure [$F_{1,66} = 73.991$, $p < 0.001$] and postnatal exposure [$F_{1,30} = 90.799$, $p < 0.001$]), indicating that females were leaner than males, as expected (data not shown).

3.2. Social behavior: sociability and preference for social novelty in the three-chamber test

Sociability and the preference for social novelty are shown as follows: **i.** The time that the animal spent in each compartment (social or novel vs non-social or non-novel) and **ii.** Sociability and novelty ratio, which was compared to the fixed value basal equal exploration (i.e., 0), indicating a social or novel preference when this ratio is positive and a non-social or non-novel preference when this ratio is negative (Figs. 2 and 3).

3.2.1. Prenatal exposure to CPF and VPA disrupts the novelty preference

Male mice's social memory was affected, particularly in that's

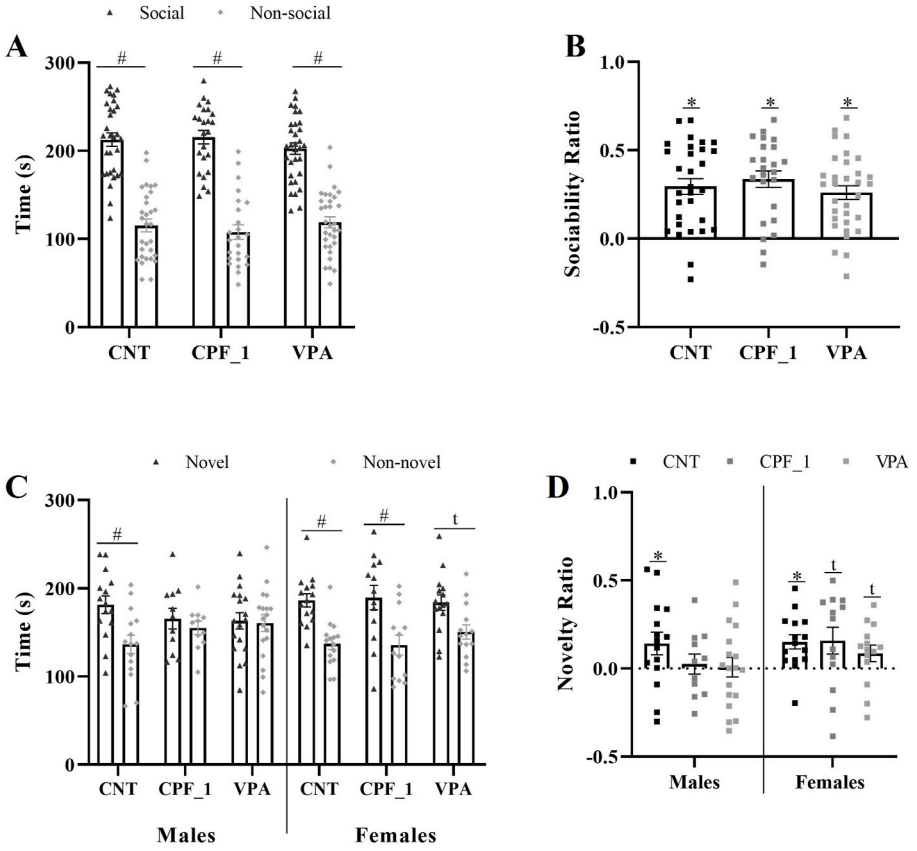

Fig. 2. Social behavioral assessment in prenatal exposure experiment. The time that the animal spends in the social or novel chamber versus the non-social or non-novel chamber (**A and C**). Sociability or novelty ratio calculated as (time spent in social or novel chamber-time spent in non-social or non-novel chamber)/total time exploring (**B and D**). The symbol # indicates the differences between the social or novel and non-social or non-novel chamber at $p < 0.05$. Differences with the chance level (i.e., 0) are represented by an asterisk, while trends are indicated with a t.

