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Exposure to chlorpyrifos during pregnancy differentially affects social behavior and GABA signaling elements in an *APOE*- and sex-dependent manner in a transgenic mouse model

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ABSTRACT

The massive use of chlorpyrifos (CPF) has been associated with an increased prevalence of neurodevelopmental disorders. Some previous studies have shown that prenatal, but not postnatal, CPF exposure causes social behavior deficits in mice depending on sex while others have found that in transgenic mice models carrying the human apolipoprotein E (*APOE*) ε3 and ε4 allele confer different vulnerabilities to either behavioral or metabolic disorders after CPF exposure. This study aims to evaluate, in both sexes, how prenatal CPF exposure and *APOE* genotype impact on social behavior and its relation to changes in GABAergic and glutamatergic systems. For this purpose, apoE3 and apoE4 transgenic mice were exposed through the diet to 0 or 1 mg/kg/day of CPF, between gestational day 12 and 18. A three-chamber test was used to assess social behavior on postnatal day (PND) 45. Then, mice were sacrificed, and hippocampal samples were analyzed to study the gene expression of GABAergic and glutamatergic elements. Results showed that prenatal exposure to CPF impaired social novelty preference and increased the expression of GABA-A α1 subunit in females of both genotypes. In addition, the expression of GAD1, the ionic cotransporter KCC2 and the GABA-A α2 and α5 subunits were increased in apoE3 mice, whereas CPF treatment only accentuated the expression of GAD1 and KCC2. Nevertheless, future research is needed to evaluate whether the influences detected in the GABAergic system are present and functionally relevant in adults and old mice.

1. Introduction

The general population is constantly exposed to a wide variety of environmental toxics including pesticides (Huang et al., 2020). Organophosphate pesticides and, chlorpyrifos (CPF) in particular, are commonly used in many countries to control plants pathogens and promote agricultural production (Darko and Akoto, 2008; Wang et al., 2008). According to the US Environmental Protection Agency, each year approximately 5.1 million pounds of CPF are handled for agricultural proposes (EPA, 2020). This massive use has been associated with a wide variety of health problems, including cognitive and motor deficits (Burke et al., 2017; Eaton et al., 2008). Although environmental regulations have been published on CPF use in the last few years, the consequences on human health will persist for decades (EFSA, 2019; EPA, 2021).

The main target of CPF is the cholinergic system by irreversibly inhibiting the activity of the acetylcholinesterase (AChE) enzyme responsible for the hydrolysis of acetylcholine (ACh) to choline and

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acetate (Casida and Quistad, 2004; Flaskos, 2012). This inhibition produces an accumulation of ACh in the synaptic cleft, which overstimulates the postsynaptic cholinergic neurons, leading to cardiac or respiratory arrest, diarrhea, sweating or convulsions (Abou-Donia et al., 2016; Garcia et al., 2003). Apart from that, CPF have other non-cholinergic targets that trigger alterations in, for example, axonal transport (Terry, 2012), mitochondrial function (Middlemore-Risher et al., 2011), oxidative stress (Eftekhari et al., 2018) and inflammation (Mohammadzadeh et al., 2018). Notwithstanding this, low doses of CPF have been reported to give no signs of neurotoxicity (Abreu-Villaça and Levin, 2017; Casida, 2017).

Moreover, there is a growing body of clinical (Lan et al., 2017, 2019) and preclinical (Furlong et al., 2014; Millenson et al., 2017; Philippat et al., 2018) studies that have associated CPF exposure with neurodevelopmental disorders such as autism spectrum disorder (ASD), characterized by difficulties in communication, and social interaction, as well as the presence of stereotyped behaviors (Eaton et al., 2008; Takumi et al., 2020). ASD prevalence has increased in the last 30 years, which some authors believe is due to a gene-environment interaction (Matsui et al., 2018; Mottron and Bzdok, 2020; Zhang et al., 2018). A recent review conducted in our laboratory (Biosca-Brull et al., 2021) showed that experimental studies provide evidence that prenatal exposure to CPF, around gestational day (GD) 12, is associated with social and cognitive alterations in rodents. However, epidemiological studies were more variable, so it was difficult to draw conclusions (Biosca-Brull et al., 2021).

The gene of apolipoprotein E (*APOE*) is polymorphic in humans, and of the three human *APOE* alleles (ε2, ε3, ε4), ε4 increases the risk of cardiovascular and cognitive impairments, and neurodegeneration (Allen et al., 1997; Getz and Reardon, 2009). We also observed that the *APOE* genetic background contributes to neurobehavioral differences in mice from the early developmental stages to adulthood (Basaure et al., 2019; Peris-Sampedro et al., 2016; Reverte et al., 2012) and modulates the effects of a variety of toxic agents such as CPF (Guardia-Escote et al., 2020), decabromodiphenyl ether (Reverte et al., 2013) or lead (Prada et al., 2016). The apoE protein has a key role in lipid transport and homeostasis in the nervous system (Yu and Foraker, 2015). The association between *APOE* and autism focuses on that apoE protein competes with Reelin (the protein of a well-established autism candidate gene) to join the very low-density lipoprotein receptor (VLDLR) and the apolipoprotein E receptor 2 (apoER2) (D'Arcangelo et al., 1999). The importance of cholesterol supply for normal brain development and function and the implication of Reelin in normal development suggest different vulnerabilities associated with *APOE* polymorphism for developing the symptomatology of autism.

On the other hand, a neurochemical hypothesis for autism points to a dysregulation of complementary systems such as gamma-aminobutyric acid (GABA) and glutamate (Ford et al., 2019; Vorstman et al., 2017). GABA neurotransmitter is synthesized from glutamate by the activity of glutamic acid decarboxylase (GAD). Both neurotransmitters are packed into synaptic vesicles and released in the synaptic cleft to join their respective receptors on the postsynaptic surface (Rowley et al., 2012). A disequilibrium between these neurotransmitters is known as excitatory/inhibitory (E/I) imbalance, suggesting an increase in glutamatergic activity alongside a decrease in GABAergic signaling, which leads to neuronal hyper-excitability (Canitano and Palumbi, 2021). The *APOE* gene interacts with the glutamatergic system (Chen et al., 2010; Zhang et al., 2020). In fact, Chen et al. (2010) associated the apoER2 with the activation of N-methyl-D-aspartate (NMDA) receptors at the neuronal surface, which are composed by two obligatory GluN1 subunits and two GluN2 or GluN3 subunits (Chazot et al., 1994; Vieira et al., 2020). Together with Reelin, apoER2 phosphorylates and enhances the activity of GluN2 subunits. Gómez-Giménez et al. (2018) demonstrated that exposure to low doses of CPF increases GABA neurotransmitter in cerebellum and hippocampus GluN2A and GluN2B NMDA receptor subunits, but only in males, indicating a sex-specific effect of CPF (Gómez-Giménez et al., 2018).

