Exposure to chlorpyrifos at different ages triggers *APOE* genotype-specific responses in social behavior, body weight and hypothalamic gene expression

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Abstract

To date, we have shown that apolipoprotein E (*APOE*) polymorphisms differentially modulate the neurobehavioral and metabolic effects of chlorpyrifos (CPF), a widely used pesticide, which is detected as residue in food. We previously reported that, after being exposed to CPF, *APOE3* subjects exhibit metabolic dysfunctions while *APOE4* subjects undergo changes in behavior. In the current study, we investigated the effects of a double exposure to CPF on social behavior and hypothalamic gene expression in apoE-targeted replacement (TR) mice. Male apoE3- and apoE4-TR mice were exposed to CPF at 0 or 1 mg/kg/day on postnatal days 10–15 and then, during adulthood (5 months of age), fed a CPF-supplemented diet (0 or 2 mg/kg/day) for 15 days. During adult exposure to CPF, body weight gain and food intake were monitored. At the end of the adult exposure period, we evaluated social behavior in a three-chamber test, as well as mRNA levels of hypothalamic neuropeptides and receptors related to social behavior and feeding control. Adult CPF exposure increased food intake in general, but only apoE4 mice increased their body weight. Postnatal CPF exposure improved preference for the social contexts in apoE4 mice while adult CPF exposure did the same in apoE3 mice. Anorexigenic-peptide and social-related behavior gene expression decreased as a result of adult CPF exposure in apoE4 mice, and *neuropeptide Y* was more expressed in apoE4 mice. These results indicate that CPF exposure produces orexigenic and metabolic effects and enlarges individual differences in social behavior, especially in apoE3 mice.

Keywords: Social behavior, feeding control, *APOE* genotype, chlorpyrifos, gene x environmental factor

1. Introduction

An increasing number of studies have focused on the association between exposure to non-persistent pesticides and medical conditions ranging from obesity to chronic and neurological diseases (Baillie-Hamilton and Phil, 2002; Dalsager et al., 2019; Wang et al., 2016). Individual factors such as age at exposure and genetic background are crucial for determining individual susceptibility to pesticide toxicity. Previous studies have indicated that exposure during developmental stages has a much greater potential risk than the same exposure in adulthood (Grandjean and Landrigan, 2014). Moreover, it is well known that the polymorphisms of the xenobiotic-metabolizing enzymes such as PON1 confer different neurobiological responses to exposure to the organophosphate pesticide (OP) chlorpyrifos (CPF) (Dardiotis et al., 2019; Marsillach et al., 2016). Nevertheless, epidemiological and model animal studies have shown that chronic exposure to CPF can lead to a wide variety of cognitive and motor activity alterations underlying neurodevelopmental disorders and neurodegenerative diseases, regardless of genetic variability (Burke et al., 2017; Mackenzie Ross et al., 2013; Sánchez-Santed et al., 2016). These deleterious effects are mainly associated with the disruption of cholinergic tone by inhibiting cholinesterase (ChE). However, several non-cholinesterase targets have also been described and it has been suggested that they contribute to the neurotoxicity of CPF (Abreu-Villaça and Levin, 2017; Burke et al., 2017). In this context, developmental exposure to CPF has been reported to affect the social behavior of wild-type mice in different ways (De Felice et al., 2014; Venerosi et al., 2015, 2012). Likewise, sex-dimorphic effects and changes in the neuropeptide expression such as vasopressin, estrogen receptor β and oxytocin in the amygdala and hypothalamus have been reported (Tait et al., 2009; Venerosi et al., 2015).

It is important to note that not only isoforms of xenobiotic-metabolizing enzymes can alter the toxic response to a pesticide. In this regard, we have reported that apolipoprotein E (*APOE*) variants influence individual susceptibility to such toxicants as polybrominated diphenyl ethers (PBDEs) (Reverte et al., 2013, 2012) and CPF (Basaure et al., 2019; Peris-Sampedro et al.,

2015a). ApoE is a lipid-transporter protein that has various neurobiological and metabolic purposes (Huang and Mahley, 2014; Mahley, 2016). The three most common *APOE* genotypes in humans are *APOE2*, *APOE3* and *APOE4*, with *APOE3* being the most frequent (Eisenberg et al., 2010). The *APOE4* genotype is considered as the major genetic risk factor for developing Alzheimer's disease (AD) (Huang and Mahley, 2014). It is well known that the cholinergic system is disrupted in AD patients. Basal differences have been found in cholinergic signaling between *APOE* variants (Dolejší et al., 2016) even at early ages (Basaure et al., 2018). Similarly, *APOE* genotype-by-age interactions have also been observed with the response to cholinergic environmental contaminants. Whereas healthy young *APOE4* carriers improved their cognitive skills after nicotine exposure, elderly smoker *APOE4-positive* carriers displayed poorer learning and memory functions than never-smokers or *APOE4-negative* carriers (Durazzo et al., 2016; Marchant et al., 2010). Along the same lines, using the mouse model apoE-targeted replacement (apoE-TR), we observed that effects of exposures to low doses of CPF in different stages of life are *APOE*-dependent. Our data suggest that apoE4 mice display more alterations in the cholinergic system and attention and inhibitory control after adulthood CPF exposure (Basaure et al., 2019; Peris-Sampedro et al., 2016). In turn, apoE3 mice show a decrease in the mRNA expression of presynaptic cholinergic elements after postnatal CPF exposure and several metabolic disruptions after adult CPF exposure (Basaure et al., 2018; Peris-Sampedro et al., 2015b; Peris-Sampedro et al., 2018)

Chronic exposure to pesticides means an important public health concern. Nowadays, the general population is continuously exposed to a long list of pesticides, of which OPs and especially CPF are the most commonly found in food (Bhandari et al., 2019; Curl et al., 2015). Throughout their lives, individuals are probably exposed to one or more pesticides more than once. However, little data are available considering multiple CPF exposures. The reported studies have used two patterns of double CPF exposure in animal models. One describes the effects of prenatal and then postnatal CPF exposure (Venerosi et al., 2012), while the other combines a 1-year chronic CPF exposure at low doses with acute high-dose of CPF administered every 2 months (Moser et al.,

2005; Padilla et al., 2005). Recently, we showed how a postnatal CPF exposure can alter the effect of an adult CPF exposure on spatial learning and memory abilities in adult mice (Basaure et al., 2019). We found that cognitive skills improved in apoE4 mice after adult exposure, but worsened in apoE3 mice after postnatal exposure. In addition, some of those deleterious effects were reverted by double exposure (Basaure et al., 2019).