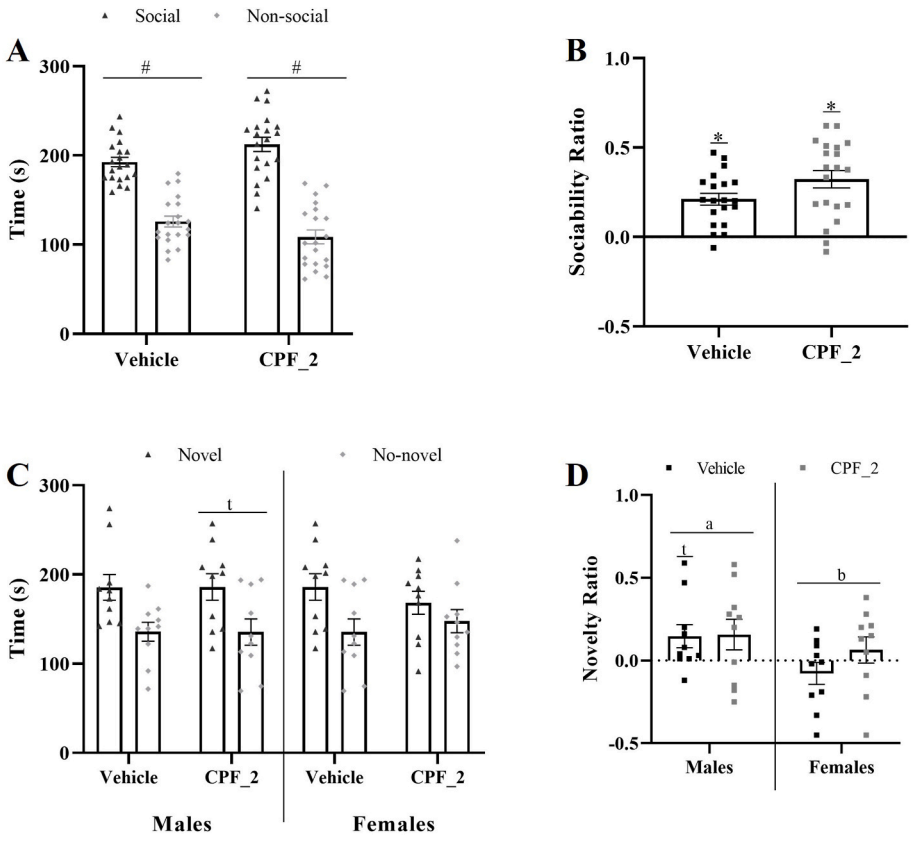

Fig. 3. Social behavioral assessment in postnatal exposure experiment. The time that the animal spends in the social or novel chamber versus the non-social or non-novel chamber (**A and C**). Sociability or novelty ratio calculated as (time spent in social or novel chamber-time spent in non-social or non-novel chamber)/total time exploring (**B and D**). The symbol # indicates the differences between the social or novel and non-social or non-novel chamber, while different letters indicate differences between sexes at $p < 0.05$. Differences with the chance level (i.e., 0) are represented by an asterisk, while trends are indicated with a t.

treated with CPF or VPA. No adverse effects were observed during the sociability preference phase, with all groups displaying a significant preference for the social stimulus. A paired sample *t*-test analysis showed a significant preference for the social stimulus versus the inanimate object (CNT: [$t_{29} = 6.642$, $p < 0.001$], CPF_1: [$t_{22} = 6.722$, $p < 0.001$] and VPA: [$t_{32} = 7.024$, $p < 0.001$]) (Fig. 2A). Similar results were observed when we evaluated the sociability ratio by conducting one sample *t*-test analysis, which also showed significant differences compared to the basal exploration in all groups (CNT: [$t_{29} = 6.662$, $p < 0.001$], CPF_1: [$t_{22} = 6.784$, $p < 0.001$] and VPA: [$t_{32} = 7.016$, $p < 0.001$]), indicating a general preference for the social stimulus (Fig. 2B).

In contrast, adverse effects on the preference for the novel stimulus were observed in the novelty phase in some treated groups. Although we did not observe general significant differences related to sex [$F_{1,65} = 1.939$, $p = 0.151$], a paired sample *t*-test analysis showed that males had a significant preference for the unfamiliar mouse, but only in the CNT groups [$t_{14} = 2.230$, $p = 0.043$], while females showed a significant preference for the novel stimulus in both the CNT [$t_{14} = 3.599$, $p = 0.003$] and CPF-treated group [$t_{12} = 2.210$, $p = 0.047$]. A tendency was observed in VPA-treated females [$t_{14} = 2.006$, $p = 0.058$] (Fig. 2C). Regarding the novelty ratio, no differences related to sex were observed [$F_{1,65} = 2.874$, $p = 0.094$], even though the one sample *t*-test analysis indicated that males in the CNT group [$t_{14} = 2.200$, $p = 0.045$] showed a novel preference, whereas CPF_1 [$t_9 = 0.157$, $p = 0.879$] and VPA-treated males [$t_{17} = 0.129$, $p = 0.899$] did not show a preference for the novel stimulus. Moreover, CNT females [$t_{14} = 3.710$, $p = 0.002$] also showed a preference for the unfamiliar mice, while in CPF_1 [$t_{12} = 2.083$, $p = 0.059$] and VPA-treated females [$t_{14} = 2.096$, $p = 0.055$], the level of statistical significance was not reached (Fig. 2D).

