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Environmental exposure to chlorpyrifos during gestation, *APOE* polymorphism and the risk on autistic-like behaviors

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ABSTRACT

Autism spectrum disorder (ASD) encompasses several neurodevelopmental conditions characterized by communication and social impairment, as well as repetitive patterns of behavior. However, it can co-occur with other mental conditions such as anxiety. The massive use of chlorpyrifos (CPF) has been linked to the increased prevalence of developmental disorders. Likewise, ASD has also been closely linked to a wide variety of genetic factors. The aims of the present investigation are to study how gestational CPF exposure and *APOE* polymorphism affects communication skills, early development and mid-term anxiety-like behaviors, as well as, changes in gene expression related to the cholinergic system. C57BL/6J and humanized apoE3 and apoE4 homozygous mice were exposed to 0 or 1 mg/kg/day of CPF through the diet, from gestational day (GD) 12–18. In addition, a group of C57BL/6J females were injected subcutaneously with 300 mg/kg/day of valproic acid (VPA) on GD 12 and 13. This group was used as a positive control for studying some core and associated autism-like behaviors. Communication skills by means of ultrasonic vocalizations and physical/motor development were assessed during the preweaning period, whereas locomotor activity, anxiety-like behaviors and the gene expression of cholinergic elements were evaluated during adolescence. Our results showed that C57BL/6J mice prenatally exposed to CPF or VPA showed a decrease in body weight and a delay in eye opening. Communication and anxiety behavior were affected differently depending on treatment, while gene expression was altered by sex and treatment. In addition, none of the parameters evaluated in apoE transgenic mice exposed to CPF were affected, but there were differences between genotypes. Therefore, we suggest that prenatal CPF exposure and VPA produce divergent effects on communication and anxiety.

1. Introduction

Organophosphate (OP) pesticides have been used worldwide to control insect pests in agricultural and residential areas. In 2003, the European Commission reported that 59.1% of the total pesticides used were OP, which amounts to 4645 tons. Of all the OP, chlorpyrifos (CPF) was the most used with 15.6% (1226 tones) (European Commission, 2007), as well as the United States (US) with 28,500 tons per year. After a considerable number of scientific reports on the health effects associated with CPF, a number of regulatory measures were introduced in both US and Europe. In brief, in 2001, the US Environmental Protection Agency (EPA) prohibited its residential use (EPA, 2002), and in the last few years, the US and the European Union have completely banned CPF, even though it is still used in developing countries (EPA, 2021; EFSA, 2019).

Although CPF is intended to have detrimental effects on pest species, some studies have reported effects on non-target organisms including humans (Eaton et al., 2008; Maggio et al., 2021; Nandi et al., 2022). Once CPF has been absorbed, cytochrome P450 metabolizes it to undergo either oxidative desulfuration which gives its active metabolite

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(CPF-oxon), or diarylation which gives 3,5,6-trichloro-2-pyridinol (TCPy) and diethyl-thiophosphate. CPF-oxon can be hydrolyzed by A- or B-esterases to TCPy and diethyl-phosphate (Chanda, 1997; Kamataki et al., 1976; Pond et al., 1998). CPF and, in particular, CPF-oxon inhibits the activity of acetylcholinesterase (AChE) by binding to its active site. This inhibition led to an accumulation of acetylcholine in the synaptic cleft and a subsequent overstimulation of the postsynaptic cholinergic receptors (Pope and Liu, 1997; Sultatos, 1994). In rodents, AChE inhibition has been described at a threshold of 1 mg/kg/day of CPF (Silva, 2020) for cholinergic signs of toxicity. Doses below this threshold produced alterations in locomotor activity (Lee et al., 2015; Silva et al., 2017), cognition (Gómez-Giménez et al., 2017; Jett et al., 2001) or anxiety-like behavior (Carr et al., 2017; Silva et al., 2017), since CPF has other targets such as the cannabinoid or endocrine system (Abreu-Villaça and Levin, 2017; Casida and Quistad, 2004; Otênio et al., 2022). In addition, several studies have demonstrated that young animals and humans are more sensitive to CPF toxicity than adults (Moser et al., 1998; Pope and Liu, 1997; Timchalk et al., 2006).

Recent investigations suggest that CPF exposure contributes to the etiology of neurodevelopmental disorders such as autism spectrum disorder (ASD) (Berg et al., 2020; Biosca-Brull et al., 2021; Perez-Fernandez et al., 2022). Autism is a heterogeneous disorder characterized by difficulties in social interaction and communication, as well as repetitive patterns of behavior (WHO, 2022). Apart from these core symptoms, ASD also presents significant impairments in the motor domain and can co-occur with other mental health conditions (i.e., anxiety) that may precede social and adaptative functioning deficits in children (Bhat, 2020; Kerns et al., 2015; Leary and Hill, 1996). On the other hand, genetics was first associated with this disorder in the 1980s, and today hundreds of genes have been suggested as risk candidates for autism (Rylaarsdam and Guemez-Gamboa, 2019). The reelin (*RELN*) gene has been closely related to ASD and other mental disorders (Scala et al., 2022). During development, RELN plays a crucial role contributing to the correct migration and positioning of neurons while it remains involved in neural plasticity in adults. This protein binds to very low-density lipoprotein and apolipoprotein E (apoE) receptor-2 (D'Arcangelo et al., 1999; Mahley, 1988) and competes with the apoE protein for binding to these receptors. The apoE protein is involved in lipid transport and metabolism and in humans, there are three major isoforms – apoE2, apoE3 and apoE4 – of which apoE3 is the most abundant in the population (Marais, 2021). In our laboratory, we have investigated the interaction between CPF exposure and the different polymorphisms of the *APOE* gene. However, results on the association between the APOE polymorphism and ASD are scarce and contradictory. Although Raiford et al. (2004) and Ashley-Koch et al. (2007) observed that the different polymorphisms of the *APOE* gene are not a risk for autism, other studies have associated the hypermethylation of this gene and the apoE2 isoform with increased risk (Hu et al., 2018; Persico et al., 2004).

Mice predominantly communicate using ultrasonic vocalizations (USVs). USVs emitted by pups while they are separated from their mother and littermates are important in terms of social motivation (Ehret, 2005). The genetic background of the animals and exposure to environmental factors highly contribute to USVs modifications. Moreover, altered USVs during the early stages of development may be an early indicator of long-term behavioral alterations. For example, changes in the total number of calls can be linked to anxiety-like behaviors (Budylin et al., 2019). Along these lines, Morales-Navas et al. (2020) observed that prenatal CPF exposure (1 mg/kg/day) reduces the number of vocalizations and increases the latency to emit the first call on postnatal day (PND) 7 in rats. These results were similar to those observed for rats treated with valproic acid (VPA), a well-established pharmacological autism model (Mabunga et al., 2015). Likewise, CD-1 mice exposed to 6 mg/kg/day of CPF during gestation also showed few vocalizations, a high latency and a tendency to hyporeflexia (Venerosi et al., 2009). On the other hand, in a study with a validated mouse model of idiopathic autism (BTBR mice) exposed to 6 mg/kg/day of CPF, De Felice et al. (2015) observed an increase in the number of USVs and a reduction in the motor activity. In terms of the *APOE* genotype, the evaluation of postnatal CPF exposure (1 mg/kg/day) by Basaure et al. (2018) showed differences between the *APOE* genotype in terms of body weight and eye opening, and a general effect of CPF.