In addition, behavioral testing plays a crucial role in evaluating autistic-like behaviors. In this sense disruption in social behavior is one of the core symptoms of autism and it can be evaluated by a wide variety of tests. The three-chamber test, developed by Crawley (2004) is one of the most commonly used method to explore social behavior in mice including mice model of autism. Social behavior, especially the ability to remember an individual (social memory), is related to the medial temporal lobe of the brain, which includes the hippocampus (Okuyama, 2018). In particular, the CA1 hippocampal region has been associated with the ability to recognize and memorize a familiar conspecific because it encodes and stores the social recognition memory (Montagrin et al., 2018). Moreover, the CA1 region is interconnected with another hippocampal subfield, the CA2 region, indicating that both subparts play an important role in the formation and consolidation of social memory (Garrido Zinn et al., 2016; Montagrin et al., 2018).

Given that CPF exposure is associated with an increase in developmental disorders, in which genetic background may be a protective or risk factor, the present study was aimed to evaluate the effect of prenatal exposure to CPF on the human *APOE3* and *APOE4* polymorphism in transgenic mice. In particular, we were looking for its effect on social behaviors and gene expression of GABAergic and glutamatergic signaling elements, as well as a possible association between prenatal exposure to CPF, *APOE* genotype and autism symptomatology.

2. Material and methods

2.1. Animals

Human apoE3 and apoE4 target replacement (TR) homozygote mice were obtained from Taconic Europe (Lille Skensved, Denmark). After a quarantine period, one male and two females were mated for 3 h. Once a vaginal plug was detected, the GD 0 was assigned. Animals were housed in plastic cages containing three to five animals until GD 12, when pregnant females were housed individually and randomly selected to receive one of the two treatments (Control [CNT] or CPF). The day of delivery was assigned as PND 0. Only litters with at least four live pups were used in this study. All mice were maintained in a 12 h light/dark automatic cycle (light ON at 8:00–20:00) with controlled temperature (22 ± 2 °C) and humidity (50 ± 10%). Food (SAFE® A04 diet, Panlab, Barcelona, Spain) and tap water were administered *ad libitum*. The present study followed the ARRIVE Guidelines (Percie du Sert et al., 2020) and complied with Spanish Royal Decree 53/2013 on the protection of experimental animals and the European Communities Council Directive (86/609/EEC). It was approved by the Animal Care and Use Committee of the Rovira i Virgili University and assigned an authorization code (number 10735) by the Government of Catalonia.

2.2. Treatment and experimental design

Pregnant female mice were exposed to 0 or 1 mg/kg/day of CPF (0,0-diethyl O-(3,5,6-trichloropyridin-2-yl) phosphorothioate) 99.5% purity provided by Sigma-Aldrich (Madrid, Spain) through the diet from GD 12 to 18. The standard food was supplemented with 15 mg CPF/kg (Pantlab, Barcelona, Spain) and calculated to deliver 1 mg/kg/day. Dams were daily monitored for body weight and food intake to verify the dose provided. The control group received the standard diet (Fig. 1). The dose was chosen because it is within the threshold for brain AChE inhibition (Silva, 2020), although it should be considered that many studies find a transient inhibition of approximately 24 h (Carr et al., 2013; Perez-Fernandez et al., 2020a, 2020b). The period of exposure was based on previous studies and include the sensitive period for adverse effects produced by valproic acid (Biosca-Brull et al., 2022; Markram et al., 2008; Schneider and Przewłocki, 2005).

Litters were kept with their mothers until PND 28. A maximum of four pups (two males and two females) per litter were separated in order

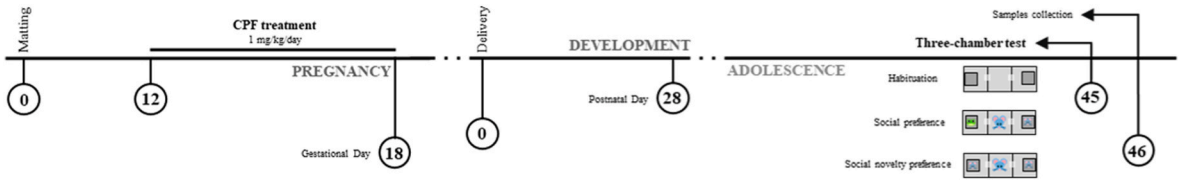

Fig. 1. Schema of the experimental design.

to assess social behavior during adolescence (PND 45). On PND 46, animals were sacrificed, and brain samples were flash-frozen and then stored at −80 °C until gene expression analysis. The unit of analysis was the litter. For this reason, values obtained from individuals of the same litter and sex were averaged to a litter value. The total number of litters and animals used are shown in Table 1.