3.2.2. Postnatal exposure to CPF did not affect social behavior

Similar to prenatal exposure, all groups showed a preference for the social stimulus, but no significant effects were observed in the social novelty preference phase. A paired sample *t*-test analysis showed significant preference for the social chamber in both vehicle [$t_{19} = 6.294$, $p < 0.001$] and CPF-treated groups [$t_{19} = 6.631$, $p < 0.001$] when the time that the animals spent in the social versus non-social chamber was compared (Fig. 3A). Moreover, the analysis of sociability ratio by one sample *t*-test also showed significant preference for the social compartment in both the vehicle [$t_{19} = 6.442$, $p < 0.001$] and CPF-treated groups [$t_{19} = 6.640$, $p < 0.001$]) (Fig. 3B), confirming that the treatment had no effect on social preference.

Regarding the novelty phase, a non-significant effect of sex was observed [$F_{2,35} = 3.139$, $p = 0.056$]. When we analyzed the time that the animal spent in the novel versus the non-novel compartment using a paired sample *t*-test, we saw a tendency for the unfamiliar mouse in CPF-treated males [$t_9 = 1.711$, $p = 0.061$], whereas others groups did not show any preference (vehicle-treated males [t7 = 1.458, $p = 0.094$], vehicle-treated females [$t_8 = -0.662$, $p = 0.263$] and CPF-treated females [$t_9 = 0.811$, $p = 0.219$]) (Fig. 3C). In the same regard, when we analyzed the novelty ratio, we observed a significant effect of sex [$F_{1,39} = 4.185$, $p = 0.048$] indicating that male mice had a greater preference for the novel stimulus in comparison to female. A one sample *t*-test showed a tendency in vehicle-treated males [$t_9 = 2.106$, $p = 0.064$], while other groups did not present any preference for the novel stimulus (vehicle-treated females [$t_9 = -1.179$, $p = 0.268$], CPF-treated males [$t_9 = 1.706$, $p = 0.122$] and CPF-treated females [$t_9 = 0.823$, $p = 0.432$]) (Fig. 3D).

3.3. Hippocampal gene expression

Prenatal, but not postnatal exposure to CPF, disrupted social behavior, especially in treated male mice. Therefore, we assessed hippocampal gene expression only in the prenatal exposure experiment.

First, we performed a screening of all evaluated genes with a PCA (Fig. 4). The results showed three main components. Principal

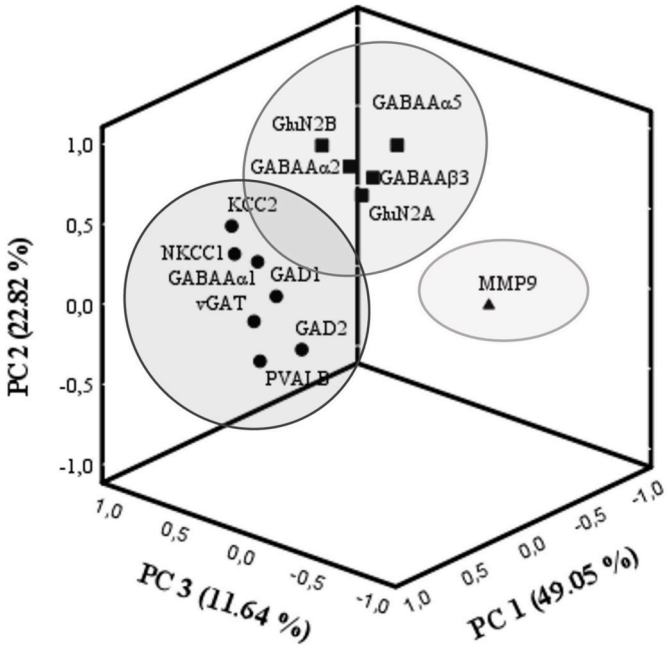

Fig. 4. Principal component analysis (PCA) of hippocampal GABA- and glutamate-related genes in adolescent mice prenatally exposed to CPF and VPA.

component (PC) 1 accounted for about 49.05% of the variance and included genes related to the GABAergic system such as GAD1 (r = 0.866), GAD2 (r = 0.655), VGAT (r = 0.804), NKCC1 (r = 0.792), KCC2 (r = 0.811), GABA-A α1 (r = 0.905) and PVALB (r = 0.565). PC 2 accounted for about 22.82% of the variance, where GABA-A α2 (r = 0.536), GABA-A α5 (r = 0.788), GABA-A β3 (r = 0.552), GluN2A (r = 0.536) and GluN2B (r = 0.560) were strongly correlated. Finally, PC 3 accounted for about 11.64% of the variance, with MMP9 (r = −0.882) being the only gene in this component. It is important to highlight that gene clustering in the same PC shares a similar gene expression pattern.