As reported above, anxiety-like behavior affect 40% of children diagnosed with autism (Kent and Simonoff, 2017). Studies with rodents observed that prenatal and postnatal exposure to CPF can increase or trigger anxiety-like behavior (Braquenier et al., 2010; Silva et al., 2017). Accordingly, Sánchez-Amate et al. (2001) suggested that CPF has an anxiogenic effect on rats exposed during adulthood and Robertson et al. (2005) demonstrated that apoE isoforms have differential effects on anxiety measures, with *APOE* ε4 carriers being the most affected individuals. Anxiety also co-occurs with Alzheimer's disease and, in this case, the apoE4 isoform is the most prevalent genetic risk factor (Safieh et al., 2019).

The aim of this investigation is to study the contribution of prenatal CPF exposure and the *APOE* genotype to the etiology of autism. To this end, communication, physical and motor development, as well as anxiety-like behaviors and the expression of genes related to the cholinergic system were first studied in a pharmacological model of autism and then, compared with C57BL/6J mice exposed to CPF. These variables were also evaluated in humanized apoE3 and apoE4 homozygous mice prenatally exposed to CPF. To the best of our knowledge, this is the first study that assesses communication, early development, anxiety behavior and the expression of cholinergic elements in apoE transgenic mice prenatally exposed to CPF and their relation to autism.

2. Material and methods

2.1. Experimental animals

Adult C57BL/6J mice from Charles Rivers Laboratories (Barcelona, Spain) and humanized apoE3-and apoE4-target replacement (TR) homozygous mice from Taconic Europe (Lille Skensved, Denmark) were used in this study. The apoE-TR mice were generated by replacing the murine *ApoE* gene with one of the two human *APOE* alleles (ε3 or ε4), without altering any endogenous regulatory sequence (Sullivan et al., 1997). After one week of quarantine, one male and two females of the same strain were mated for 3 h. The presence of a vaginal plug was assigned as gestational day (GD) 0. All the mice were housed in plastic cages containing between two and five animals until GD 12, when pregnant females were housed individually. Pregnant C57BL/6J mice were randomly assigned to one of the three treatments (control [CNT], CPF or VPA), whereas human apoE-TR were randomly selected to receive one of the two treatments (CNT or CPF). The day of delivery was assigned as PND 0, and the number of litters and live pups were recorded. Only litters with at least four live pups were used in this study. 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 (SAFE® A04 diet, Panlab, Barcelona, Spain) and tap water were administered *ad libitum*. The present study was approved by the Animal Care and Use Committee of the Rovira i Virgili University and the Government of Catalonia (Catalonia, Spain) (number 10735) and conducted following the ARRIVE Guidelines (Percie du Sert et al., 2020) and 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).

2.2. Chemicals, treatment, and experimental design

Two parallel experiments were conducted in this study (Fig. 1). In the first experiment, we exposed C57BL/6J mice to either 0 or 1 mg/kg/day of CPF (0,0-diethyl O-(3,5,6-trichloropyridin-2-yl) phosphorothioate) (Sigma-Aldrich, Madrid, Spain) through a supplemented diet from GD 12 to 18. We included a group treated with a subcutaneous

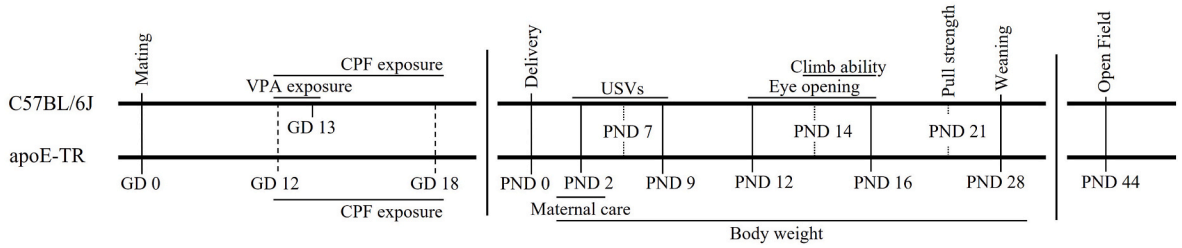

Fig. 1. Experimental schema of C57BL/6J and apoE-TR mice. A group of C57BL/6J mice were exposed to 300 mg/kg/day of VPA by a subcutaneous injection on GD 12 and 13. Pregnant females of both strains were orally exposed to CPF (1 mg/kg/day) between GD 12 and 18. USVs were assessed on PND 2, 7 and 9. Physical and motor development were assessed from PND 2 to 28, whereas locomotor activity and anxiety-like behavior were evaluated during adolescence.

injection of 300 mg/kg/day of VPA (2-propypentanoic acid sodium) (Sigma-Aldrich, Madrid, Spain), administered on two consecutive days (GD 12 and 13). This group was used as a pharmacological animal model for autism (Sakai et al., 2018). In the second experiment, apoE-TR mice were exposed to 0 or 1 mg/kg/day of CPF through a supplemented diet from GD 12 to 18. In both experiments, standard food was supplemented with 15 mg CPF/kg chow (Panlab, Barcelona, Spain). The body weight and food intake of dams were monitored daily to adjust the amount of food and achieve a daily dose of 1 mg/kg of CPF. All animals were provided with regular chow *ad libitum*.

During the preweaning period, communication skills were evaluated, in both strains, on PND 2, 7 and 9. After USV analysis on PND 2, maternal care and nest quality were evaluated. Physical and motor development of the litter were assessed until weaning on PND 28. At weaning, mice were assigned to groups of two to five animals of the same sex and treatment per cage. The unit of analysis was the litter. Measures obtained from individuals of the same litter and sex were subjected to statistical analysis. All the animals and litters used in this study are summarized in Table 1.

2.3. Pre-weaning assessment

2.3.1. Litter characteristics and maternal care

The day of delivery (PND 0), we recorded the number of living and dead pups. The mortality between PND 0 and 2 was recorded to calculate the viability index (live pups PND 2/litter size PND 0) (Basaure et al., 2018).

Nest quality and maternal care were assessed on PND 2, after pup communication has been analyzed. First, we rated the quality of the nest as described in Table 2. Afterwards, the dam was removed from the cage while the pups were weighed and placed in the corner opposite the nest. The dam was then returned to the home cage and the time that she took to collect the first pup (latency) and the total time to collect all the pups were recorded for a maximum of 3 min. In addition, we calculated a new parameter named efficiency (time to collect all the pups - latency)/(number of pups in the litter) (Reverte et al., 2014a).

Table 1
Number of animals and litters used in this study.

	C57BL/6J			apoE3		apoE4	
	CNT	CPF	VPA	CNT	CPF	CNT	CPF
Ultrasonic vocalizations							
Litters	15	14	18	12	13	16	13
Males	15	12	14	12	12	15	12
Females	12	12	15	12	12	15	12
Maternal care							
Litters	15	15	15	15	13	15	15
Physical and motor development							
Litters	10	10	8	8	10	10	10
Open field							
Litters	18	13	21	12	16	19	15
Males	14	10	16	12	12	15	11
Females	15	13	10	10	12	17	12

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

Table 2
Assessment of maternal care and physical and motor development.