Using an experimental design involving a similar postnatal plus adult exposure to CPF in adult male apoE3- and apoE4-TR mice (Basaure et al., 2019), in the present study we have examined the effects of social abilities and hypothalamic-mRNA levels of oxytocin, oxytocin-receptor, vasopressin, vasopressin receptor, estrogen receptor α and estrogen receptor β. Moreover, on the basis of our previous results on the metabolic imbalance caused by *APOE*-CPF interaction (Peris-Sampedro et al., 2015b, 2015a, 2018), we addressed this by investigating components involved in anorexigenic and orexigenic pathways of the hypothalamus such as pro-opiomelanocortin, cocaine- and amphetamine-related transcript protein, agouti-related protein and neuropeptide Y mRNA level, evaluating the body weight and food intake as well.

2. Material and methods

2.1 Animals

Human apoE3- and apoE4-TR homozygote mice were generated as previously described (Sullivan et al., 1997) and obtained from Taconic Europe (Lille Skensved, Denmark). After a quarantine period, females were mated with males of the same genotype. The day of delivery was designated as postnatal day (PND) 0. Only litters with 2-4 pups were used. Only male mice were used in this study. All of the mice had free access to water and given normal food (rodent chow from Panlab, Barcelona, Spain). The animal room was maintained at a temperature of 22 ± 2°C, a relative humidity of 50 ± 10% and a 12-h light/dark automatic light cycle (light: 08:00–20:00 h). The use of animals and the experimental protocols were approved by the Animal Care and

Use Committee of the Rovira i Virgili University (Tarragona, Spain) and were conducted in accordance with Spanish Royal Decree 53/2013 on the protection of experimental animals, and the European Communities Council Directive (2010/63/EU).

2.2. Experimental design

The current protocol had been already used in our research group (Basaure et al., 2019). CPF ([O,O-diethyl O-(3,5,6-trichloropyridin-2-yl) phosphorothioate] provided by Sigma-Aldrich, Madrid, Spain) was administered in two different periods of the mice's lifespan. The first period was from PND 10 to 15 (the postnatal treatment) while the second period was at 5 months of age for 15 consecutive days (the adult treatment). On PND 10, the litters were randomly assigned to either the treated postnatal-CPF group or the control group. Litters were weaned on PND 28 and housed in cages with peers of the same genotype and sex (4 animals per cage). Subsequently, at 5 months of age, each group was further divided into two, and randomly assigned to either the treated or non-treated adult-CPF. Hence, apoE3 and apoE4 male mice were divided into the following subgroups: 0-CPF (treated with vehicle from PND 10 to 15); P-CPF (treated with CPF from PND 10 to 15); A-CPF (treated with vehicle from PND 10 to 15 and treated with CPF at 5 months of age for 15 days); and A+P-CPF (treated with CPF from PND 10 to 15 and at 5 months of age for 15 days) (Fig. 1). The housing protocol followed the same line during the adult treatment. For the postnatal treatment, CPF was dissolved in corn oil and adjusted in order to administer 1 mg/kg in 1 µL/g of body weight. Thus, pups were treated with an oral dose of 0 or 1 mg/kg using a micropipette. For the adult treatment, CPF was administered daily at 4 g/mouse of CPF-supplemented rodent chow. As described in previous studies (Basaure et al., 2017; Peris-Sampedro et al., 2018) the standard food was supplemented with 15 mg CPF/kg chow (Panlab, Barcelona, Spain) to deliver 2 mg/kg/day. In order to verify the dose mice were exposed to every day, the body weight of the mice and the food intake during the adult treatment period were monitored weekly (1.98±0.06 mg/kg of CPF). These data were also used to analyze the Food Efficiency Ratio (FER), which was defined as follows: body weight gain (g/week)/food intake

(g/week) x 100. Both doses of CPF were already used in previous studies (Basaure et al., 2018, 2019; Guardia-Escote et al., 2018; Peris-Sampedro et al., 2015a, 2015b, 2018).

At the end of the adult treatment period, the mice were divided in two groups. One group (n=76) was used to evaluate social behavior. The other group (n=36) was sacrificed to assess gene expression. Number of animals used is described in Table 1. Sacrifice was performed by exsanguination under isoflurane anesthesia. Mice were decapitated and the brain removed, to obtain the hypothalamus samples. All samples were stored at −80ºC until analysis.

2.3. Social behavior

At 5 months of age and 15 days after the adult treatment started, a total of 76 male mice (8-11=n/group) were used to evaluate sociability and the preference for social novelty in a three-chamber apparatus similar to that undertaken by Crawley (2004). Briefly, the apparatus consisted of a rectangular box (45 x 15 x 30 cm) with three interconnected chambers (15 x 15 x 30 cm). The floor and walls were made of transparent Plexiglas (PL10011, Polimark®, Monza, Italy). Additionally, two identical black metal wire cups (7 x 7 cm) were placed upside down in each of the lateral chambers in which unknown mice or an inanimate object (i.e. red plastic frog, 2.5 x 2.5 cm) were placed when required. To assess sociability, an age-, sex-, *APOE* genotype- and size-matched unknown mouse was placed in one wire cup (social chamber) and an inanimate object in the other one (non-social chamber). To test preference for social novelty, a different unknown mouse was placed in the wire cup that had previously been used for the inanimate object (novel chamber) and the first unknown mouse was left in its original wire cup (non-novel chamber). Before assessing social behavior, the mice were habituated to the apparatus with the empty wire cups for 10 min. Habituation, sociability and preference for social novelty were then assessed sequentially. In both social phases, animals were allowed to move freely for a total of 6 min in the apparatus. At the end of each phase, the mice were gently guided to the central chamber where they stayed until the next phase started. To prevent olfactory cue bias, the apparatus and

the wire cups were cleaned with 70% ethanol solution between each mouse. Mice were recorded by a video camera (Sony CCD-IRIS), and the recordings were then computerized through a video-tracking program (Etho-Vision© XT 11.5, Noldus Information Technologies, Wageningen, The Netherlands). The time spent in each chamber during habituation and the two social conditions was quantified. Then, in these three phases, we evaluated the exploratory activity, which was calculated as follows: time spent in one lateral chamber + time spent in the other lateral chamber. To assess social interactions in both social conditions, we analyzed two social variables, which were defined as follow: social index = [(time spent in social chamber - time spent in non-social chamber)/exploratory activity in sociability phase]; and novelty index = [(time spent in novel chamber - time spent in non-novel chamber)/exploratory activity in novelty phase].