3.3.1. Genes clustered in PC 1

Prenatal exposure to CPF modulates RNA expression in treated females (Fig. 5). All GABAergic-related genes clustered in PC 1 (GAD1, GAD2, VGAT, NKCC1, KCC2, GABA-A α1 and PVALB) are part of the GABA components in synapses, which leads to a phasic response. A Kruskal-Wallis test indicated that CPF-treated females showed an increase in GAD1 expression in comparison to CNT males (p = 0.034), CPF-treated males (p = 0.037) and VPA-treated females (p = 0.011), whereas a non-clear increase was observed with respect to CNT females (p = 0.072) (Fig. 5A). On the other hand, a multivariate analysis of all the genes clustered in PC 1 only showed an interaction between sex and treatment [$F_{2,33}$ = 3.832, p = 0.034] in GABA-A α1 subunit. *Post-hoc* analysis indicated a non-significant increase in females, between CNT and CPF (p = 0.092), as well as a tendency between both treated groups (p = 0.056) (Fig. 5F).

3.3.2. Genes clustered in PC 2

GABAergic- (GABA-A α2, GABA-A α5 and GABA-A β3 subunits) and glutamatergic- (GluN2A and GluN2B) related genes were clustered in PC 2 (Fig. 6). The composition of ionotropic GABA receptors establishes their distribution and regulation. In PC 2, we found an extrasynaptic subunit (i.e., GABA-A α5) that leads to tonic activation; a postsynaptic and presynaptic subunit (GABA-A α2) that, respectively, leads to phasic activation or acts as controllers of GABA release (autoreceptors), and a GABA-A β3 subunit, which is located in postsynapse and extrasynapse. Moreover, the ionotropic glutamatergic (NMDA) receptor subunits, which were mainly postsynaptic receptors, were also clustered in PC 2.

We observed a general increase in treated males of all genes clustered in PC 2 (Fig. 6). Regarding $GABA_A$ subunits, a multivariate analysis of all genes clustered in this component showed a general trend of treatment in the GABA-A α2 [$F_{2,33}$ = 3.316, p = 0.051] and GABA-A β3 subunits [$F_{2,33}$ = 3.170, p = 0.057]. Moreover, we observed an interaction between sex and treatment in the GABA-A β3 subunit [$F_{2,33}$ = 3.877, p = 0.033], whereas GABA-A α2 showed a tendency [$F_{2,33}$ = 3.237, p =