2.3. Behavioral assessment: three-chamber test

The three chamber test was used, as on other occasions in our laboratory, to assess social behavior (Basaure et al., 2019; Biosca-Brull et al., 2022). Males and females of 45 days of age were placed in a Plexiglas box (60 × 30 × 30 cm) with three interconnected chambers (20 × 30 × 30 cm) and two doors in the middle walls so that the mice could move freely between compartments. Both lateral chambers contain an empty wire cup (7 × 7 cm). Before starting the test, the mice were brought to the testing room and left undisturbed for 10 min. Then, one mouse was placed in the central compartment and allowed to move freely for 10 min. After the habituation phase, sociability preference was evaluated by placing an inanimate object in one of the two wire cups (i.e., red plastic frog, 2.5 × 2.5 cm), while an unfamiliar mouse of the same sex and age was placed in the other (social chamber). Finally, we evaluated the social novelty preference by replacing the inanimate object in the non-social chamber with an unfamiliar mouse of the same sex and age (novel chamber). The familiar mouse was kept in the same wire cup in the now non-novel chamber. In the last two phases, animals had 6 min to explore freely. At the end of the test, the Plexiglas box was cleaned with ethanol 70% in order to avoid olfactory clues. The time that the subject mouse spent in each compartment was recorded by a video camera (Sony CCD-IRIS) and computerized by a video-tracking program (Etho-Vision©, Noldus Information Technologies, Wageningen, The Netherlands). We used this recording to evaluate the preference for the social or novel stimulus, assessing the time that the animal spends in the social or novel chamber versus the time in the non-social or non-novel chamber. We also evaluated other social variables such as sociability ratio ([time that the animal spends in the social chamber - time in the non-social chamber]/Total time exploring) and novelty ratio ([time that the animal spends in the novel chamber - time in the non-novel chamber]/Total time exploring). A positive ratio indicates a preference for the social or novel stimulus. "Total time exploring" refers to the sum of time in both lateral compartments. Those animals that explored one of the three chambers for less than 60 s, in the social preference phase, were removed from the study. Only one apoE3-treated male mouse was removed.

Table 1
Animals used in this study. The number in parentheses refers to the total number of animals, whereas the other number refers to the litters used.

	Social behavior		Gene expression	
	CNT	CPF	CNT	CPF
apoE3				
Males	8 (14)	9 (12)	5 (5)	5 (5)
Females	8 (13)	8 (13)	6 (6)	6 (6)
apoE4				
Males	10 (15)	9 (12)	6 (6)	6 (6)
Females	9 (17)	8 (14)	6 (6)	6 (6)

CNT-Control; CPF-Chlorpyrifos.

2.4. Gene expression analysis

Hippocampal RNA was extracted with the SPEEDTOOLS Total RNA Extraction Kit from Biotools (Madrid, Spain). After each extraction, the concentration and purity of RNA was measured by a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Then, complementary RNA (cDNA) was synthesized from 0.70 μg of RNA samples using a Maxima First Strand cDNA Kit for RT-qPCR (ThermoFisher Scientific, Waltham, MA, USA). Real-time polymerase chain reaction (qPCR) analysis, which included triplicates and was performed with Maxima SYBR Green/ROX qPCR Master Mix (2X) Kit (ThermoFisher Scientific, Waltham, MA, USA) and the 7900HT Fast Real-Time PCR System (ThermoFisher Scientific, Waltham, MA, USA), was used to assess the gene expression of GABA- and glutamate-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 retinoic-acid related orphan receptor alpha (*Rora*), which is an hormone-dependent transcription factor that could help understand the differences between sexes. Each sample was then normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) (ΔCt) and standardized to the average of the apoE3 male control group (ΔΔCt) to assess the relative gene expression levels with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Primer sequences are described in detail in Table 2.

2.5. Statistical analysis

Statistical analysis was conducted by SPSS 27.0 software (IBM Corp. Chicago, IL, USA). A three-way analysis of variance (ANOVA) was conducted to assess the general effects of sex, treatment or genotype, and their interactions. Similarly, the general effects of variables that were evaluated over time (body weight) were assessed with repeated measures ANOVA (RMANOVA). The unit of analysis was for all cases the litter. A two-sample *t*-test was used so that the significant effects of prenatal treatment, genotype, and sex could be better analyzed when it was appropriate. The sociability and novelty ratio were assessed with a one-sample *t*-test. As a general gene screening, a principal component analysis (PCA) of ΔCt was conducted. Correlations between genes were also assessed using Pearson or Spearman coefficients, depending on the homogeneity of the sample. The Levene test was used to study the homogeneity of variances. The Kruskal-Wallis or Mann-Whitney *U* test were performed when was appropriate. All data are presented as the mean ± S.E.M. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Body weight

Body weight was measured at three points in time: birth, the day of weaning and before social behavior was assessed. A three-way RMANOVA using the day as the within factor and sex, genotype and

Table 2
Primers used in 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)
Rora	RORA	CCACCTACTCCTGTCCTCGTCAG	CTTCTGCACCTCGGCGTACAAG	Qin et al. (2021)

treatment as the between factor showed an increase in weight over the days [$F_{2,31} = 3358.879$, $p < 0.001$] and an interaction between PND and genotype [$F_{2,31} = 6.786$, $p = 0.004$] and PND and sex [$F_{2,31} = 41.746$, $p < 0.001$]. In order to define the general effect, each PND was studied with a two-sample *t*-test for each genotype and sex. The *APOE4* genotype showed a lower body weight on the day of weaning (PND 28 [$t_{38} = 3.488$, $p = 0.001$], while the females showed a lower body weight on the day of behavioral testing (PND 45) [$t_{38} = 7.897$, $p < 0.001$] (Fig. 2A and B).

3.2. Social behavior in a three-chamber test

The results of social behavior are represented as a sociability or novelty ratio in which a value of 0, known as a chance value, indicates that mice spend the same time in each chamber. Positive ratios indicate a preference for the social or novel stimulus, while negative ratios represent a preference for the non-social or non-novel stimulus (Fig. 3).

All groups showed a preference for the unfamiliar mouse in the sociability preference phase. A three-way ANOVA (sex, genotype, and treatment) showed a general effect of sex [$F_{1,68} = 4.335$, $p = 0.042$], which indicates that males had a greater preference for the unfamiliar mouse than females (Fig. 3A). The one-sample *t*-test analysis comparing the sociability ratio to the chance level showed significant social preference for all groups evaluated (males: apoE3-CNT [$t_7 = 3.693$, $p = 0.004$], apoE3-CPF [$t_8 = 8.432$, $p < 0.001$], apoE4-CNT [$t_9 = 5.341$, $p < 0.001$], apoE4-CPF [$t_8 = 11.935$, $p < 0.001$] and females: apoE3-CNT [$t_7 = 3.477$, $p = 0.005$], apoE3-CPF [$t_7 = 4.525$, $p = 0.001$], apoE4-CNT [$t_8 = 2.999$, $p = 0.009$], apoE4-CPF [$t_7 = 2.887$, $p = 0.012$]) (Fig. 3A).