Test	Day of evaluation (PND)	Measure/score
Body weight	2, 7, 9, 14, 16, 21, 23, 28	Weight (g)
Maternal care	2	Latency (s) to collect the first pup and time (s) taken to collect all pups
Nest quality	2	0 = no nest, 1 = not all the pups are in the nest, 2 = the nest is well-defined
Eye opening	12–16	0 = both eyes are closed, 1 = one eye open and one closed, 2 = both eyes are open
Climb ability	14–16	0 = no movement, 1 = less than half of the grid, 2 = more than half of the grid
Pull strength	21	Force (g)

2.3.2. Evaluation of the litter's physical and motor development

On the basis of previous studies conducted in our laboratory (Basaure et al., 2018; Reverte et al., 2014a), the developmental timeline of mice was monitored between PND 2 and 28 to evaluate physical and motor development at different timepoints (see Table 2). Physical development was assessed by body weight and eye opening, whereas motor development was evaluated by the climb ability and pull-strength force. To assess climbing ability, we placed a metal grid (24 × 24 cm) at an angle of 45° in a plastic cage with the pups at the bottom, while to measure force we placed the pups near the grid of a grip strength meter and gently pulled it backwards by the tail three time recording the highest value (Ugo Basile, Gemonio, Italy). The value for each pup within the litter was used to calculate the mean value of the litter.

2.3.3. Communication assessment: ultrasonic vocalizations

Communication was assessed by recording mice USV using a SeaWave software (Gianni Pavan©, Pavia, Italy) and an UltraMic 250 (Dodotronic, Italy) in a sound-attenuating chamber.

The USV call rate increases during the first 5–6 days, peaking around PND 6–7. Then, starts to decrease until it completely disappears (Elwood and Keeling, 1982). In order to determine the best days to carry out the test, we recorded mice communication for 3 min on PND 2, 4, 6, 8 and 10. Our data, together with that reported by Branchi et al. (2001), led us to select PND 2, 7 and 9 as the most sensitive days to observe the complete profile of vocalizations.

USVs were recorded in one male and one female from each litter. Animals were moved to the testing room in their home cage 5 min before the start of the test. Then, we placed a random pup in the sound-attenuated chamber for 5 min. The distance between the microphone and the pup was set at 10 cm (Scattoni et al., 2008). Then, the chamber was cleaned with ethanol 70% to prevent olfactory clues. A pup from the same litter but opposite sex was randomly selected and placed into the chamber to follow the same protocol. This protocol was performed on every day of the evaluation. Changes over the 5-min period, total

number of calls, the average of duration, frequency and intensity, and the latency to emit the first call were assessed.

2.4. Behavioral assessment in adolescent mice: open field test

Locomotor and anxiety-like behaviors were assessed at PND 44 in both strains using an open field test. Briefly, the apparatus consists of an open wooden box measuring 60 × 60 cm and with a 50 cm high wall. The field was divided into two zones: the center zone covering an area of 30 × 30 cm in the middle of the field and the periphery which covers the rest of the space. Five minutes before starting the test, the animals were transported to the testing room. Then, they were placed in the center of the arena and allowed to explore freely for 30 min. After the test had finished, the open box was cleaned with ethanol 70% to prevent olfactory clues. Locomotor activity was evaluated by recording the total distance traveled in periods of 5 min, while anxiety-like behavior was assessed by measuring the time that the animals spent in the center zone in comparison to the periphery, the velocity at which they moved and the distance they covered. To this end, we used a video camera (Sony CCD-IRIS) and a video-tracking program (EthoVision XT 11.5, Noldus Information Technologies, Wageningen, The Netherlands) (Kraeuter et al., 2019).

2.5. Sacrifice and sampling

Biological samples were obtained at two different points of time (PND 2 and PND 46). On PND 2, four animals from different litters, sex and experimental group were sacrificed by decapitation, while on PND 46 six animals from different litter sex and experimental group were sacrificed with anesthesia. In both cases, brain samples were flash-frozen and stored at −80 °C until AChE activity (PND 2) and gene expression (PND 46) were evaluated.

2.6. Determination of brain AChE activity

Brain samples were weighed and homogenized in cold PBS 0.1 M at pH 8 with 1% Triron X-100. Then, homogenates were centrifuged at 2000 g for 10 min at 4 °C and the supernatant removed for analysis. AChE activity was measured in duplicates and determined spectrophotometrically using a semiautomatic COBAS MIRA analyzer (Hoffman-La Roche & Co., Basel, Switzerland) and an updated version of the Ellman method (Ellman et al., 1961; Peris-Sampedro et al., 2015b). The enzyme activity was calculated relative to the protein concentration, which was assessed by the Lowry method (Lowry et al., 1951) described in our previous study (Biosca-Brull et al., 2022). Brain AChE activity was expressed in U/mg of protein.

2.7. Gene expression analysis

Hippocampal tissue was used to extract RNA with the SPEDDTOOLS Total RNA Extraction Kit from Biotools (Madrid, Spain). Concentration and purity of the RNA extracted was measured after each extraction using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Then, we synthesized complementary RNA (cDNA) using a Maxima First Strand cDNA Kit for RT-qPCR (ThermoFisher Scientific, Waltham, MA, USA) (0.42 μg of RNA for C57BL/6J mice and 0.70 μg of RNA for apoE-TR mice). Gene expression analysis of choline related genes such as choline acetyltransferase (*Chat*), solute carrier family 18-member A3 (*Slc18a3*), cholinergic receptor nicotinic alpha 4 (*Chrna4*) and alpha 7 (*Chrna7*) subunits and cholinergic receptor muscarinic 1 (*Chrm1*) and 2 (*Chrm2*) subunits were assessed by real-time polymerase chain reaction (qPCR). In both cases, the qPCR was performed with Maxima SYBR Green/ROX qPCR Master Mix (2X) Kit (ThermoFisher Scientific, Waltham, MA, USA). In C57BL/6J mice, we included duplicates, and the cycle threshold (Ct) was calculated using the Rotor-Gene Q Real-Time PCR 2.0 software (Qiagen Inc., Hilden, Germany), while in apoE-TR mice we included triplicates and calculated the Ct using 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Finally, each sample was normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) (ΔCt) and standardized to the average of C57BL/6J male CNT or C57BL/6J female CNT group and apoE3 male CNT or apoE4 male CNT group (ΔΔCt) to assess the relative gene expression levels in accordance to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The sequence of primers used are described in detail in Table 3.