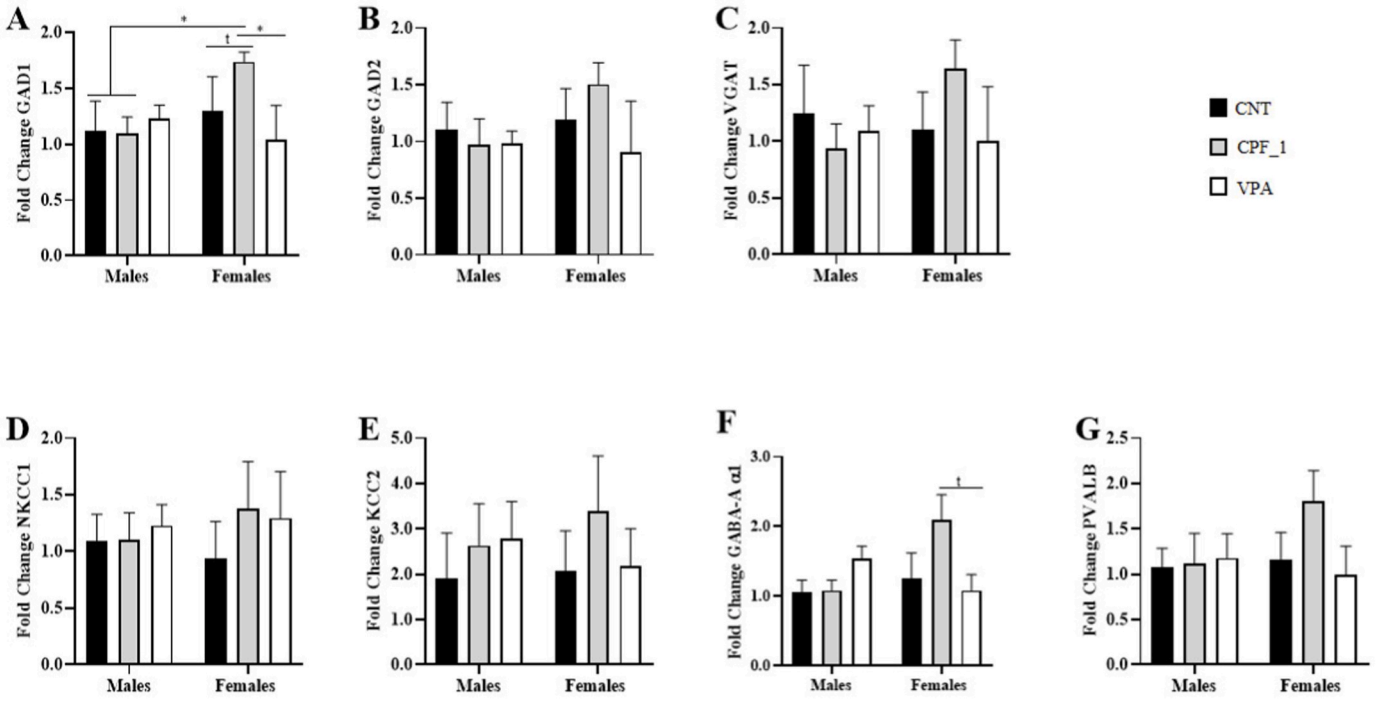

Fig. 5. Hippocampal relative gene expression related to PC 1 of the PCA. GAD1**(A)**. GAD2 **(B)**. VGAT **(C)**. NKCC1 **(D)**. KCC2 **(E)**. GABA-A α1 **(F)** and PVALB **(G)**. An asterisk indicates differences between treatments at $p < 0.05$, while tendencies are indicated with a t.

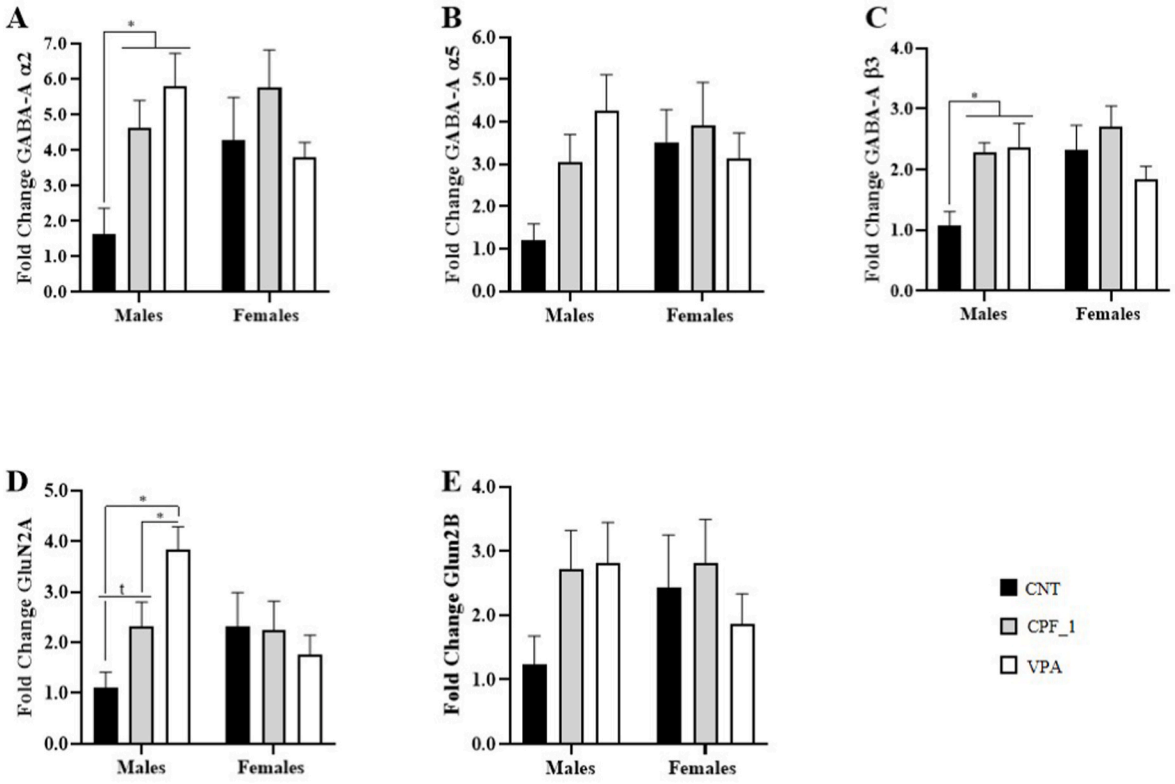

Fig. 6. Hippocampal relative gene expression related to PC 2 of the PCA. GABA-A α2 **(A)**. GABA-A α5 **(B)**. GABA-A β3 **(C)**. GluN2A **(D)**. GluN2B **(E)**. An asterisk indicates differences between treatments at $p < 0.05$, while tendencies are indicated with a t.

