Learning, memory and the expression of cholinergic components in mice are modulated by the pesticide chlorpyrifos depending upon age at exposure and apolipoprotein E (*APOE*) genotype

Pia Basaure [a,b,c], Laia Guardia-Escote [a,c,d], María Cabré [a,d], Fiona Peris-Sampedro [a,e], Fernando Sánchez-Santed [f], José L. Domingo [c], María Teresa Colomina [a,b,c]*

[a] Research in Neurobehavior and Health (NEUROLAB), Universitat Rovira i Virgili, Tarragona, Spain

[b] Department of Psychology and Research Center for Behavior Assessment (CRAMC), Universitat Rovira i Virgili, Tarragona, Spain

[c] Laboratory of Toxicology and Environmental Health, School of Medicine, IISPV, Universitat Rovira i Virgili, Reus, Spain

[d] Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Tarragona, Spain

[e] Department of Physiology/Endocrinology, Institute of Neuroscience and Physiology, The Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden

[f] Department of Psychology and CIAIMBITAL, Almeria University-ceiA3, Almeria, Spain

** Corresponding author:* Dr. Maria Teresa Colomina. Phone: (34) 977 550875; Fax. (34) 977 558088. E-mail: mariateresa.colomina@urv.cat

Abstract

Polymorphisms of the apolipoprotein E (*APOE*) gene differentially affect neurobiological functions and cognitive performance and confer different vulnerabilities to subclinical exposures to chlorpyrifos (CPF), a pesticide used worldwide. The data reported on this topic suggest a complex interaction between cholinergic signaling and the *APOE* genotype. To gain greater functional insight into this interaction, we evaluated spatial learning and memory and hippocampal cholinergic expression in young apoE3 and apoE4 transgenic mice exposed to CPF. Male and female mice were exposed to CPF at 0 or 1 mg/kg on postnatal days 10-15 and then, exposed to CPF at 0 or 2 mg/kg for 60 days at 5 months of age. At 6 months of age, mice were tested for spatial skills in a Barnes maze. At the end of the task, animals were sacrificed and gene expression of cholinergic components was assessed in the hippocampus. Our results show that apoE4 female mice performed worse in the spatial task, while postnatal CPF impaired escape strategies and spatial memory in apoE3 mice. In turn, CPF in adulthood improved spatial abilities in apoE4 female mice. Regarding gene expression, choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT) expression were increased in apoE4 mice. Postnatal exposure to CPF increased ChAT mRNA levels in apoE4 mice, whereas adult exposure to CPF induced changes in acetylcholinesterase-S, α7- and α4-subunit nicotinic receptor expression in apoE4 females. The current findings provide new insights into *APOE*-dependent cholinergic signaling, which directly affects the response to CPF cholinergic insult, especially in *APOE4* subjects.

Keywords: apolipoprotein E, pesticide, cholinergic system, sex differences, learning, memory

Introduction

Apolipoprotein E (apoE) is a lipid-transport protein widely expressed in the central nervous system (CNS), and involved in several neurobiological processes (Bales 2010; Huang and Mahley 2014). In humans, the apoE encoding gene (*APOE*) is polymorphic, being the most common alleles *ε3* and *ε4* (Roses 1996). Besides influencing the onset of metabolic and cardiovascular diseases, *APOE* genotype confers different risks of several neurological and psychiatric disorders (Villeneuve et al. 2014; Forero et al. 2016). Evidence from a variety of studies suggests that there are complex interactions between age, sex and *APOE* genotype. For instance, several studies have identified differences in spatial learning and memory task performance between *APOE* targeted replacement (apoE-TR) mice at young ages (Reverte et al. 2012; Liraz et al. 2013; Rodriguez et al. 2013; Peris-Sampedro et al. 2015a). Data from human studies indicate that *APOE4* women carriers have a higher risk of cognitive decline with age than men (Holland et al. 2013; Lin et al. 2015; Riedel et al. 2016). In this line, *APOE4* is well known to be the strongest genetic risk factor for developing mild cognitive impairment (MCI) and Alzheimer's disease (AD) (Bales 2010; Xu et al. 2013). Certainly, a prime warning sign of AD is the disrupted hippocampal-based memory (Schliebs and Arendt 2011). This deterioration, along with a loss of cholinergic activity in the CNS observed in AD patients and aging animal models have led to the "cholinergic hypothesis" (Bartus 2000). Therefore, the first drugs used to treat mild and moderate AD aimed to increase the amount of acetylcholine (ACh) in the synaptic cleft by inhibiting acetylcholinesterase (AChE) (Zemek et al. 2014).

A much debated question is whether *APOE4*-related cognitive deficit is due to alterations in the cholinergic neurotransmission. Although various human studies have suggested differences between *APOE4* carriers and non-carriers (Cohen et al. 2003; Lai et al. 2006; Eggers et al. 2006), others have observed none (Corey-Bloom et al. 2000; Reid et al. 2001). More consistently, cholinergic profiles have been reported in apoE-TR mice. For example, a different cholinergic compensation between *APOE* variants after cerebral lesions (Bott et al. 2016) as well as a reduced release of ACh, caused by a decrease in vesicular acetylcholine transporter (VAChT) in 12-month apoE4 mice (Dolejší et al. 2016). Indeed, 15- and 30-day-old apoE3 and apoE4 mice displayed significant differences in forebrain mRNA levels of VAChT, α7-subunit nicotinic receptor (nAChR), AChE isoforms AChE-S and AChE-R (Basaure et al., 2018). Furthermore, it is not clear whether the effects of cholinergic agonists depend on *APOE* genotype. Although some epidemiological-

based studies have described beneficial cognitive effects on a young *ε4* population after a nicotine treatment (Marchant et al. 2010; Evans et al. 2013), others have found no differences in the response to treatments for AD with ChE inhibitors between *APOE4-positive* and -*negative* carriers (Rigaud et al. 2000, 2002). In relation to this, we have found that *APOE* polymorphisms elicit different responses to chlorpyrifos (CPF) in apoE-TR mice (Basaure et al., 2018; Peris-Sampedro et al., 2018, 2016, 2015a, 2015b).