2.8. Statistical analysis

Data were analyzed using the SPSS 27.0 software (IBM Corp. Chicago, IL, USA). A three-way analysis of variance (ANOVA) was done to analyze the general effects of sex, treatment and genotype, as well as their interactions. In those cases in which a variable was assessed over time, effects were evaluated by using repeated measures ANOVA (RMANOVA). The number of pups in each litter was used as a co-variable to analyze maternal care latency and all the developmental landmarks evaluated, while in the study of litter characteristics (litter size and viability index) the co-variable was the age of the dams. The homogeneity of variance was assessed by the Levene test. Parametric data was further analyzed by one-way ANOVA followed by a *post-hoc* Tukey or a two-sample *t*-test, while non-parametric data was analyzed by the Kruskal-Wallis or Mann-Whitney *U* test to assess differences between groups when necessary. The results are represented as mean values ± S.E.M and with the statistical significance set at $p < 0.05$.

3. Results

3.1. AChE activity was unaffected by prenatal exposure or APOE genotype

The activity of AChE was assessed on PND 2 in the brains of pups from both strains. No signs of AChE inhibition or differences between control and exposed groups were observed in either C57BL/6J or apoE-TR groups (data not shown). Therefore, prenatal exposure to low doses of CPF (1 mg/kg/day) during the late gestation of mice did not affect the activity of the AChE enzyme in pup brains.

3.2. Prenatal treatment with CPF and APOE genetic background do not alter the litter characteristics or maternal care

The analysis of litter size and viability index using a one-way (treatment) or two-way (treatment and genotype) ANOVA showed that neither the treatment nor the genotype affected these parameters (data not shown).

As far as maternal behaviors are concerned, all the dams evaluated showed a well-defined nest with all the pups inside (data not shown). The latency to collect the first pup and the total time corrected by the latency and the number of pups in each litter were analyzed using a one-way (treatment) ANOVA in C57BL/6J mice and a two-way (treatment and genotype) ANOVA in apoE-TR mice, but no differences between treatment or genotype were observed (data not shown).

3.3. Assessment of litter development

The influence of the treatment, sex and genotype on the early development of mice was determined by analyzing physical and motor endpoints between PND 2 and PND 28 (Figs. 2 and 3).

3.3.1. Prenatal CPF and VPA exposure reduces body weight and delays eye opening in C57BL/6J mice

The analysis of body weight with a two-way RMANOVA (sex and treatment), showed an increase in weight with time [$F_{7,70} = 609.595$, $p < 0.001$] and an interaction between PND and treatment [$F_{14,142} =$

Table 3
Sequence of primers used in this study.

Mus musculus gene	Article Symbology	Forward primer	Reverse primer	Source
Chat	ChAT	ATTTTGCCCGCACAGGGGAC	CGCCTCGTCCAGAGTATCGGT	García-Gómez et al. (2016)
Slc18a3	VAChT	CAGCTTTGGAAGCCTAGTGG	AGGAGTAGGAGTGCGTCGAA	Nagy and Aubert (2012)
Chrna4	α4 nAChR	GTTCTATGACGGAAGGGTGCAGTGGACA	GGGATGACCAGCGAGGTGGACGGGATGAT	Léna et al. (1999)
Chrna7	α7 nAChR	GTGGAACATGTCTGAGTACCCCGGAGTGAA	GAGTCTGCAGGCAGCAAGAATACCAGCA	Léna et al. (1999)
Chrm1	M1 mAChR	TGACAGGCAACCTGCTGGTGCT	AATCATCAGAGCTGCCCTGCGG	Laspas et al. (2015)
Chrm2	M2 mAChR	CGGACCACAAAAATGGCAGGCAT	CCATCACCACCAGGCATGTTGTTGT	Laspas et al. (2015)
Gapdh	–	ACAACTTTGGCATTGTGGAA	GATGCAGGGATGATGTTCTG	Yao et al. (2016)

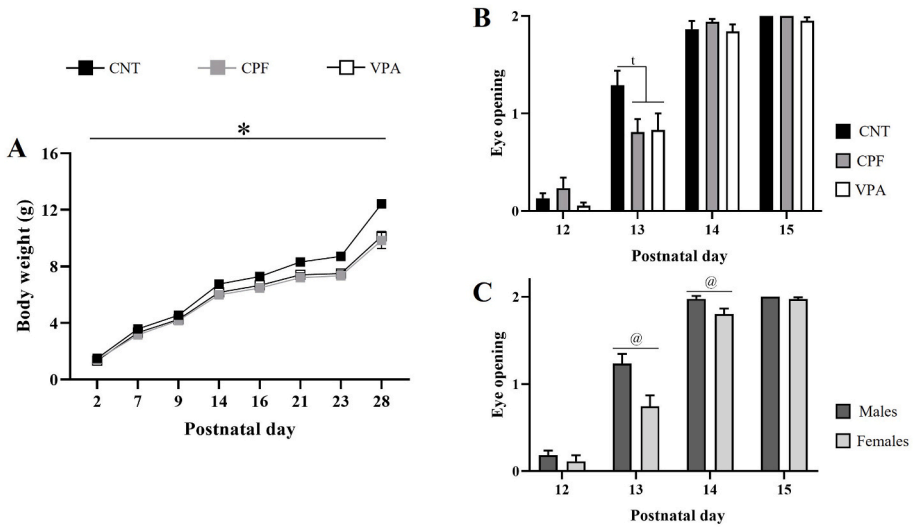

Fig. 2. Physical development of C57BL/6J mice. Body weight from PND 2 to 28 (**A**) and eye opening from PND 12 to 15 according to treatment (**B**) and sex (**C**). Symbols indicate significant differences between treatments (*) and sex (@) at $p < 0.05$, while t indicates a tendency.

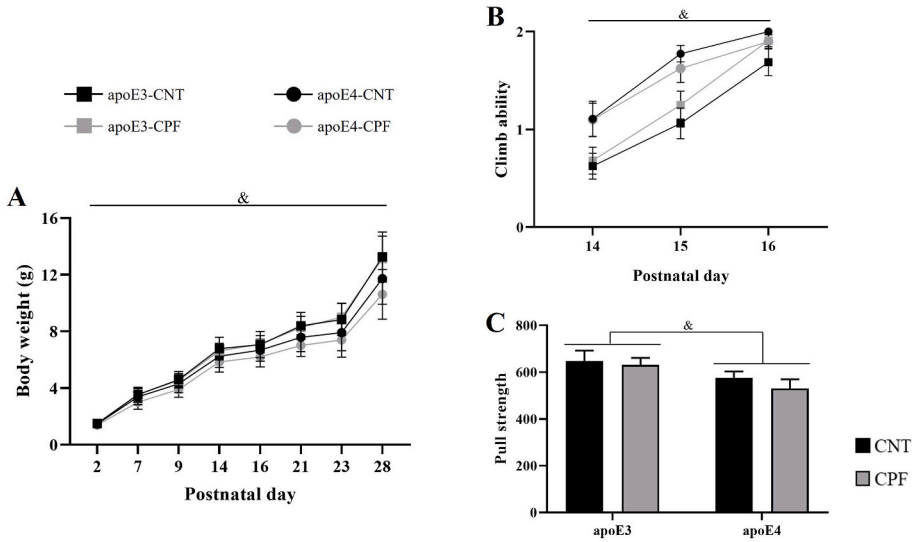

Fig. 3. Physical and motor development in apoE-TR mice. Body weight from PND 2 to 28 (**A**), climbing ability from PND 14 to 16 (**B**) and pull strength on PND 21 (**C**). The symbol & indicates significant differences between genotypes at $p < 0.05$.