Regarding the social novelty preference phase, a three-way (sex, genotype, and treatment) ANOVA showed a general effect of sex [$F_{1,68} = 9.525$, $p = 0.003$], and treatment [$F_{1,68} = 4.132$, $p = 0.046$], and an interaction between sex and treatment [$F_{1,68} = 4.191$, $p = 0.045$], indicating that male mice spent more time with the unfamiliar mouse than with the familiar one (Fig. 3B). Notably, prenatal CPF exposure affects social behavior in a sex-dependent manner, and neither apoE3 or apoE4 exposed females showed any preference for the novel subject. One-sample *t*-test analysis of the chance level indicated that all male groups showed a general preference for the new stimulus (apoE3-CNT [$t_7 = 3.312$, $p = 0.013$], apoE3-CPF [$t_8 = 2.805$, $p = 0.023$], apoE4-CNT [$t_9 = 3.456$, $p = 0.007$], apoE4-CPF [$t_8 = 6.335$, $p < 0.001$]), while only females in the apoE4-CNT group showed a significant preference [$t_8 = 3.438$, $p = 0.009$]. However, apoE3-CNT females tended to show an interest in the unfamiliar mouse, which was not observed in the treated group [$t_7 = 1.543$, $p = 0.083$] (Fig. 3B).

3.3. Hippocampal gene expression

The expression of GABA and glutamate system genes was evaluated. Firstly, we performed a general screening of all the genes selected by means of a PCA analysis (Fig. 4). This analysis groups the genes into different principal components (PC). Each PC clusters the genes in terms of the expression patterns, so the genes in the same PC have a similar pattern and correlate positively between them (Supplementary Table 1). PC 1 explained 37.65% of the variance and included genes from the two systems evaluated: GABA-A α2 ($r = 0.948$), GABA-A α5 ($r = 0.825$), GABA-A β3 ($r = 0.849$), KCC2 ($r = 0.412$), PVALB ($r = -0.575$), GluN2A ($r = 0.911$) and GluN2B ($r = 0.894$). PC 2 explained 22.56% of the variance and was strongly correlated with the genes involved in the synthesis and release of GABA: GAD1 ($r = 0.850$), GAD2 ($r = 0.890$) and VGAT ($r = 0.899$). Finally, PC 3 explained 10.81% and included NKCC1 ($r = 0.791$), GABA-A α1 ($r = 0.838$) and RORA ($r = 0.660$).

3.3.1. PC 1 cluster: GABAergic and glutamatergic system-related genes and ionic cotransporter KCC2

Among the genes clustered in PC 1, we found two ionotropic glutamate N-methyl-D-aspartate (NMDA) receptor subunits, a GABAergic interneuron (PVALB), an ionic cotransporter (KCC2) and some ionotropic GABA receptor subunits (GABA-A α2, GABA-A α5 and GABA-A

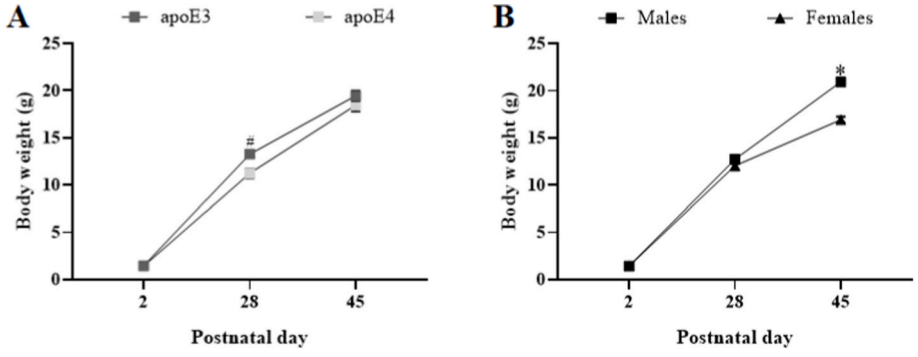

Fig. 2. Body weight depending on *APOE* genotype (**A**) or sex (**B**) at PND 2, 28 and 45. Symbols indicate differences between sexes (*) and genotypes (#) at $p < 0.05$.

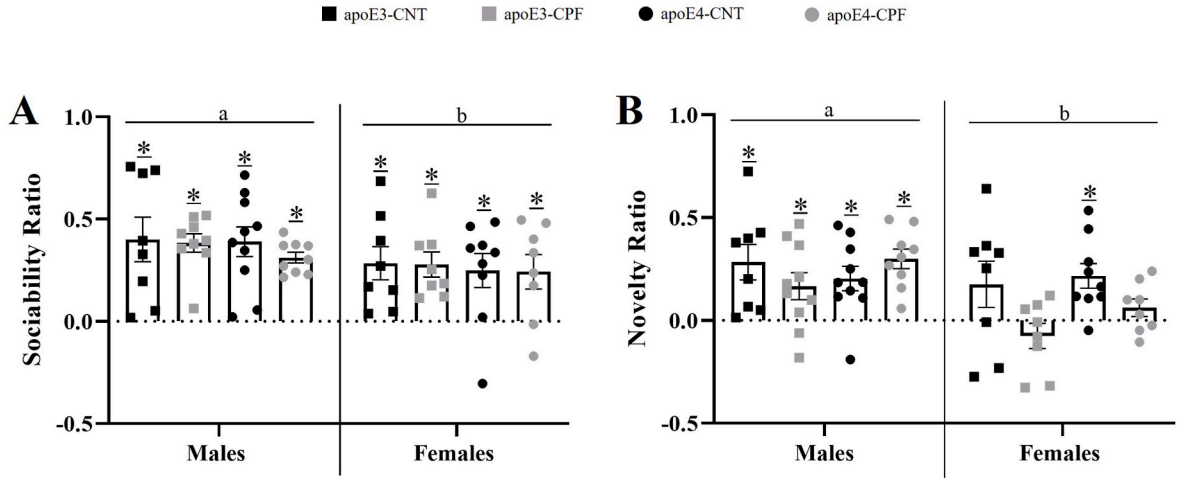

Fig. 3. Sociability and social novelty preferences assessed by the three-c~~h~~amber test at PND 45. Sociability (**A**) and Novelty ratio (**B**) calculated as (time spent in (social or novel) chamber – time in (non-social or non-novel) chamber)/total time exploring. An asterisk indicates differences compared to the chance level (i.e., 0, equal time in each right or left compartment) (*), while different letters indicate differences between sexes at $p < 0.05$.