2.4. Gene expression

Hypothalamic gene expression of oxytocin (*Oxt*), oxytocin receptor (*Oxtr*), arginine vasopressin (*Avp*), arginine vasopressin receptor 1A (*Avpr1a*), estrogen receptor α (*Esr1*), estrogen receptor β (*Esr2*), pro-opiomelanocortin *(Pomc)*, cocaine- and amphetamine-related transcript protein *(Cartpt)*, agouti-related protein *(Agrp)* and neuropeptide Y *(Npy)* (n=4-5/group) was determined by real-time polymerase chain reaction (qPCR) analysis. The full process was performed with RNase-free tubes and pipette tips, and the surfaces were cleaned with RNaseZap solution (ThermoFisher Scientific, Waltham, MA, USA). Total RNA of the whole hypothalamus was extracted with the SPEEDTOOLS Total RNA Extraction kit (Biotools, Madrid, Spain). RNA concentration and purity were determined with a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). The Maxima First Strand cDNA Synthesis Kit for RT-qPCR (ThermoFischer Scientific, Waltham, MA, USA) was used to synthesize cDNA from 1 μg RNA samples. The cDNA samples were distributed in a 72-well rotor in duplicate to carry out the qPCR reactions in the Rotor-Gene Q Real-Time PCR cycler (Qiagen Inc., Hilden, Germany) with the Maxima SYBR Green/ROX qPCR Master Mix (2X) kit (ThermoFisher Scientific, Waltham, MA, USA). The primer sequences used are shown in Supplementary

material (Table S1). The Rotor-Gene Q 2.0 software was used to calculate the comparative cycle threshold (Ct). Each sample was normalized against its *Gapdh* expression to determine the relative gene expression levels, and $2^{-\Delta\Delta Ct}$ was calculated for purposes of analysis.

2.5. Statistics

Statistical analyses were performed using the SPSS Statistics 25.0 software (IBM Corp, Chicago, IL, USA) and MATLAB R2018a (The Mathworks Inc., Natwick, MA, USA). Genotype, postnatal treatment (P-CPF) and adult treatment (A-CPF) were analyzed as different factors, in order to address the following questions: were social behaviors and/or gene expression affected by (i) postnatal exposure to CPF alone? (ii) adult exposure to CPF alone? (iii) a mixture of both postnatal and adult exposure to CPF? (iv) genotype and postnatal or adult exposure to CPF? Body weight was analyzed by means of a repeated measure multivariate Analysis of Variance (RMANOVA). A three-way Analysis of Variance (ANOVA) was used to evaluate both social behaviors and gene expression. Principal Component Analysis (PCA) was used to explore gene expression data. A one-sample *t* test was applied to compare the social variables to a fixed neutral level (i.e., 0). This fixed neutral level was defined by the chance of being in both lateral chambers at the same time, showing interest by neither social/novelty nor non-social/non-novelty scenario. When appropriate, differences between groups were assessed by the *post-hoc* Tukey test. The homogeneity of variance was determined using the Levene test. All data were expressed as mean values ± SE. Statistical significance was set at $p<0.05$.

3. Results

3.1. Body weight, body weight gain, food intake and food efficiency ratio

Body weight during the adult treatment period (Fig. 2) was analyzed by a RMANOVA (genotype x P-CFP x A-CPF), using the time (baseline week, when mice started the adult treatment, and the

two weeks of adult treatment) as the within-subject factor. We found an effect of the time [$F_{2,67}$=16.38, p<0.001], an interaction between time and genotype [$F_{2,67}$=8.03, p=0.001] and an interaction between time and A-CPF [$F_{2,66}$=10.86, p<0.001]. It indicates that although all mice gained weight over time, this increase depended on the genotype and whether the animals were treated during adulthood or not. In addition, a general effect of genotype [$F_{1,68}$=16.60, p<0.001] and P-CPF [$F_{1,67}$=4.11, p=0.047] was observed. Overall, apoE3 mice weighed more than apoE4 mice, while mice exposed to postnatal CPF started the adult treatment with a higher body weight than the non-exposed mice [$F_{1,75}$=5.83, p=0.018]. However, adult-treated groups began to gain weight at two weeks on the adult treatment [$F_{1,75}$=4.47, p=0.038]. This increase was more evident in apoE4 mice [$F_{3,39}$=3.08, p=0.040].

A three-way ANOVA (genotype x P-CFP x A-CPF) was used to evaluate body weight gain, food intake and FER during the two weeks of adult treatment. As far as weight gain is concerned, there was an overall effect of genotype [$F_{1,75}$=10.93, p=0.002] and A-CPF [$F_{1,75}$=15.72, p<0.001], as well as an interaction between genotype and A-CPF [$F_{1,75}$=5.12, p=0.027]. These results indicate that adult treatment promoted an increased weight gain, particularly in apoE4 mice (Table 2).

Food intake was influenced by P-CPF [$F_{1,75}$=15.72, p<0.001] and A-CPF [$F_{1,75}$=84.20, p<0.001]. Food intake was also affected by interactions between genotype and A-CPF [$F_{1,75}$=4.79, p=0.032], P-CPF and A-CPF [$F_{1,75}$=6.41, p=0.014], and genotype, P-CPF and A-CPF [$F_{1,75}$=12.45, p=0.001]. As shown in Table 2, food intake was increased by adult treatment, regardless of the genotype. To better analyze the interactions with the genotype, additional analysis of the treatment groups indicated that A-CPF and A+P-CPF apoE3 groups consumed more food than P-CPF apoE3 mice (p<0.005). Likewise, A-CPF and A+P-CPF apoE4 groups presented a higher food intake than 0-CPF apoE4 mice (p<0.005).

In the case of FER, genotype [$F_{1,75}$=9.68, p=0.003] and A-CPF [$F_{1,75}$=19.01, p<0.001] were observed to have the main effects. Food efficiency was increased in apoE4 mice in the adult

treated groups (Table 2). A more detailed analysis showed that the A+P-CPF apoE4 group significantly increased the FER in comparison to both P-CPF and control apoE4 mice ($p<0.05$) (Table 2).

3.2. Social behavior

3.2.1. Exploratory activity in the three-chamber apparatus in the different phases.

Exploratory activity was analyzed by a three-way ANOVA (genotype x P-CPF x A-CPF) for each one. Throughout habituation, activity was similar in all the groups. However in the social phase, genotype [$F_{1,76}=67.85$, $p<0.001$], A-CPF [$F_{1,76}=11.81$, $p=0.001$], the interaction between genotype and P-CPF [$F_{1,76}=8.14$, $p=0.006$] as well as the interaction between genotype, P-CPF and A-CPF [$F_{1,76}=4.44$, $p=0.039$] influenced activity levels. On the other hand, differences during the novelty phase were only affected by genotype [$F_{1,76}=79.73$, $p<0.001$]. As shown in Fig. 3, levels of activity during the habituation were similar between apoE3 and apoE4 mice. However, apoE3 mice increased their activity in both the social and the novelty phases, when the new stimuli were presented in the wire cups. Treatment effects were only noticed in the sociability phase, being P-CPF apoE4 mice those who showed the lowest activity of all the apoE4 groups (P-CPF vs 0-CPF: $p=0.009$; P-CPF vs A-CPF: $p<0.001$; P-CPF vs A+P-CPF: $p=0.017$).