2.602, $p = 0.002$] (Fig. 2A). Subsequent analysis with one-way ANOVA (PND 2, 7, 9, 16, 23 and 28) or the Kruskal-Wallis test (PND 14 and 21) revealed differences between CNT and CPF (PND2 ($p = 0.031$), PND 7 ($p = 0.013$), PND 14 ($p = 0.004$), PND 16 ($p = 0.001$), PND 21 ($p < 0.001$), PND 23 ($p < 0.001$), PND 28 ($p < 0.001$)) and also between CNT and VPA (PND2 ($p < 0.001$), PND 14 ($p = 0.001$), PND 16 ($p = 0.005$), PND 21 ($p = 0.001$), PND 23 ($p < 0.001$) and PND 28 ($p < 0.001$)), with CNT being the heaviest group over time (Fig. 2A).

Eye opening scores were analyzed from PND 12 to 16 using a two-way RMANOVA (sex and treatment). Maturation of this parameter was observed over time [$F_{4,48} = 409.877$, $p < 0.001$], and by PND 15 both eyes were completely open in all groups. We also found an interaction between PND and treatment [$F_{8,98} = 2.891$, $p = 0.006$] and PND and sex [$F_{4,48} = 2.769$, $p = 0.038$] (Fig. 2B and **C**). Analysis with the Kruskal-Wallis test showed a downward trend on PND 13 ($p = 0.057$) with the lowest eye opening score for CPF- and VPA-treated groups

(Fig. 2B). Differences between males and females were assessed by the Mann-Whitney *U* test (PND 13 and 15) and a two-sample *t*-test (PND 12 and 14). We observed that males opened their eyes earlier than females, and this was significant on PND 13 ($p = 0.005$) and PND 14 ($p = 0.017$) (Fig. 2C).

No effects of sex or treatment were observed on climbing ability and pull strength force (data not shown).

3.3.2. Physical and motor development of apoE-TR mice was not affected by prenatal exposure to CPF

Body weight was analyzed with a three-way RMANOVA (sex, treatment and genotype). It showed a progressive increase in weight over time [$F_{7,83} = 857.260$, $p < 0.001$], and an interaction between PND and genotype [$F_{7,83} = 5.034$, $p < 0.001$], and PND and sex [$F_{7,83} = 2.423$, $p = 0.026$] (Fig. 3A). A two-sample *t*-test with the genotype as independent variable indicates that apoE4 had lower body weight throughout the period (PND 2 [$t_{95} = 2.957$, $p = 0.004$], PND 7 [$t_{95} = 3.095$, $p = 0.003$], PND 9 [$t_{95} = 3.841$, $p < 0.001$], PND 14 [$t_{95} = 3.841$, $p < 0.001$], PND 16 [$t_{95} = 3.936$, $p < 0.001$], PND 21 [$t_{95} = 5.527$, $p < 0.001$], PND 23 [$t_{95} = 5.255$, $p < 0.001$] and PND 28 [$t_{95} = 5.542$, $p < 0.001$]) (Fig. 3A).

Treatment, sex and genotype have no effect on the time at eye opening (data not shown).

In terms of motor development, a three-way ANOVA (sex, treatment and genotype) revealed a progressive improvement in climbing ability [$F_{2,}69 = 74.524$, $p < 0.001$] and an interaction between PND and genotype [$F_{2,69} = 5.045$, $p = 0.009$]. Further analysis with a two-sample *t*-test (PND 14 and 15) or the Mann-Whitney *U* test (PND 16) revealed that apoE4 mice achieved top scores earlier than apoE3 mice, which indicates different rhythms of motor maturation (PND 14 [$t_{73} = -2.869$, $p = 0.055$], PND 15 [$t_{76} = -3.951$, $p < 0.001$] and PND 16 ($p = 0.040$)) (Fig. 3B). In addition, a three-way ANOVA (sex, treatment and genotype) showed an overall effect of genotype [$F_{1,72} = 5.497$, $p = 0.022$] on the pull strength, being the highest values for the *APOE3* genotype (Fig. 3C).

3.4. Assessment of USVs

Mouse USVs were analyzed in periods of 1 min (data not shown) to study changes over the 5-min period and in terms of the total number of vocalizations, the average changes in duration, frequency and intensity, and the latency to the first vocalization (Figs. 4 and 5).

3.4.1. CPF and VPA produced opposite effects on communication skills in C57BL/6J mice

As reported above, USVs were recorded on three different days. On PND 2, a two-way (sex and treatment) ANOVA showed a general effect of treatment on the total number of calls [$F_{2,79} = 8.657$, $p < 0.001$], duration [$F_{2,79} = 4.830$, $p = 0.011$] and intensity [$F_{2,79} = 5.359$, $p = 0.007$] of vocalizations (Fig. 4A, C and 4D). The subsequent analysis with a Kruskal-Wallis test showed that VPA-treated animals emitted fewer vocalizations (CNT *vs* VPA ($p < 0.001$), CPF *vs* VPA ($p < 0.001$)) with less duration (CPF *vs* VPA ($p = 0.011$)) and intensity (CPF *vs* VPA ($p = 0.014$)), especially in comparison with the animals treated with CPF (Fig. 4A, C and **4D**).

However, these differences disappeared on PND 7 (data not shown), but on PND 9 we observed an overall effect of treatment on the total number of vocalizations [$F_{2,78} = 11.032$, $p < 0.001$], latency [$F_{2,78} = 4.590$, $p = 0.013$], duration [$F_{2,78} = 4.709$, $p = 0.012$] and intensity [$F_{2,78} = 3.942$, $p = 0.024$] of calls (Fig. 4A–D). The analysis with the Kruskal-Wallis test showed differences in the total number of vocalizations between CNT and VPA ($p = 0.036$), CPF and VPA ($p < 0.001$) and CNT and CPF ($p = 0.046$) (Fig. 4A). In addition, a significant difference between CPF and VPA ($p = 0.004$) was observed in the latency to the first vocalization (Fig. 4B), while differences in call intensity were observed between CNT and CPF and between CPF and VPA at $p = 0.001$ (Fig. 4D). The duration of USVs was also analyzed by one-way ANOVA (treatment) [$F_{2,78} = 4.449$, $p = 0.015$]. *Post-hoc* analysis showed significant differences between the CNT and VPA-treated group ($p = 0.017$) (Fig. 4C). These results indicate that the treatment with VPA reduces the number, duration and intensity of USVs and increases the time to emit the first call, which results in inefficient communication, while CPF does the opposite.