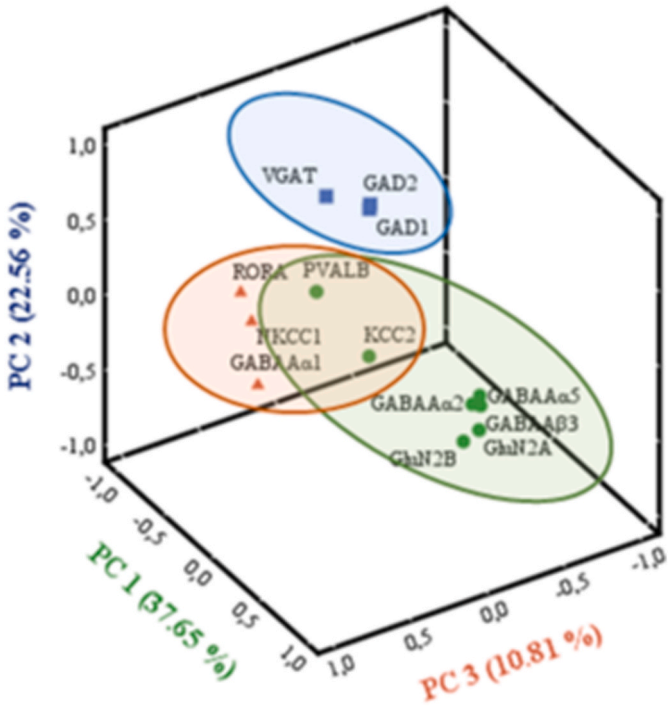

Fig. 4. Principal component analysis (PCA) of hippocampal gene expression related to GABAergic and glutamatergic systems, as well as RORA gene in adolescent homozygous apoE3-and apoE4-TR mice exposed prenatally to CPF.

β3). As far as the GABA receptor is concerned, the GABA-A α5 subunit is located extrasynaptically causing a tonic inhibition, the GABA-A α2 subunit is found in postsynaptic and presynaptic locations causing a phasic inhibition or acting as GABA controller, whereas the GABA-A β3 subunit is in postsynaptic or extrasynaptic locations.

Differences in the GABA system were observed depending on the *APOE* genotype and treatment, while the glutamate system showed differences depending on sex and genotype. A three-way (sex, genotype, and treatment) ANOVA showed that the ionic cotransporter KCC2 (Fig. 5A), GABA-A α2 and α5 subunits (Fig. 5D and **F**) were significantly influenced by the genotype (KCC2 [$F_{1,45} = 4.233$, $p = 0.047$], GABA-A α2 [$F_{1,45} = 8.465$, $p = 0.006$] and α5 [$F_{1,45} = 4.741$, $p = 0.036$]), indicating an increase of those elements in apoE3 mice in comparison with the *APOE* ϵ4 carriers (Fig. 5B, E and 5G). Moreover, an interaction between genotype and treatment [$F_{1,45} = 4.277$, $p = 0.045$] was ~~also~~ observed, indicating a greater expression of the ionic cotransporter in those apoE3 mice treated with CPF [$t_{20} = -2.475$, $p = 0.013$]. It was also observed a non-significant trend of treatment [$F_{1,45} = 3.590$, $p = 0.066$] (Fig. 5C).

On the other hand, a three-way (sex, genotype and treatment) ANOVA analysis of ionotropic glutamate receptor subunits showed a non-significant interaction between genotype and sex [$F_{1,45} = 3.496$, $p = 0.069$] (Fig. 5H). Supplementary Table 2 shows the mean ± S.E.M of the genes that did not show significant differences.

3.3.2. PC 2 cluster: GABAergic system-related genes

As reported above, PC 2 covers those genes involved in the synthesis (GAD1 and GAD2) and the release (VGAT) of GABA to the synapse.

The expression of GAD1 depends on genotype and CPF exposure. A three-way (sex, genotype and treatment) ANOVA for each gene showed an overall effect of the genotype [$F_{1,45} = 4.963$, $p = 0.032$] and a non-statistical trend of the treatment [$F_{1,45} = 3.761$, $p = 0.060$] in GAD1 expression, indicating that apoE3 mice presented higher expression levels than apoE4 mice (Fig. 6A–C). Supplementary Table 2 shows the mean ± S.E.M of the genes that did not show significant differences.

3.3.3. PC 3 cluster: GABAergic system-related genes, ionic cotransporter NKCC1 and RORA gene

The PC 3 cluster included the main ionotropic postsynaptic GABA subunit receptor (GABA-A α1), the ionic cotransporter NKCC1 and RORA.

The effects of prenatal CPF exposure on the GABAergic system depend on sex. A three-way (sex, genotype, and treatment) ANOVA showed an interaction between sex and treatment [$F_{1,45} = 4.442$, $p = 0.042$] in the GABA-A α1 subunit (Fig. 7A), indicating that prenatal CPF exposure increases GABA-A α1 subunit expression in females [$t_{22} = -2.195$, $p = 0.019$] (Fig. 7B). Supplementary Table 2 shows the mean ± S.E.M of the genes that did not show significant differences.

3.3.4. GABA and glutamate developmental switch

As well as PCA, we also used the NKCC1/KCC2 ratio (Fig. 8) to explore the GABA switch from excitatory to inhibitory. It is well known that NKCC1 expression is increased during brain development, when GABA is excitatory, but when GABA switches to inhibitory (mature neurons), the KCC2 cotransporter is overexpressed. Statistical analysis showed a trend in the interaction between genotype and treatment [$F_{1,45} = 3.574$, $p = 0.066$] in the NKCC1/KCC2 ratio.