3.2.2. Sociability and preference for novelty in the three-chamber apparatus

In order to analyze sociability and preference for social novelty, social and novelty index were compared with chance exploration (i.e., 0 s) in each group by means of a one-sample *t* test (Fig. 4). Thus, a high positive index indicated a social/novelty preference and a high negative index indicated a non-social/non-novelty behavior. In the sociability phase (Fig. 4A), A-CPF apoE3 and A+P-CPF apoE3 showed a significant preference for the social stimulus (A-CPF: [$t_7=4.12$, $p=0.004$]; A+P-CPF: [$t_9=3.06$, $p=0.014$]). Moreover, P-CPF apoE4 and A+P-CPF apoE4 groups

showed a tendency to prefer the social chamber (P-CPF: [t_9=2.16, p=0.058]; A+P-CPF: [t_8=2.19, p=0.059]). In turn, the analysis in the novelty phase indicated that A-CPF apoE3, A+P-CPF apoE3 and P-CPF apoE4 showed a significant preference for the social novel stimulus (A-CPF apoE3 [t_7=3.23, p=0.014]; A+P-CPF apoE3 [t_9=3.97, p=0.003]; P-CPF apoE4 [t_9=2.55, p=0.031]) (Fig. 4B).

To better understand differences between groups, the influence of the genotype, the postnatal exposure to CPF and the adult exposure to CPF involved in both social indexes were analyzed by a three-way ANOVA (genotype x P-CPF x A-CPF) (Fig. 4). In the sociability phase, an interaction between genotype and P-CPF was observed [$F_{1,76}$=6,04, p=0.016], indicating that the two genotypes were affected differently by the postnatal exposure to CPF. P-CPF apoE3 mice showed less preference for sociability, in contrast to P-CPF apoE4 mice, which showed more preference (Fig. 4A). Meanwhile, in the novelty phase, two interactions were found: one between genotype and A-CPF [$F_{1,76}$=6.04, p=0.016] and the other between genotype, P-CPF and A-CPF [$F_{1,76}$=4.35, p=0.041]. Further statistical tests revealed differences between P-CPF apoE3 and A+P-CPF apoE3 mice (p=0.022) in their preference for the social novel stimulus (Fig. 4B).

In summary, the effect of CPF treatment depended on the genotype. Thus, while adult exposure to CPF increased the interest that apoE3 mice had for social stimuli, it seemed to have no effect on apoE4 mice. Instead, postnatal exposure to CPF increased social and novelty interest in apoE4 mice, but decreased social and novelty interest in apoE3 mice. Interestingly, the influence of both postnatal and adult exposure to CPF accentuated differences between subjects.

3.3. Hypothalamic gene expression

In order to detect gene targets affected by CPF exposure, hypothalamic genes involved in social behavior and feeding control were measured 15 days after the adult treatment started.

Firstly, all genes were analyzed together by a PCA (Fig. 5). The results revealed three main components. PC1 explains about 54.5% of the variance in our data, which is mostly related to the social genes. *Oxt* (r=0.814), *Oxtr* (r=0.697), *Avp* (r=0.874), *Avpr1a* (r=0.888) and *Esr1* (r=0.643) were positively correlated with PC1, as well as *Pomc* (r=0.817) and *Cartpt* (r=0.769). PC2 describes about 14.4% of the variance, where *Npy* (r=0.941) and *Agrp* (r=0.784) were strongly correlated. PC3 explains 10.6%, where only *Esr2* was strongly correlated (r=0.930). Thus, in the mice of the current study, genes clustered in each PC vary together, sharing a specific expression pattern.

Social behavior-related genes and feeding control-related genes were individually analyzed by a three-way ANOVA (genotype x P-CPF x A-CPF).

3.3.1. Social behavior-related genes

The expression of *Oxt* and *Oxtr* was affected by an interaction between genotype and A-CPF (*Oxt* [$F_{1,35}$=5.41, p=0.027]; *Oxtr* [$F_{1,35}$=5.98, p=0.021]). As shown in Fig. 6, *Oxt* (Fig. 6A) and *Oxtr* (Fig. 6B) presented similar expression profiles. While both mRNAs decreased in apoE4 mice after adult exposure to CPF, they increased in apoE3 mice after the same exposure.

Similar expression profiles were also observed for *Avp* and *Avpr1a*. In the case of *Avp*, we found an effect of the genotype [$F_{1,35}$=6.43, p=0.017] and A-CPF [$F_{1,35}$=5.99, p=0.021], and an interaction between genotype and A-CPF [$F_{1,35}$=15.45, p=0.001]. However, *Avpr1a* expression was affected by A-CPF [$F_{1,35}$=10.87, p=0.003], and the interaction between genotype and A-CPF [$F_{1,35}$=11.84, p=0.002]. In detail, a further analysis revealed that *Avp* expression significantly decreased in adult-treated apoE4 mice, in comparison to the other groups (Fig. 6C). It was also found that expression of *Avp* in P-CPF apoE3 mice was lower than in A+P-CPF apoE3 mice (p=0.038) (Fig. 6C). Likewise, adult-treated apoE4 had a lower *Avpr1a* expression than adult-untreated apoE4 mice (Fig. 6D).

In contrast, *Esr1* and *Esr2* had different expression profiles (Fig. 7). In the analysis of *Esr1*, a general effect of A-CPF [$F_{1,35}$=5.14, p=0.031] and an interaction between genotype and A-CPF [$F_{1,35}$=8.24, p=0.008] were observed. As shown in Fig. 7A, *Esr1* expression was significantly lower in adult-treated apoE4 mice compared to their counterparts. In the case of *Esr2*, no significant differences were found (Fig. 7B).

3.3.2. Feeding control-related genes

The expression of *Pomc* and *Cartpt* was influenced by an interaction between genotype and A-CPF (*Pomc* [$F_{1,35}$=8.17, p=0.008]; *Cartpt* [$F_{1,35}$=4.88, p=0.036]). As shown in Fig. 8, *Pomc* (Fig. 8A) and *Cartpt* (Fig. 8B) displayed similar expression profiles. Adult treated apoE4 groups exhibited always a lower expression compared to untreated apoE4 peers.