3.4.2. Prenatal exposure to CPF does not affect communication skills in apoE-TR mice

Although, on PND 7 we observed significant differences in some USV parameters between genotypes (Fig. 5), these effects were not observed

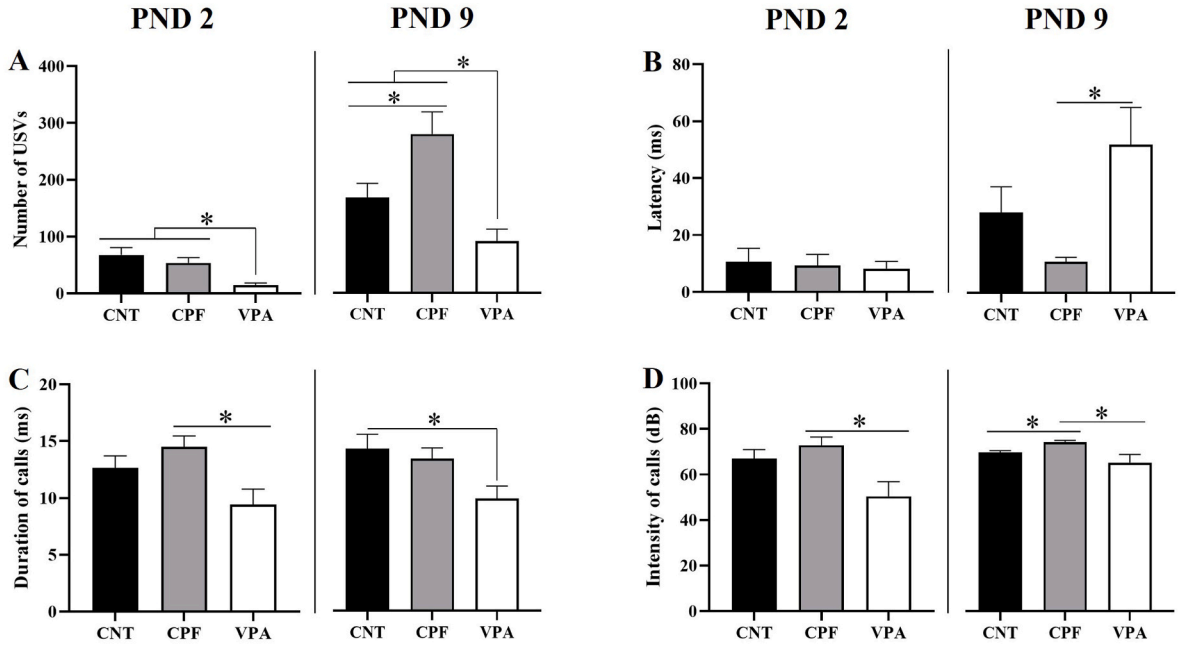

Fig. 4. Communication assessment by measuring USVs on PND 2 and 9 in C57BL/6J mice. Average of total number of calls (**A**), latency (**B**), duration (**C**) and intensity (**D**). An asterisk indicates significant differences between treatments at $p < 0.05$.

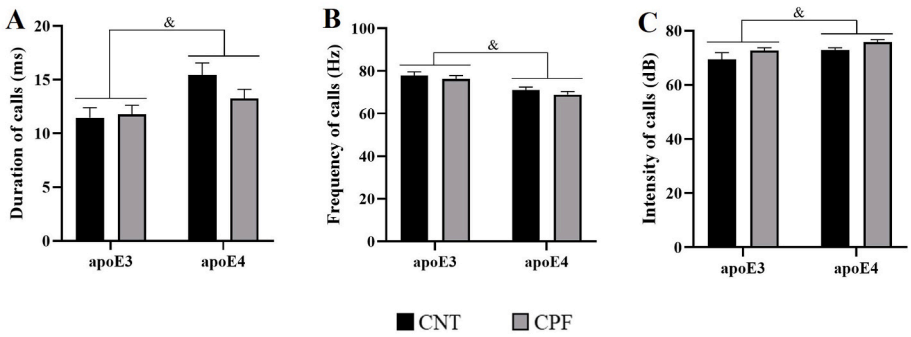

Fig. 5. Communication assessment by measuring USVs on PND 7 in apoE-TR mice. Average duration (**A**), frequency (**B**) and intensity (**C**). The symbol & indicates significant differences between genotypes at $p < 0.05$.

on PND 2 and disappeared by PND 9 (data not shown). On PND 7, a three-way ANOVA (sex, treatment and genotype) showed an overall effect of genotype in all the parameters, except for the total number of vocalizations and latency (duration [$F_{1,101} = 7.771$, $p = 0.006$], frequency [$F_{1,101} = 19.877$, $p < 0.001$] and intensity [$F_{1,101} = 5.157$, $p = 0.025$]). The *APOE4* genotype shows an increase in the duration and intensity of calls, whereas frequency decreased (Fig. 5).

3.5. Anxiety-like behavior in an open field test

3.5.1. C57BL/6J mice treated with VPA showed anxiety-like behavior

General activity in the open field test was analyzed by a two-way RMANOVA (sex and treatment). The total time (30 min) was divided into six periods of 5-min. A progressive decline in locomotor activity was observed over time [$F_{5,45} = 5.753$, $p < 0.001$] (data not shown). In addition, the total average of distance traveled and the velocity in the central area were also assessed (Fig. 6). A two-way ANOVA (sex and treatment) showed an overall effect of treatment on the total average of distance [$F_{2,54} = 3.953$, $p = 0.026$] and velocity [$F_{2,54} = 4.975$, $p = 0.011$] (Fig. 6). Subsequent analysis by a one-way ANOVA (treatment) revealed significant differences between CPF and VPA (total average distance traveled ($p = 0.011$) and velocity in the center ($p = 0.005$)) (Fig. 6), with the group treated with CPF traveling longer distances at greater velocity. Nevertheless, no differences were observed with the CNT group.

3.5.2. APOE4 genotype showed more anxiety-like behavior than APOE3

As reported above, locomotor activity was assessed by three-way RMANOVA (sex, treatment and genotype). Both apoE3 and apoE4 mice habituated to the novel space regardless of the treatment [$F_{5,51} = 20.358$, $p < 0.001$]. However, interaction between time and treatment [$F_{5,51} = 2.443$, $p = 0.046$] was observed. This indicates that the CNT group presented greater activity than the treated groups, and that differences were significantly different at 10 [$t_{61} = 2.453$, $p = 0.017$] and 25 min [$t_{61} = 2.298$, $p = 0.025$] of the test (Fig. 7A). The distance and velocity in the central zone were analyzed using a three-way ANOVA (sex, treatment and genotype), which revealed an overall effect of the genotype (distance in the center [$F_{1,62} = 5.423$, $p = 0.024$] and velocity in the center [$F_{1,62} = 5.918$, $p = 0.018$]), with apoE4 mice traveling the shortest distance more slowly in the center. This may indicate a more anxious phenotype in those mice carrying the *APOE* ε4 allele.

3.6. Cholinergic genes were differently expressed according to sex and genotype

Based on the results obtained in development, USVs and anxiety behavior, we decided to analyze the results of gene expression in relation to sex and genotype, in C57BL/6J and apoE-TR mice, respectively.

Statistical analysis using a one-way ANOVA (treatment) showed a significant difference in VChAT expression in C57BL/6J female mice [$F_{2,16} = 4.235$, $p = 0.036$]. Subsequent *post-hoc* analysis indicated differences between CNT and VPA ($p = 0.035$) (Fig. 8).

Likewise, the analysis of the same cholinergic-related genes in homozygous mice for the ε3 and ε4 alleles disclose statistically significant differences in apoE4 mice. A two-way ANOVA (sex and treatment) showed an overall effect of sex in α4 nAChR [$F_{1,23} = 5.024$, $p = 0.037$] (Fig. 9), indicating that regardless of treatment, ε4 carriers had higher expression levels of this nicotinic receptor than males.