0.054]. In both GABA subunits, *post-hoc* analysis showed significant differences in males, between CNT and both treated groups (GABA-A α2 subunit: CPF_1 ($p = 0.024$) and VPA ($p = 0.004$) and GABA-A β3 subunit: CPF_1 ($p = 0.011$) and VPA ($p = 0.007$)) (Fig. 6A and C).

However, in terms of glutamatergic expression, we only observed an interaction between sex and treatment [$F_{2,33} = 4.962$, $p = 0.014$] in the GluN2A subunit (Fig. 6D). *Post-hoc* analysis showed significant differences in males between VPA and CNT ($p = 0.001$), VPA and CPF_1 ($p = 0.023$), as well as a tendency between CNT and CPF_1 ($p = 0.079$) (Fig. 6D).

3.3.3. Genes clustered in PC 3

The MMP9, a protease that mediates extracellular matrix degradation, was the only gene that clustered in PC 3. Although, results showed an upregulation of this gene, we did not observe significant differences due to their high variability (data not shown).

3.4. Hippocampal PVALB protein expression

Previous gene expression analysis showed an increase in GABAergic interneurons and synapse elements, but only in CPF-treated females. Intending to find a possible correlation between GABA upregulation and GABA neurons, we evaluated PVALB protein levels (Fig. 7). However, we did not find significant differences.

4. Discussion

In the present investigation, we assessed the effects that low doses of CPF can have on social behavior when administered over different lifetime periods (prenatal or postnatal), as well as the effects of CPF on GABAergic and glutamatergic synapses by studying the gene expression, looking for an association between prenatal CPF exposure and neurodevelopmental disorders. For this reason, we also included mice prenatally exposed to VPA, as a mice autism model.

Adolescent mice did not show deficits in sociability in either experiment (prenatal or postnatal), but differences were observed when we evaluated the preference for the novelty phase. Males treated during prenatal period with CPF or VPA did not show a preference for the novel stimulus. Similarly, Lan et al. (2019) exposed mice between GD 12 and GD 15 to subtoxic doses of CPF ranging from 2 to 5 mg/kg/day. Then, they assessed their social behavior by conducting a Three-Chamber test in adult mice (PND 90), where only the males showed a reduction in the

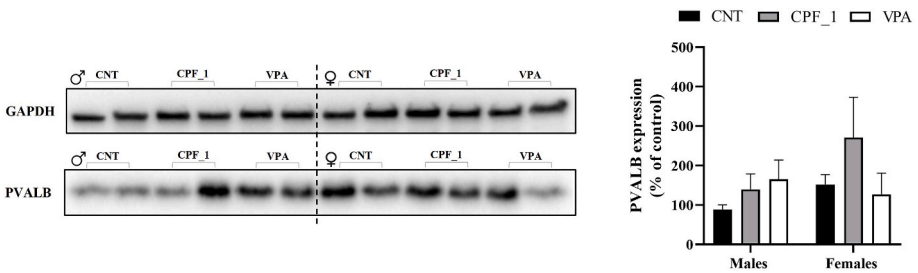

Fig. 7. Hippocampal relative PVALB protein expression.

innate preference for a conspecific (Lan et al., 2019). This suggests that prenatal exposure to CPF can disclose social behavior in the early stages of development which may persist into adulthood. Nevertheless, it should be borne in mind that our experiment was conducted with low doses of CPF according to the European Food Safety Authority (EFSA), which has established an acceptable daily intake of 0.001 mg/kg/day, whereas Lan et al. (2019) exposed mice to higher doses that cause adverse effects that perpetuate over time. On the other hand, a study conducted by Perez-Fernandez et al. (2020a) assessed postnatal exposure to CPF in rats by using the same protocol as our postnatal experiment and by evaluating social behavior at two different ages (adolescence and adulthood). In accordance with our results, Perez-Fernandez et al. (2020a) did not find social impairment on PND 35 (adolescence), but did observe differences during adulthood (around 5 months of age) where treated male rats reduced their novelty exploration. However, it must be noted that the difference between the two species in postnatal experiments could generate discrepancies in the results. These findings indicate that the CPF exposure period could condition adverse effects, suggesting that prenatal exposure could affect social behavior from an early stage, whereas postnatal exposure could disclose long-term social deficits. Anyway, further studies are needed to confirm this hypothesis. Nevertheless, it is clear that CPF exposure affects rodents' social behavior in a sex-dependent manner, with males being the most affected sex, in accordance with the incidence of autism in humans.