Otherwise, while we observed an interaction between P-CPF and A-CPF [$F_{1,35}$=4.50, p=0.043] in *Agrp* expression, a general effect of genotype [$F_{1,35}$=9.28, p=0.004], and an interaction between P-CPF and A-CPF [$F_{1,35}$=7.20, p=0.012] were found for *Npy*. In the case of *Agrp*, adult treatment with CPF reduced its expression in apoE4 mice, while double-treated or postnatal-treated groups did not show this trend (Fig. 8C). On the other hand, *Npy* expression was higher in apoE4 mice than in apoE3 mice (Fig. 8D). Further analysis indicated that P-CPF apoE3 mice had a lower expression of *Npy* than A+P-CPF apoE3 mice (p=0.034) (Fig. 8D).

4. Discussion

In the current investigation, we assessed the effects of different exposures to low doses of CPF on social behavior in adult male mice and its interaction with the *APOE* genotype. *APOE* polymorphisms have shown differences in several behavioral and metabolic conditions. Therefore, this study also included the expression of genes related to social and feeding behavior.

Briefly, apoE4 mice were lighter than apoE3 mice. Postnatal CPF exposure increased body weight and adult CPF exposure enhanced food intake. Moreover, adult CPF exposure induced a sharp increase in the body weight of apoE4 mice, which also affected their food efficiency ratio. However, apoE4 mice did not increase their body weight more than control apoE3 mice, and exposed apoE3 mice remained the heaviest group. Regarding social behavior, apoE3 mice displayed greater exploratory activity. Adult CPF exposure caused a marked preference for the social context in apoE3 mice while postnatal CPF exposure had the same effect in apoE4 mice. It is worth noting that the genes studied can be organized into two profiles: the first one consists of the social behavior-related genes (*Oxt*, *Oxtr*, *Avp*, *Avpr1a* and *Esr1*) and two feeding control-related genes, (*Pomc* and *Cartpt*), and the second profile consists of the feeding control-related genes *Npy* and *Agrp*.

To date, a number of studies have attempted to shed light on how *APOE* polymorphisms influence body mass index (BMI), food consumption and metabolic responses to environmental stimuli (Arbones-Mainar et al., 2016; Grootendorst et al., 2005; Peris-Sampedro et al., 2018; Segev et al., 2016). In the current study, apoE-TR mice showed basal differences in body weight, with apoE3 being the heaviest. This is in agreement with previous rodent and human investigations, which have associated BMI with *APOE* genotypes in the following order: *APOE2>APOE3>APOE4* (Grootendorst et al., 2005; Tejedor et al., 2014; Volcik et al., 2006). However, we recently found that apoE3 mice weighed more than apoE4 and C57BL/6 after 8-13 weeks of adulthood exposure to CPF (Peris-Sampedro et al., 2015a, 2015b), and also manifested disruption of insulin pathways in the liver (Peris-Sampedro et al., 2018). In this case, the mice exposed to postnatal CPF weighed more at the age of adult treatment. Similar findings were also reported by Lassiter and Brimijoin (2008) in rats. In this regard, it has been suggested that developmental exposure to CPF induces a reprogramming of body weight control and metabolic functions (Lassiter and Brimijoin, 2008; Meyer et al., 2004). On the other hand, although in the present study adult exposure increased food intake regardless of the genotype, adult-treated apoE4 mice significantly increased body weight and subsequently the FER.

Even though food overconsumption might be causing weight gain, body weight control is not simply a simple result of the balance between energy intake and expenditure. In fact, several metabolic and behavioral mechanisms contribute to this balance and settling a stable body weight over relatively long periods of time (Baillie-Hamilton and Phil, 2002). The main integrative circuit of feeding behavior in mammals is located in the hypothalamus and contains two types of neuron. The first type expresses NPY and AgRP, the orexigenic peptides. The second type expresses POMC and CART, the anorexigenic peptides (Berthoud and Morrison, 2007; Zheng and Berthoud, 2008). In our case, the variations detected in body weight are in accordance with the observed gene expression profiles. With regard to it, the expression of the orexigenic-peptide *Npy* was greater in apoE4 mice, with the double-exposed apoE4 group being the most affected. Even so, *Agrp* expression appeared to have diminished only in the adult single-exposed apoE4 group. In turn, the expression of anorexigenic-peptide genes was only diminished in apoE4 mice after adult exposure to CPF. Recently, Herman et al. (2017) reported that a disruption in the cholinergic innervation of the basal forebrain reduced *Pomc* transcript levels, while also caused a marked increase in food consumption in mice. In the present case, we have found various differences in cholinergic expression in other brain areas between apoE3- and apoE4-TR mice, as well as changes after CPF exposure (Basaure et al., 2019, 2018; Guardia-Escote et al., 2018). It would be interesting to explore whether the alterations produced by CPF exposure in the cholinergic inputs into the hypothalamus are affecting feeding and metabolic control. In parallel, social behavior modulators such as OXT, OXTR, AVP , AVP and ESRα are also involved in feeding control behavior, and diminish food consumption through different pathways. OXT and OXTR signaling act as modulators of meal size (Olson et al., 1991; Ong et al., 2017). AVP and AVPR activation decrease food intake (Freeman et al., 2018; Pei et al., 2014) and ESRα deletion in POMC neurons triggers hyperphagia (Xu et al., 2011).-In this study, a decreased expression of *Oxt, Oxtr, Avp, Avpr1a* and *Esr1* genes in adult-exposed apoE4 mice could also have contributed to the observed increase in food intake. Interestingly, these genes displayed e same expression pattern of the ~~other~~ anorexigenic-peptide genes studied. However, the differences in *Oxt*

expression were more evident between the adult-exposed apoE3 and apoE4 groups. Therefore, we suggest that these variations may be more related to other effects observed in this study, such as social behavior.