4. Discussion

In the current study, we evaluate the potential risk associated with gestational exposure to CPF of developing ASD-like symptomatology in various animal models. We included the VPA pharmacological autism model, and a transgenic mouse model carrying the human *APOE* alleles ϵ3 and ϵ4 to study genetic vulnerability and interactions with the pesticide in males and females. In particular, we studied how prenatal CPF exposure and *APOE* genotype affect early communication and developmental landmarks, which are core and associated symptoms of ASD. To this end, we designed two experiments that exposed C57BL/6J

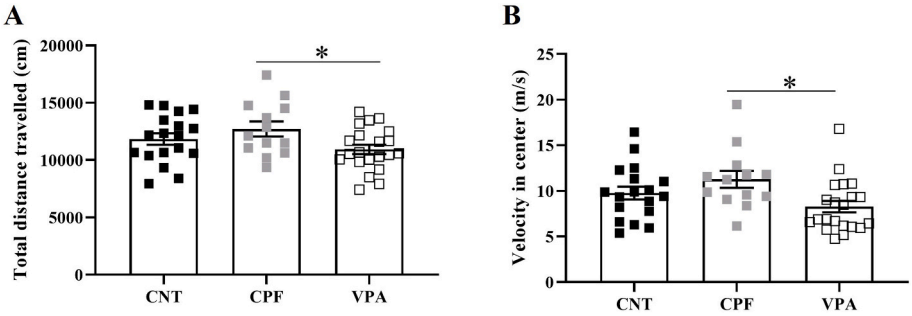

Fig. 6. Evaluation of anxiety behavior in adolescent C57BL/6J mice. Total average distance traveled (**A**) and velocity in the central area (**B**) of the open field. An asterisk indicates significant differences between treatments at $p < 0.05$.

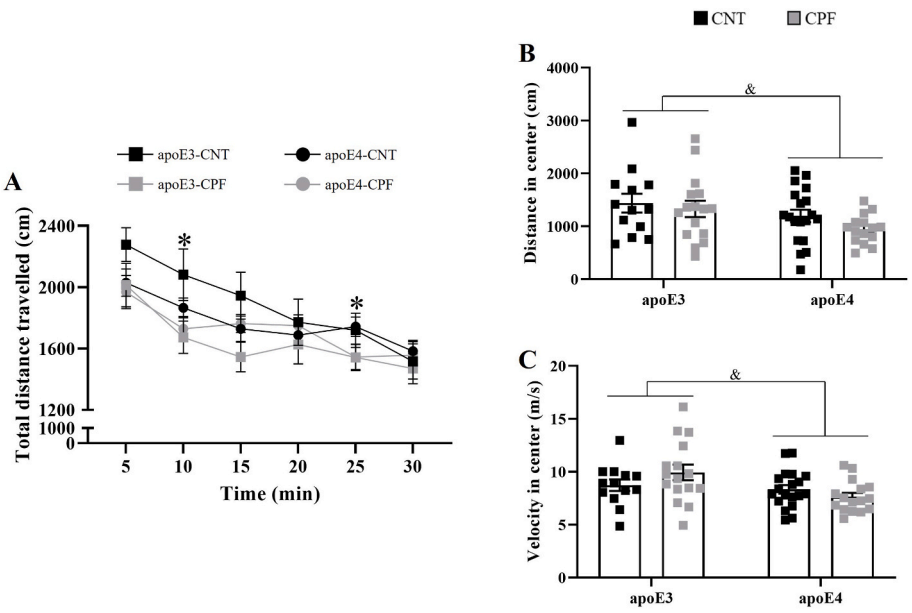

Fig. 7. Evaluation of locomotor activity and anxiety behavior in adolescent apoE-TR mice. Total distance traveled during the 30 min of the test in six periods of 5-min (**A**). Distance (**B**) and velocity (**C**) in the central area of the open field. The symbol & indicates significant differences between genotypes at $p < 0.05$.

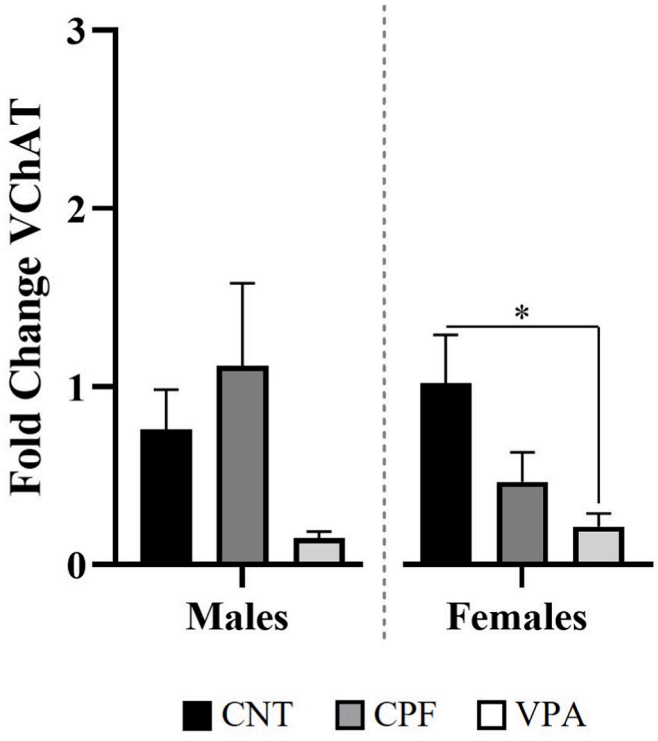

Fig. 8. Expression of VChAT in C57BL/6J. An asterisk indicates significant differences between treatment at $p < 0.05$.

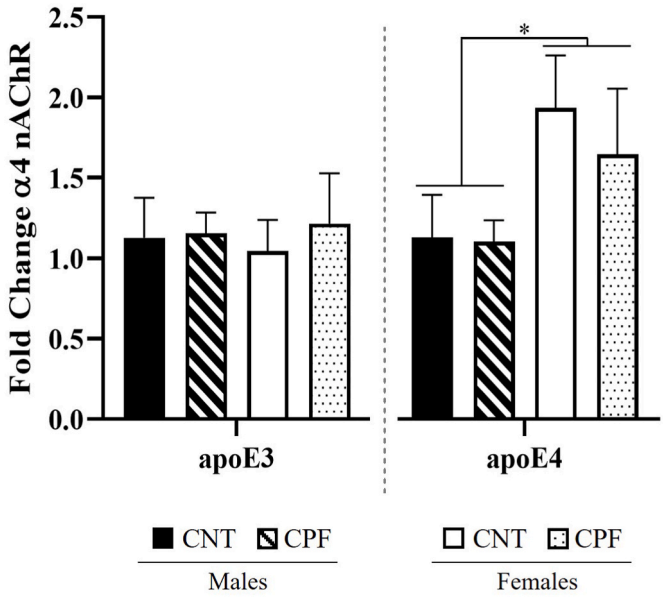

Fig. 9. Expression of α4 nAChR in apoE-TR mice. The symbol & indicates significant differences between genotypes at $p < 0.05$.

mice to VPA or CPF and apoE-TR mice to CPF during gestation. Communication and physical/motor development were evaluated in the preweaning period, while locomotor activity, anxiety behavior and expression of genes related to cholinergic system were assessed in adolescence. In C57BL/6J mice, we observed that both CPF and VPA decreased body weight and delayed eye opening. VPA-treated animals showed communication deficits after birth and anxiety-like behavior in adolescence, especially when we compared with CPF treated animals, while prenatal CPF affected both communication and anxiety but in the opposite way. Prenatal CPF exposure did not alter any of the parameters evaluated in the *APOE* genotype, but significant differences were observed between ε3 and ε4 homozygous carriers. Finally, cholinergic system was observed to be differently expressed depending on sex in C57BL/6J mice and genotype in apoE-TR mice.