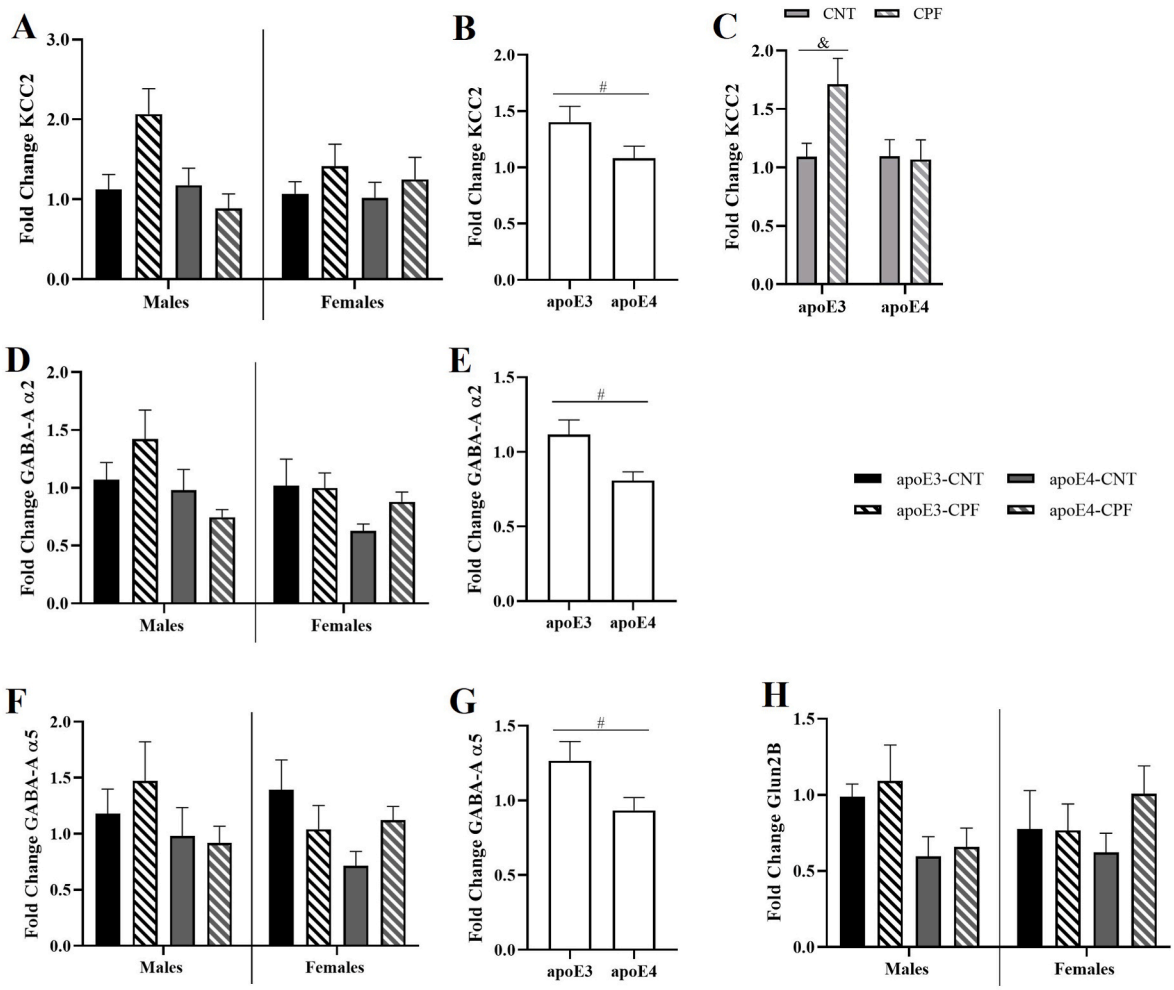

Fig. 5. Hippocampal gene expression clustered in PC 1. KCC2 (**A to C**), GABA-A α2 (**D and E**), GABA-A α5 (**F and G**) and GluN2B (**H**)~~and~~. Symbols indicate differences between genotype (#) and treatment (&) at $p < 0.05$.

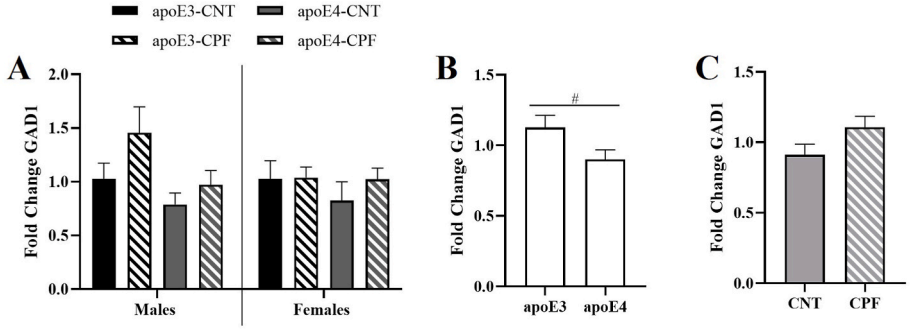

Fig. 6. Hippocampal gene expression clustered in PC 2. GAD 1 (**A to C**). The symbol # indicates differences between genotypes at $p < 0.05$.

Similarly, in the glutamatergic system, the GluN2A/GluN2B ratio is evaluated as a control of developmental maturation. The GluN2B subunit is highly expressed throughout embryonic life, while the expression of the GluN2A subunit increases during adulthood. This ratio has been observed to increase in mature neurons, although no differences were showed in this study [$F_{1,45} = 1.343$, $p = 0.254$] (Supplementary Fig. 1).

4. Discussion

The current study was aimed to provide new insights into the impact of *APOE* and gestational CPF exposure on developmental disorders. Hence, we investigated prenatal exposure to CPF in a humanized transgenic mouse model expressing the two human *APOE* alleles ε3 and ε4. We focused primarily on social behavior as an indicator of autistic-like behavior, along with hippocampal gene expression of the principal excitatory and inhibitory neurotransmitters. We observed a clear disruption in social novelty preference in females exposed to CPF regardless of the genotype, but no effects in males. Remarkably, the expression of some elements of the GABA and glutamate systems differed between genotypes, and there were some interactions between genotype and sex. CPF exposure mainly affects the expression of GABA elements and showed sporadic interactions with both genotype and sex.