Mice are essentially social animals, so social interactions and conspecific recognition enable them to establish hierarchies or identify new pairs (Berry and Bronson, 1992). To the best of our knowledge, this is the first study evaluating the influence of the *APOE3* and *APOE4* genotype on social outcomes. Nevertheless, human and animal model studies have already reported that aggressive behaviors are more common in *APOE4* carriers than in non-carriers (Chan and Shea, 2007; Craig et al., 2004; van der Flier et al., 2006), which suggests that social interactions might also be regulated by the *APOE* genotype. Using the three-chamber paradigm, we found that apoE3 mice were very willing to explore the apparatus in social and novelty sessions, which indicated that they were more aroused after stimulation. In fact, adult-exposed apoE3 mice exhibited the most socially proactive behavior. On the other hand, although the postnatal-exposed mice were the most prosocial among the apoE4 mice, apoE4 mice showed less activity in both contexts. In this case, we cannot rule out the possibility that the apparatus with the object and unknown animals could have triggered an anxiety-like response. Indeed, while some anxious contexts might encourage some animals to actively escape from an aversive stimulus, they might cause others to freeze or decrease their general activity (Pawlak et al., 2008). With respect to this, several studies have revealed that apoE4-TR mice display an anxiety-like phenotype (Meng et al., 2017; Reverte et al., 2013, 2012). Despite differences in exploratory activity, both apoE3 and apoE4 mice showed an interest in social scenarios. It is noteworthy that the postnatal-treated apoE4 group showed prosocial behavior, but also the lowest activity.

Social behavior is associated with a greater preference for social stimuli and new encounters, and encompasses social motivation and affiliation behavior (Crawley, 2004; Kohls et al., 2012). Surprisingly, the effect of CPF exposure on social behavior depended on the genotype. Different effects of developmental exposure to low doses of CPF have been reported. While some studies

have shown no effect on social behaviors in males after postnatal exposure to either 1 or 3 mg/kg of CPF (Ricceri et al., 2003), others have observed greater social interest after perinatal exposure (Venerosi et al., 2015) and a deficit of social preferences after prenatal exposure (Lan et al., 2019, 2017). These discrepancies could also be explained by the fact that different strains of mice were used in those studies. As discussed above, differences between apoE-TR mice might be partly related to *APOE*-mediated regulation in cholinergic status. In relation to this, Karvat and Kimchi (2014) described that donepezil, an AChE inhibitor, improved new social interactions in a model of autism. Along the same lines, prenatal exposure to CPF has been associated with a reversion in inherent social abnormalities (De Felice et al., 2015; Mullen et al., 2013). Likewise, social motivation deficits caused by disruption to cholinergic innervations have also been observed after selective damage to the nucleus basalis magnocellularis in rats (Savage et al., 2011). Given the involvement of the cholinergic system in cognitive flexibility and the appraisal of new stimuli (Pepeu and Giovannini, 2004), social behavior and motivation, we can hypothesize that our results are related to the shifts induced by CPF exposure in the cholinergic system tone. Taken together, all factors, age at CPF exposure and *APOE* genotype give rise to a myriad of dissimilar effects.

As above discussed, the age at which animals are exposed, the length of the exposure and the doses of CPF are also issues to bear in mind. However, there is very little information in the scientific literature on the question of multiple exposures or exposures to complex mixtures, as well as the harm they can cause. For instance, in previous studies we observed that both postnatal (Guardia-Escote et al., 2018) and adult exposure to CPF (Peris-Sampedro et al., 2015a) trigger effects on spatial learning and memory functions, which are different from those of a double exposure, one postnatal plus the other in adulthood (Basaure et al., 2019). This means an important concern given the long list of environmental compounds and continuous multiple exposures throughout an individual's life (Grandjean and Landrigan, 2014). Although both apoE3- and apoE4-TR mice did not show basal differences in social interest, CPF administration clearly led to the emergence of two different populations within each genotype, depending on the age at CPF exposure. Thus, exposure to CPF increases the basal differences between prosocial

and unsocial groups. We can also infer that exposure to CPF throughout life would result in several genotype-mediated fluctuations in the desire to seek social contact. In turn, the regulation of hypothalamic neuropeptides such as OXT and AVP and their receptors plays an important role in social and reproductive outcomes. Both systems facilitate pair-bonding, maternal care and social recognition (Donaldson and Young, 2008). In parallel, ESRα ~~α~~ and ESRβ are also important for social interactions mediated by OXT and AVP signaling (Murakami et al., 2011). Likewise, gene expression related to social behavior is also modified by CPF exposure. The literature describes long-term changes in AVP levels after a prenatal plus postnatal double exposure to CPF (Tait et al., 2009), an increased expression of *Esr2* in the hypothalamus and a decreased *Oxt* expression in the amygdala after perinatal exposure from gestational day 15 to PND 14 (Venerosi et al., 2015). In the current investigation, although the differences within the same genotype did not always reach a significance level, it is clear that the most prosocial groups such as adult-treated apoE3 or postnatal-treated apoE4 mice are associated with high *Oxt* and *Avp* expression. In fact, expression of *Avp, Avpr1a, Oxt and Esr1* was decreased only in adult-exposed apoE4 mice. We can suggest that this general decrease is associated with some kind of disruption in affiliative social behavior in apoE4 mice. These observations about gene expression require further investigations into the social interactions that promote parenting and attachment (Panksepp et al., 2007). Even so, gene expression and social assessment outcomes suggest that not only social interest can be modified, but also the establishment of relations between conspecifics. Likewise, previous studies from our group have also described that CPF triggers sex-specific effects on cognitive functions in apoE-TR mice (Basaure et al., 2019; Guardia-Escote et al., 2018). Therefore, additional studies using female mice will be needed, in order to develop a full picture of the vulnerability to CPF depending on *APOE* genotype in social behavior as well as the metabolic status.

5. Conclusion

Genetic background is an important factor that leads to differential vulnerability upon exposure to environmental toxicants. In the present investigation, the effects of CPF exposure caused differences in both *APOE3* and *APOE4* variants. Body weight is altered by adult exposure to the pesticide, with postnatal exposed subjects being more susceptible to this adult exposure, especially apoE4 mice. Social behaviors are also affected, and individual differences within the population are enlarged. It should be noted that postnatal exposure to CPF impairs sociability in apoE3 but improves it in apoE4, while adult exposure improves sociability only in apoE3 mice. These changes run in parallel with those observed in gene expression in the hypothalamus especially in apoE4 mice. Likewise, we continue to stress that toxicological studies need to be carried out with different animal models to emulate human population susceptibility to environmental threats.

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Figure captions

Fig. 1. Schematic illustration of the experimental design displaying age of first and second period of exposure to CPF, doses used and treated groups. Male apoE3- and apoE4-TR mice were treated with either CPF 0 or 1 mg/kg/day on PND 10-15, the first period of treatment (gray). Then, mice were treated with either CPF 0 or 2 mg/kg/day for 15 days at 5 months old, the second period of treatment (black). Thus, mice were grouped as follows: 0-CPF (treated with vehicle from PND 10 to 15); P-CPF (treated with CPF from PND 10 to 15); A-CPF (treated with vehicle from PND 10 to 15 and treated with CPF at 5-months-of-age for 15 days); and A+P-CPF (treated with CPF from PND 10 to 15 and at 5-months-of-age for 15 days).