Since no effect was observed on AChE activity, we can confirm that the dose used in this study had no toxicity or clinical effects, as Silva (2020) reported in her review. However, it must be taken into account that the time elapsed between maternal exposure and AChE activity determination is long enough for any effect to have disappeared (Basaure et al., 2018; Carr, 2001; Morales-Navas et al., 2020; Perez-Fernandez et al., 2020a, 2020b). Likewise, it is important to highlight that in our study we find little differences between genotype, sex or treatment in the expression of cholinergic system, although this is the

main target of CPF. The maturation of the cholinergic system begins after birth, concretely, during the first week of age (Leonzino et al., 2016), even though, transcription of the VChAT gene starts earlier having more than half of the adult levels at birth, while ChAT levels are relatively low at that time (Abreu-Villaça et al., 2011). This could explain the decrease in VChAT expression in VPA-treated females compared to CNTs.

Our results were unable to detect any maternal behavior that could explain differences between pups in USVs, motor and physical development or behavior later in life. However, we observed that body weight from PND 2 to 28 was lower in C57BL/6J mice exposed to both CPF and VPA. In addition, these treated animals showed a delay in eye opening, but no motor alterations. Although the literature about the influence of prenatal CPF exposure on fetal growth is scarce, a study on pregnant women living in New York associated the levels of CPF in umbilical cord with lower body weight and length at birth (Whyatt et al., 2004). On the other hand, treatment with VPA is associated with body weight gain in adolescents (Biton et al., 2003; Wirrell, 2003) and adults (Mattson et al., 1992), but Espinosa et al. (2008) did not observe any increase in the body mass index of prepubertal children. In our study, apoE3 mice showed higher body weight but poorer climbing ability, even though motor deficits were reversed as the animals grew (PND 21), since the mice carrying the *APOE* ε3 allele had greater strength than the *APOE* ε4 carriers. Published data indicate that the *APOE3* genotype is the most vulnerable to metabolic alterations such as obesity (Arbones-Mainar et al., 2008; Johnson et al., 2017; Peris-Sampedro et al., 2015a, 2018; Tejedor et al., 2014), so this could explain the differences observed in body weight.

Communication skills are affected by strain, age, sex and environmental factors (Caruso et al., 2022; Sasaki et al., 2020; Scattoni et al., 2009). Rodent USVs increase during the first PNDs which is when pups are strictly dependent on their mothers for survival, but progressively decrease as they develop physically and in terms of motor functions (Caruso et al., 2020). In CD-1 mice exposed to 6 mg/kg/day of CPF during late gestation, Venerosi et al. (2009) found that the number and duration of calls decreased, but the latency to the first vocalization increased on PND 10. Similar results in rats were found by Morales-Navas et al. (2020). In this case, the rats were exposed to 1 mg/kg/day of CPF during the late gestation. A decrease in the total number of calls and an increase in the latency were observed on PND 7. Furthermore, this study compared the results with a pharmacological model of autism, showing similarities between CPF- and VPA-treated group (Morales-Navas et al., 2020). In disagreement with these studies, our results showed opposite effects for CPF and VPA in C57BL/6J mice and no effect of CPF in apoE-TR mice. These discrepancies may be related to differences in the species or strain used, as well as the administered dose of CPF. However, increased USV rates have also been reported in several studies with idiopathic and knockout mouse models of autism (De Felice et al., 2015; Picker et al., 2006; Scattoni et al., 2008; Tsai et al., 2012). For this reason, we should point out the need to investigate the USV profile in *APOE* genetic background to determine whether some apoE isoforms follow an idiopathic communication profile or whether there are different rhythms of maturation, given that this is the first study to assess communication skills in apoE-TR mice.

Likewise, several studies have used developmental USVs to predict later-life anxious behaviors (Budylin et al., 2019; Lukas and Wöhr, 2015; Yamauchi et al., 2022). Although the literature about USVs and late anxiety-like behaviors is limited in terms of CPF and the *APOE* genotype, it is well known that treatment with VPA increases anxiety levels in rodents (Olexová et al., 2016; Schneider et al., 2006, 2008). In agreement with this, our VPA-treated mice showed a reduction in center velocity, indicating that they showed inactive behaviors in the inner zone as a sign of anxiety, but these effects were only significant compared to the CPF group. Furthermore, several studies observed that CPF exposure during late gestation or the first postnatal weeks can induce long-term alterations in terms of anxious behaviors in rodents (Braquenier et al., 2010; Ribeiro-Carvalho et al., 2020; Venerosi et al., 2010). However, the results obtained in this study show no differences between CNT and CPF-exposed mice. Our results on the *APOE* genotype are in agreement with those of Reverte et al. (2014b), which show that the *APOE* genetic background confers different anxiety behavior, with the ε4 allele being the most affected. However, it was not affected by CPF treatment.

In summary, prenatal and early postnatal are probably the most important stages in the development of a human or animal. Exposure to environmental toxics during these periods can have short-, mid- and long-term adverse effects related to neurodevelopmental disorders, and these effects may be influenced by the genetic background. Our results showed that communication and anxiety behaviors in animals after prenatal exposure to CPF differ from those observed in animals after exposure to VPA, whereas both treatments reduced body weight and delayed eye opening in C57BL/6J mice. On the other hand, we observed basal differences between *APOE* genotypes in all the variables evaluated, but they were not affected by treatment with CPF. The expression of cholinergic elements was affected depending on sex in C57BL/6J mice and *APOE* genotype. For this reason, we should point out that there are small differences in the maturation profile between species that could be conditioning later behavior, so further research is needed to evaluate the possible long-lasting effects of CPF in adults.

Author contributions

Judit Biosca-Brull: Methodology, Formal analysis, Writing – Original Draft. **Pia Basaure:** Methodology, Writing – Review & Editing. **Laia Guardia-Escote:** Methodology, Writing – Review & Editing. **Maria Cabré:** Methodology, Writing – Review & Editing. **Jordi Blanco:** Methodology, Writing – Review & Editing. **Miguel Morales-Navas:** Writing – Review & Editing. **Fernando Sánchez-Santed:** Conceptualization, Writing – Review & Editing. **Maria Teresa Colomina:** Conceptualization, Methodology, Writing – Review & Editing, Supervision, Project administration, 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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