Behavioral outcomes observed in CPF- and VPA-treated males are related to nervous system development. Prenatal exposure takes place from GD 12 to GD 18. This timeframe refers to humans' second and third trimester of pregnancy (Azad et al., 2017) and encompasses the onset of neurogenesis, which starts at embryonic day 11 and ends before adolescence (around PND 20). Each brain region has a stage of neuron production. For example, in the cerebral cortex, neurogenesis occurs from GD 12 to GD 17, while in the hippocampus, neuronal production occurs in two stages; between GD 12 and GD 17 (neurogenesis of the CA2 region and pyramidal cells) and from GD 17 to PND 15 (neurogenesis of dentate gyrus) (Chen et al., 2017). Furthermore, this prenatal exposure encompasses other key brain developmental processes such as synaptogenesis or neuronal migration, which begin around GD 11 (Chen et al., 2017). Postnatal exposure starts on PND 10 and ends on PND 15, the equivalent to human brain development between the third trimester and the first month of age (Richard and Flamant, 2018). In mice, this timeframe covers the late stages of neuronal migration and synaptogenesis, in addition to developmental brain processes such as gliogenesis or myelination (Schepanski et al., 2018).

A great number of cases of developmental disorders, including autism, lack etiology or genetic basis. Recent biological evidence suggests that the autistic population presents alterations in the E/I balance, and more specifically, their glutamatergic system is upregulated, whereas their GABAergic system is downregulated (Uzunova et al., 2016). To find a plausible association between pesticides and autism, we evaluated GABA- and glutamate-related genes only during the prenatal exposure experiment because this is where we found evidence for deficits in social behavior.

The GABA neurotransmitter has different important functions during development. It is involved in neuronal migration, neuronal differentiation and synapse formation (Ben-Ari, 2002). To the best of our knowledge, this is the first time that a study has covered the hippocampal expression of a large variety of GABAergic genes in relation to CPF exposure and the evaluation of behavior related to autism. Although general upregulation of GABAergic synaptic genes was observed in CPF-treated females, only the GAD1 and GABA-A α1 subunit showed significant differences. GAD1 and GAD2 are the major rate-limiting enzymes that regulate GABA synthesis from a pool of L-glutamate (Tao et al., 2018). In contrast to our results, postmortem studies carried out on humans with autism showed a downregulation of both genes in the frontal cortex (Zhubi et al., 2017) and cerebellum (Fatemi et al., 2002), indicating a deficiency in GABA availability. GAD1 is located in interneurons and cytoplasm and is present during development in order to maintain metabolic activity, whereas GAD2 could be more involved in the vesicular synthesis of GABA and it seems to be present when synaptic inhibition is frequent (mature nervous system) (Fatemi et al., 2002). Thus, the overexpression of the inhibitory system observed in the current study suggests that prenatal CPF exposure increases the production of GABA neurotransmitter in females. In addition, prior to neuron maturation, GABA is excitatory and exerts a depolarizing action related to a high expression of neuronal co-transporter NKCC1. In adult neurons, GABA switches from excitatory to inhibitory action and, consequently, GABAergic neurons exert a hyperpolarizing action producing an upregulation of the co-transporter KCC2 and a downregulation of NKCC1 (Lemonnier et al., 2017). This switch from excitatory to inhibitory GABA transmission during the second week following birth coincides with the increase in cholinergic activity, which builds to adult levels during the third week following birth (Abreu-Villaça et al., 2011; Leonzino et al., 2016). Considering that ChE is the main target for CPF, the current study suggests that CPF exposure may interfere with GABA switching on the central nervous system. GABA's E/I effects are mediated by GABA-A receptors, whose expression varies over the course of development (Kang and Barnes, 2013). In our study, GABA-A α1-containing receptors were upregulated in CPF-treated females. These receptors are predominant in synapsis, giving rise to a phasic response which is fundamental for information transfer in the brain (Farrant and Nusser, 2005). The GABA-A α1 subunit was decreased in the parietal cortex (Fatemi et al., 2009) and cerebellum (Yip et al., 2007) of the autistic population, but little is known about its expression in the hippocampus. Therefore, the results obtained from synaptic GABAergic gene expression, suggest that CPF acts in a sex-dependent manner. CPF exposure increases GAD1 expression in females, suggesting an overproduction of the GABA neurotransmitter which is related to the increase observed in the GABA-A α1 subunit. Therefore, this indicates an increment in inhibitory neuronal activity, maybe because of an increase in GABAergic neurons.