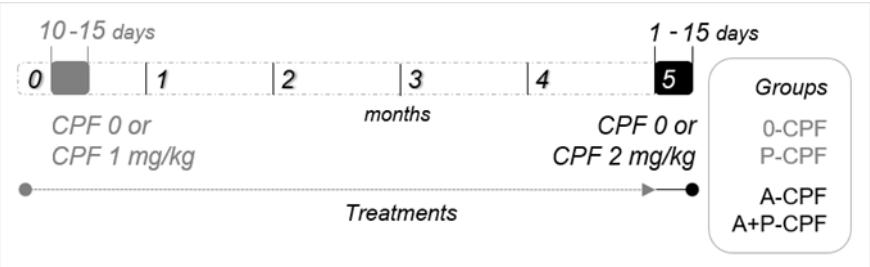

Fig. 2. Body weight status during adult exposure to CPF and 5 months after postnatal CPF exposure. Differences between apoE3- and apoE4-TR mice are represented with different letters. The symbol # indicates differences between the A+P-CPF apoE4 group and the other apoE4 groups at $p<0.05$.

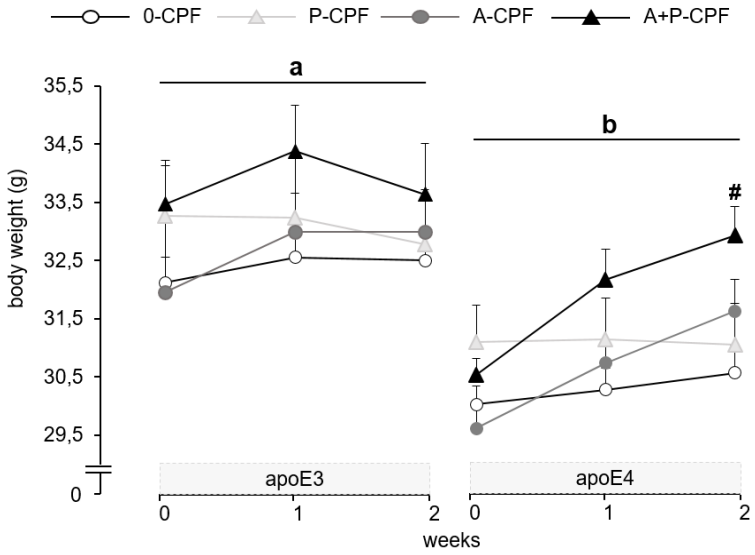

Fig. 3. Exploratory activity in the habituation, sociability and preference for social novelty phases in the three-chamber test at the end of the adult exposure to CPF and 5 months after postnatal CPF exposure. Differences between apoE3- and apoE4-TR mice in the sociability and novelty phases are represented with different letters. The symbol # indicates differences between the P-CPF apoE4 group and the other apoE4 groups at $p<0.05$.

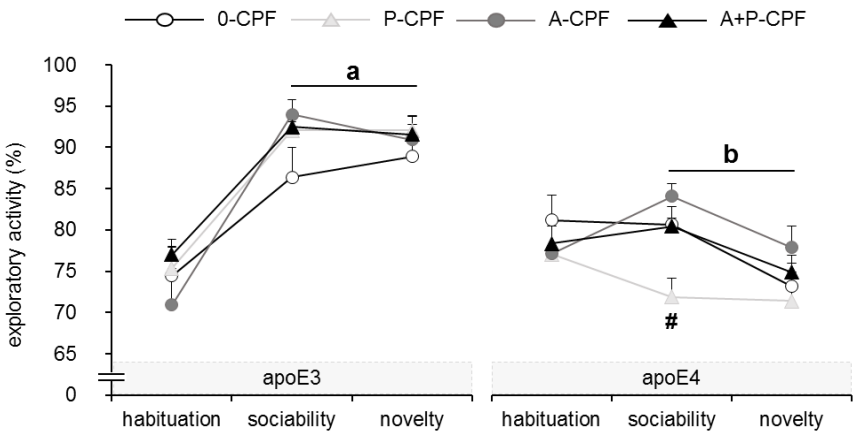

Fig. 4. Sociability and preference for social novelty assessed by the three-chamber test at the end of the adult exposure to CPF and 5 months after postnatal CPF exposure. Social index (A) is calculated as follows: (time spent in social chamber - time spent in non-social chamber)/exploratory activity in sociability phase. Novelty index (B) is calculated as follows: (time spent in novel chamber - time spent in non-novel chamber)/ exploratory activity in novelty phase. Differences between each group and the chance level (i.e., 0) are represented by an asterisk. The symbol # indicates differences between the P-CPF apoE3 and the A+P-CPF apoE3 group at $p<0.05$.

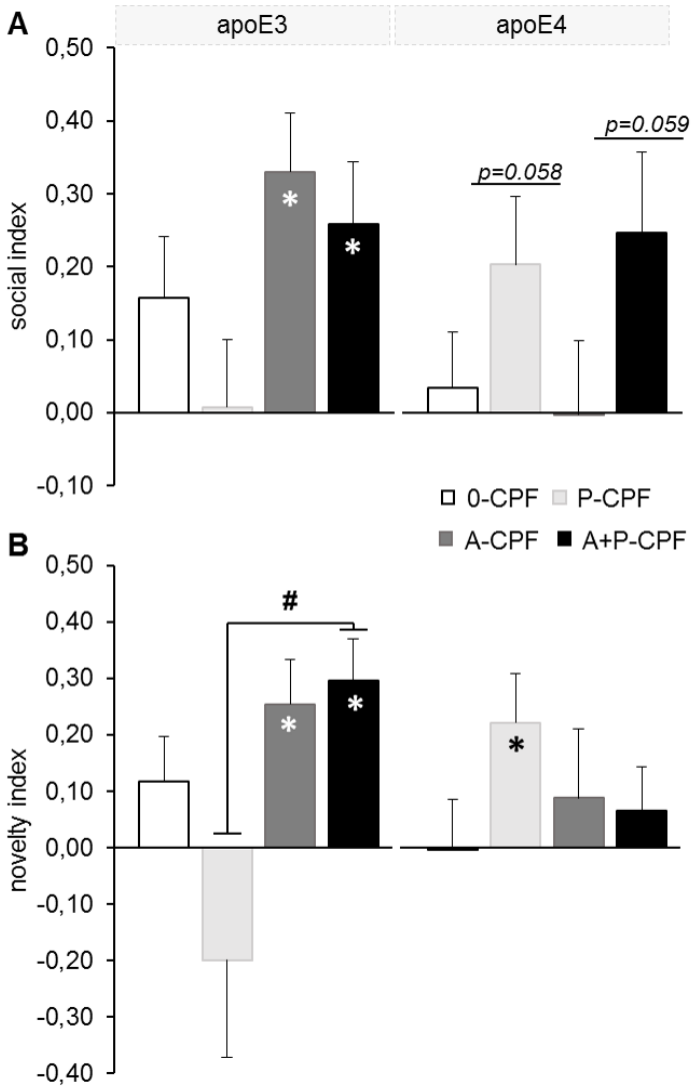

Fig. 5. Relative gene expression in the hypothalamus of elements related to social behavior and feeding control at the end of adult exposure to CPF and 5 months after postnatal CPF exposure**.** PC1 components were *Oxt*, *Oxtr*, *Avp*, *Avpr1a*, *Esr1*, *Pomc* and *Cartpt*. PC2 components were *Agrp* and *Npy*. The PC3 component was *Esr2*.