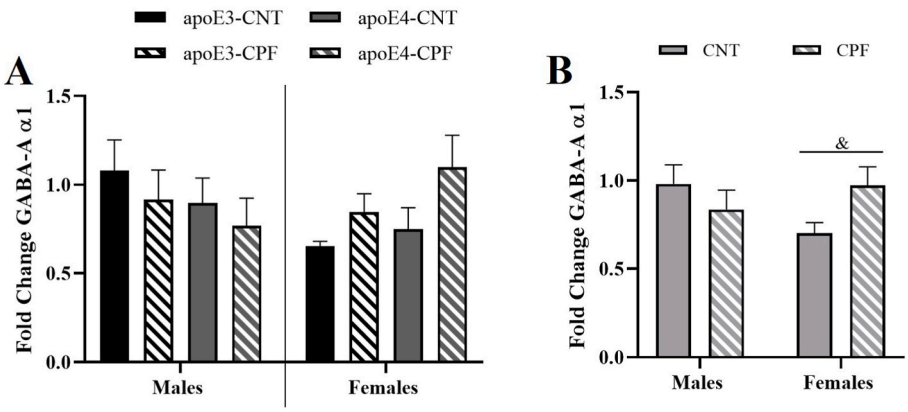

Fig. 7. Hippocampal gene expression clustered in PC 3. GABA-A α1 (**A and B**). The symbol & indicates differences between treatments at $p < 0.05$.

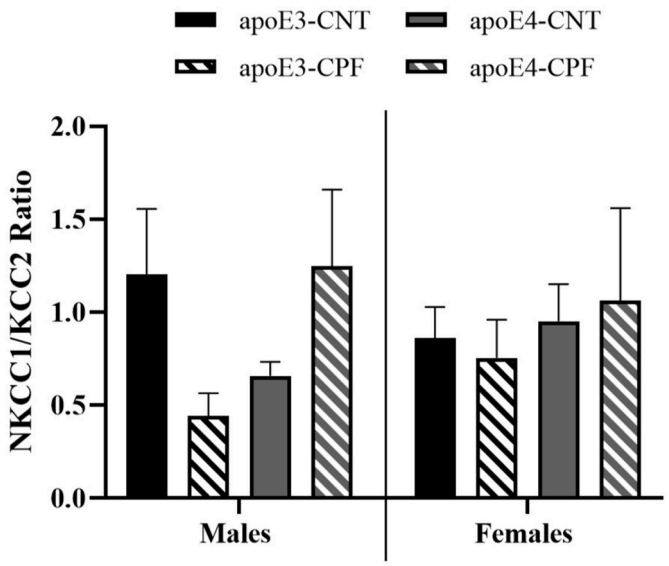

Fig. 8. Hippocampal gene expression of GABA cotransporters ratio.

Developmental exposure to the pesticide CPF has been associated with changes in social behavior in rodents. However, studies are not always consistent and both increased and decreased social activity has been reported. Studies conducted in C57BL/6J mice showed that prenatal exposure to doses below the threshold of observable signs of CPF toxicity evoked sex-dependent deficits in social behavior (Biosca-Brull et al., 2022; Lan et al., 2019). In this respect, Biosca-Brull et al. (2022) observed that the adolescent male offspring of dams treated with low doses of the pesticide (1 mg/kg/day) during gestation presented a decrease in the preference for the novel stimulus, but no effects in females. Another study conducted in C57BL/6J mice prenatally exposed to the pesticide at a dose of 5 mg/kg/day showed altered adult social behavior in male mice (a deficit in both innate and learned social preference), suggesting that alterations in social behavior were maintained over time (Lan et al., 2019). On the other hand, when CD-1 mice were prenatally exposed to CPF social investigation and ultrasonic vocalizations (USV) increased in females, while no effects were found in males (De Felice et al., 2014; Venerosi et al., 2006). Moreover, exposure during the perinatal period (from GD 15 to PND 14) in CD-1 mice has shown enhanced social recognition in males (Venerosi et al., 2015). Thus, it seems that the period of CPF exposure and the animal model plays an important role in behavioral social outcomes.

In this sense, our results showed that CPF exposure during gestation affects social behavior in adolescent homozygous females carrying the human *APOE* ε3 and ε4 allele, but no effects were observed in males. In contrast, a previous study done in our laboratory with homozygous apoE3-and apoE4-TR male mice exposed to 1 mg/kg/day of CPF from PND 10 to 15 and then, at 5 months of age, re-exposed to the same doses for 15 days, showed that postnatal exposure to CPF enhanced the preference for the social stimulus in apoE4 males, whereas adult exposure enhanced this preference in apoE3 males (Basaure et al., 2019). These apparent discrepancies between postnatal and prenatal exposure may be because the exposure occurs during two different critical windows of the development of the central nervous system (CNS). It must also be taken into account that the period for social behavior assessment was different. In fact, we primarily considered prenatal exposure from GD 12 to 18 because this period corresponds to the second and third trimester of pregnancy in humans (Azad et al., 2017). In rodents, our prenatal exposure coincides with the beginning of CNS development. Processes such as neurogenesis begin on GD 9.5 and extend to PND 15 (Rice and Barone, 2000) and the peak of neuron formation in the hippocampus is well established between GD 14 and 17 (Semple et al., 2013).

As mentioned above, the GABA switch relies on the expression of both NKCC1 and KCC2 cotransporters. During brain maturation, NKCC1 is strongly expressed and causes chloride ions to enter through GABA-A receptors and increases the chloride concentration inside the neurons. Then, KCC2 becomes dominant and reduces intracellular chloride concentration by driving ion exit (Ben-Ari et al., 2012). In the present study, we did not observe any significant result, just a trend suggesting that the expression of both ionic cotransporters could be differently regulated by the *APOE* genetic background and prenatal CPF exposure. In this sense, whether differences related to basal GABA signaling between apoE3 and apoE4 isoforms, corresponds to differences on the maturation patterns during development (Basaure et al., 2018; Reverte et al., 2014) must be further explored. However, statistical values indicated that this parameter should be further studied.