Another gene expression pattern was observed in CPF- and VPA-treated males. Glutamatergic receptor subunits (GluN2A and GluN2B) and GABAergic receptor subunits (GABA-A α2, GABA-A α5 and GABA-A β3) were significantly increased compared to control males. Studies of GABA-A receptor subunits in rats brains showed that α2, α3, α5 and β3 predominate during embryonic development, while α1 and β2 increase after birth (Laurie et al., 1992). Similarly, during postnatal development, the GABA-A receptors (α1, α4, β1 and β2 subunits) increase in the mice's hippocampus (Kim et al., 2015). The GABA-A α2 subunit is present in axon terminals of the hippocampus (pre-synapsis), acting as an autoreceptor that regulates the release of GABA in the synapse (Ruiz and Kullmann, 2013), even though some postsynaptic α2 subunits were also found (Farrant and Nusser, 2005). In addition, the GABA-A α5 subunit is predominantly or exclusively extra-synaptic, giving rise to a tonic response which is evident in certain embryonic regions before synaptic formation (Farrant and Nusser, 2005), while the GABA-A β3 subunit could be postsynaptic and extra-synaptic (Herd et al., 2008). The GABA-A α2 subunit has been associated with disorders of altered neuronal excitability such as epilepsy (Butler et al., 2018), with which ASD shares neurological mechanisms. In relation to this, Perez-Fernandez et al. (2020b) found a significant upregulation of the α2 subunit in the frontal cortex of rats exposed postnatally to CPF. In the same lines, GABA-A α5 and GABA-A β3 subunits are clustered in chromosomes 7 and 15 (mouse and humans, respectively) (Kang and Barnes, 2013). Defects in that part of the genome are associated with Prader-Willi and Angelman syndrome, two disorders that share symptoms with autism, making both subunits important candidates for neurodevelopmental disorders. Therefore, as we explained above, the upregulation in the treated males of the three GABA subunits (α2, α5 and β3) would suggest that CPF and VPA affect the GABA system similarly during early developmental stages. Indeed, prenatal CPF exposure

affects GABAergic neurotransmission in both sexes, but effects do not point in the same direction.

Glutamate ionotropic NMDA receptors, GluN2A and GluN2B subunits, have a similar molecular structure and function (Myers et al., 2019). These subunits appear at different stages during development. GluN2B is expressed early, whereas the GluN2A subunit is expressed postnatally (Myers et al., 2019). These subunits have been studied concerning social behavior. In this sense, Jacobs et al. (2015) found that mice's forebrain GluN2A overexpression impairs social memory, while overexpression of the GluN2B subunit enhances social memory, suggesting that GluN2B is crucial for social memory formation and consolidation. In this regard, we observed a significant upregulation in the GluN2A subunit (CPF and VPA treated male mice), which could be related to deficits in the preference for novelty that we observed in our experiment. Nevertheless, we did not find any differences in the GluN2B subunit. The increase in GluN2A may suggest an imbalance between these two subunits, leading to an E/I disequilibrium, which could be a consequence of a delay or incorrect switch during development.

Regarding the GABA system, we also evaluated the expression of PVALB to test a possible increase in GABAergic interneurons which had been involved in the consolidation of short- and long-term memory formation (Nahar et al., 2021). In fact, it has been suggested that PVALB coordinates neuronal communication after novel experiences (Ognjanovski et al., 2017). Furthermore, the activity of excitatory neurons is modulated by these inhibitory interneurons, which control postsynaptic activation (Filice et al., 2020). Studies done in postmortem brains of humans with autism observed a decrease in PVALB mRNA levels in cerebellar Purkinje cells (Soghomonian et al., 2017) and the cerebral cortex (Schwede et al., 2018). In the same way, mouse models expressing an autism phenotype, showed a decrease in PVALB cells in the hippocampus, striatum and cortex, which is related to PVALB downregulation (Peñagarikano et al., 2015). Moreover, Lauber et al. (2018) found a decrease in PVALB protein expression, only in the striatum. Despite this, our gene expression analysis did not show significant differences in both PVALB expression and protein levels. However, a non-significant increase in CPF-treated females was observed, suggesting that CPF enhances the inhibitory system in females, whereas, in male's treatment seems to not affect PVALB gene and protein expression. These findings indicate that CPF's toxic effects involve different pathways in males and females.

In conclusion, the results of the current study show that the period in which exposure to CPF takes place plays a key role in determining the adverse effects produced in the short, medium or long term. Our results also suggest that CPF exposure during pregnancy could lead to social behavioral deficits in a sex-dependent manner. The same-sex bias was also observed in gene expression, suggesting that females treated with CPF presented an upregulation in the GABAergic system, whereas treated males showed a more immature pattern in both the GABA and glutamate systems, which could be compatible with an increase in neuronal excitability.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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