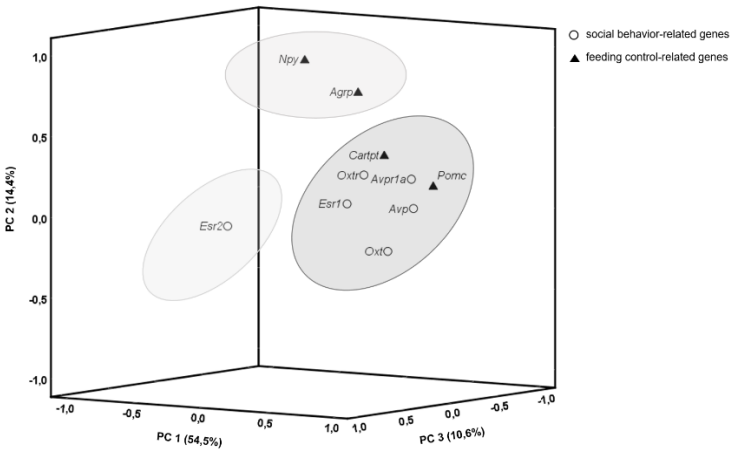

Fig. 6. Relative gene expression in the hypothalamus of *Oxt* (A), *Oxtr* (B), *Avp* (C) and *Avpr1a* (D) at the end of the adult exposure to CPF and 5 months after postnatal CPF exposure**.** Differences between groups are represented with different letters. The symbol # indicates differences between the P-CPF apoE3 and the A+P-CPF apoE3 group at $p<0.05$.

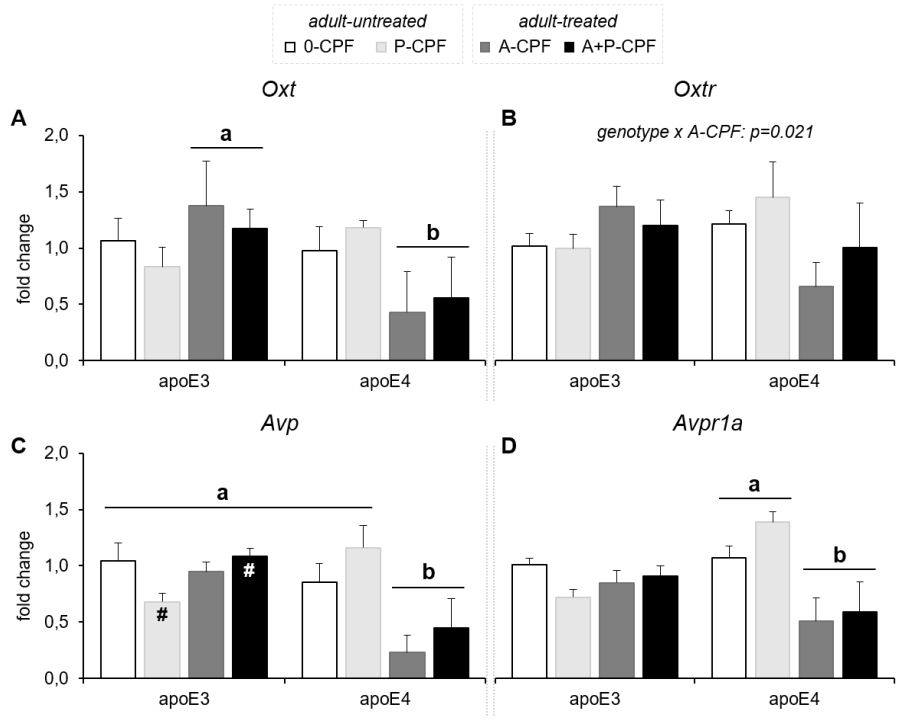

Fig. 7. Relative gene expression in the hypothalamus of *Esr1* (A) and *Esr2* (B) at the end of the adult exposure to CPF and 5 months after postnatal CPF exposure**.** Differences between groups are represented by an asterisk at $p<0.05$.

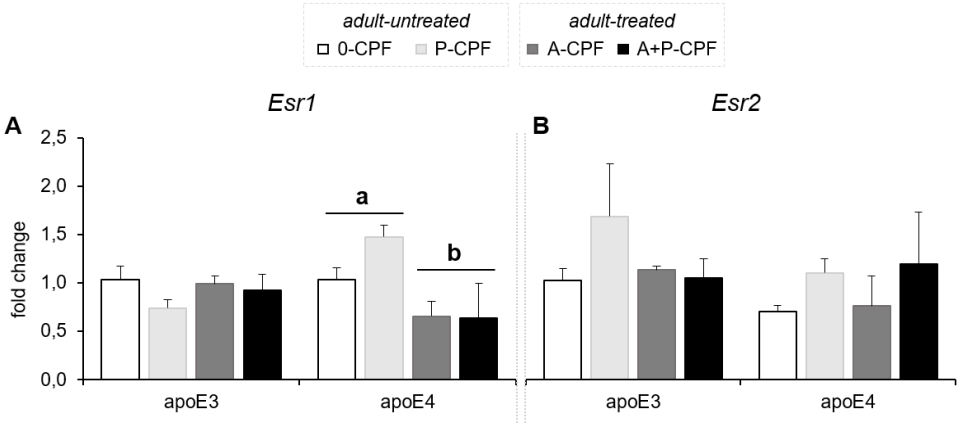

Fig. 8. Relative gene expression in the hypothalamus of *Pomc* (A), *Cartpt* (B), *Agrp* (C) and *Npy* (D) at the end of the adult exposure to CPF and 5 months after postnatal CPF exposure**.** Differences between groups are represented with different letters. The symbol # indicates differences between the P-CPF apoE3 and the A+P-CPF apoE3 group at $p<0.05$.