To the best of our knowledge, this is the first investigation to focus on assessing the hippocampal gene expression of a wide variety of GABA-related genes in relation to *APOE* genotype, CPF exposure and social behavior. Hippocampus plays an important role in social behavior (Garrido Zinn et al., 2016; Montagrin et al., 2018). This structure together with other brain regions such as amygdala, hypothalamus, medial prefrontal cortex or anterior cingulate cortex contributes to the formation and consolidation of social memory (Barak and Feng, 2016; Tavares et al., 2015). The GABA neurotransmitter is highly present in various brain regions and it has a variety of important functions during development (Represa and Ben-Ari, 2005). GABA activity is mediated by GABA-A receptors. These ionotropic receptors are composed of different subunits, which condition their location and function (Fritschy and Panzanelli, 2014). Postsynaptic subunits such as GABA-A α1, α2, α3 and γ2 are related to fast and high-amplitude responses, whereas extrasynaptic subunits such as GABA-A α4, α5 and α6 are related to persistent

low-amplitude responses (Fritschy and Panzanelli, 2014). The expression levels in each of these subunits change depending on the period of development and the brain region studied (Yu et al., 2006). In rats, Yu et al. (2006) showed an increase of α2, α3 and α5 GABA-A subunits in the hippocampus during the first postnatal week, while in the adult brain these subunits decrease. In addition, the α1 subunit had lower expression levels in the neonatal brain, but reached high levels in adulthood (Lopez-Tellez et al., 2004; Yu et al., 2006). This indicates that an increase in the expression of both α2 and α5 could be associated with the formation of the inhibitory circuits in the hippocampus, promoting the switch of GABA during the first week of age. Our current findings showed that the *APOE3* genotype increased both α2 and α5 GABA-A subunits, indicating that GABA maturation in this genotype may occur earlier than in apoE4, in agreement with the results obtained for the chloride cotransporters ratio. In addition, an increase in GABA-A α1 subunit was observed in treated females, suggesting an upregulation of the GABA-A receptor or other direct or compensatory effect on the GABAergic system. However, we want to highlight that this subunit was the only modified of all the signaling elements evaluated in that group making difficult to draw solid conclusions between GABAergic system and social behavior. Although the statistical analysis conducted did not showed any significant effect of sex, our results suggest that both α2 and α5 subunits, as well as the ionic cotransporter KCC2 are differently regulated by sex. However, our sample size for this analysis is too small to detect significant differences.

The *APOE3* genotype also showed an increase in GAD1 expression due to CPF exposure. The GAD enzyme is responsible for the synthesis of GABA from glutamate. It is located in the GABAergic presynaptic neurons and, in particular, the GAD1 isoform is located in the cell soma (Naseri et al., 2017). GAD1 is expressed at low levels in fetuses, but expression increases during development, reaching its highest levels in adulthood (Hyde et al., 2011). The inhibition of this enzyme was strongly associated with a reduction in GABA release (Engel et al., 2001), indicating that GAD1 is important in the *novo* synthesis of GABA and plays an important role in the maintenance of inhibitory neurotransmission. Thus, our results suggest that CPF exposure contributes to the early maturation of the GABA system and increases the production of this neurotransmitter in apoE3 mice.

Inhibitory and excitatory neurotransmitters must be in balance if the brain is to develop properly. The main excitatory neurotransmitter in the adult CNS is glutamate which has a great variety of receptors (Thoreson, 1999). In the current study, we have focused on two ionotropic NMDA receptor subunits, which are predominant in the hippocampus (GluN2A and GluN2B subunits) (Shipton and Paulsen, 2014). The expression of both GluN2A and GluN2B subunits is regulated during development. GluN2B is highly expressed at birth but starts to decrease in the adult brain. On the other hand, the expression of GluN2A increases over the years, reaching its highest levels in adult life (Acutain et al., 2021). The switch between these subunits is commonly used as a parameter indicating synapse maturation, but in our study no differences were observed in the GluN2A/GluN2B ratio. In our results we observed that the expression of GluN2B increased in apoE3 males and decreased in apoE4 males, but no variations were observed in females. In accordance with this, there are different studies that associated the apoE4 isoform with a down-regulation of the expression of the NMDA receptor subunits. Reelin activates the Src family non-receptor tyrosine kinases (SFKs) by binding to ApoE receptors. SKFs then phosphorylates and activates the GluN2 subunits of the NMDA receptors. However, the apoE3 and apoE4 isoforms differs in a single nucleotide which alter their innate intracellular trafficking properties. While the apoE3 isoform is readily endocytosed and recycled, the apoE4 remains in endosomes for a long period of time. This increases the possibility that ApoE receptor remains in the intracellular compartments being unable to interact with reelin and consequently activate glutamate receptors, reducing its expression in *APOE* ϵ4 carriers (Chen et al., 2010; Heeren et al., 2004; Liu et al., 2015; Zhang et al., 2020).

In conclusion, the results of the current study show that the long-lasting effects of prenatal exposure to low doses of CPF depend on *APOE* genotype and sex. Prenatal CPF exposure affects social behavior in a sex-dependent manner. Furthermore, gene expression analysis indicates differences between both genotypes in the expression of GABAergic system components, with higher levels in the apoE3 than in apoE4 mice, while females seem to be equally affected, especially in the expression of GABA-A α1 subunit. Nevertheless, we would like to point out that future research is needed to study the contribution of the *APOE* genotype to GABAergic and glutamatergic functions and related behaviors.

Credit author statement

Judit Biosca-Brull: Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing Laia Guardia-Escote: Methodology, Investigation, Writing – review & editing Pia Basaure: Methodology, Investigation, Writing – review & editing Maria Cabré;: Methodology, Investigation, Resources, Writing – review & editing Jordi Blanco: Methodology, Investigation, Resources, Writing – review & editing Cristian Pérez-Fernández: Writing – review & editing Fernando Sánchez-Santed: Writing – review & editing José L. Domingo: Writing – review & editing Maria Teresa Colomina: Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2023.115461.

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