Briefly, CPF has been the most used organophosphate pesticide worldwide for the last decades. It is a cholinesterase (ChE) inhibitor agent, which has been associated with long- and short-term deficits in cognitive functions (Sánchez-Santed et al. 2016; Abreu-Villaça and Levin 2017). Impairments in learning and memory caused by low-dose CPF exposure have been described in rodents exposed during the perinatal period (Jett et al. 2001; Johnson et al. 2009; Turgeman et al. 2011), and adulthood (Yan et al. 2012; López-Granero et al. 2014; Basaure et al. 2017). Several studies have raised the concern that postnatal CPF exposures at doses that do not cause ChE inhibition could trigger alterations in cholinergic neurotransmission and contribute to the emergence of cognitive shortfalls (Jett et al. 2001; Qiao et al. 2003; Oriel and Kofman 2015). In our previous investigations, apoE3 male mice exposed to CPF for 13 weeks showed impaired retention in a spatial task compared to CPF-fed apoE2 and apoE4 (Peris-Sampedro et al. 2015a), while adult apoE4 female mice exposed to CPF at 3.75 mg/kg/day for 4 weeks reversed their inherent lack of inhibitory control (Peris-Sampedro et al. 2016). Furthermore, when apoE-TR mice were exposed to CPF from postnatal day (PND) 10 to 15, VAChT expression only decreased in apoE3 mice (Basaure et al., 2018).

As a result of the large-scale and indiscriminate use of all types of drugs and environmental toxic compounds, the patterns of exposure throughout life are likely to be chronic, prolonged and to include repeated exposures. In the case of CPF, exactly how prior contact influences response to subsequent exposures is not clear yet. Since CPF-induced long-lasting changes might affect multiple neurochemical and detoxifying systems, this prior contact is particularly important in the case of early exposures (Qiao et al. 2004; Rhodes et al. 2004; Abreu-Villaça and Levin 2017) throughout an individual's life. To address this environmental concern, we designed the current study to assess spatial learning and memory and cholinergic changes in the hippocampus of young apoE3- and apoE4-TR mice after two exposures to CPF,

one during the postnatal period and the other during adulthood. Gene expression in hippocampus of choline acetyltransferase (ChAT), VAChT, the α4- and α7-subunit nAChRs and AChE isoforms was also analyzed.

Material and methods

Animals

Male and female apoE-TR homozygous mice, for the human *ε3* and *ε4* alleles (Taconic Europe, Lille Skensved, Denmark) were used in this study. These mice have a C57BL/6 background and express functional human apoE isoforms (Sullivan et al. 1997). After a quarantine period, female mice were mated with males of the same genotype. The day of delivery was designated as PND 0, and only litters with 6–8 pups of both sexes were used. All animals were allowed free access to water and food (Panlab rodent chow, 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 the Spanish Royal Decree 53/2013 on the protection of experimental animals, and the European Communities Council Directive (2010/63/EU).

Chemicals and treatments

CPF [O,O-diethyl O-(3,5,6-trichloropyridin-2-yl) phosphorothioate] was supplied by Sigma-Aldrich Co. LLC. (Madrid, Spain). CPF was administered at two different periods during the lifespan, the first period was during development, from PND 10 to 15 (named *postnatal treatment*), and the second exposure was initiated at 5 month of age and lasted for 8 weeks (named *adult treatment*). On PND 10, the litters were randomly assigned to either the control group or treated group for the postnatal treatment, and 5 months later two males and two females from each group were randomly assigned to either the control group or treated group for the adult treatment. For the postnatal treatment, CPF was dissolved in corn oil and adjusted to administer 1 mg/kg in 1 μL per g of body weight. Pups received an oral dose of 0 or 1 mg/kg with a

142 micro-pipette. For the adult treatment, rodent chow was supplemented with CPF at 15 mg CPF/kg chow
143 (Panlab, Barcelona, Spain) to deliver 2 mg/kg/day as previously described (Basaure et al., 2017; Peris-
144 Sampedro et al., 2018). In order to check if the mice were receiving the estimated dose, body weight and
145 food intake were weekly monitored to further calculate the real ingested dose, which was: 2.04 ± 0.08
146 mg/kg of CPF. The doses administered and the treatment periods were similar to those previously applied
147 in our lab, both for the postnatal exposure to CPF (Basaure et al. 2018; Guardia-Escote et al. 2018) and for
148 the adult exposure to CPF (Peris-Sampedro et al. 2015b, a, 2018). Thus, apoE3 and apoE4 male and female
149 groups were subdivided into the following subgroups: 0-CPF (exposed to vehicle from PND 10 to 15), P-
150 CPF (CPF-treated from PND 10 to 15), A-CPF (CPF-treated at 5-months-of-age for 8 weeks) and A+P-
151 CPF (CPF-treated from PND 10 to 15 and at 5-months-of-age for 8 weeks) (Fig. 1).
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153 **- Insert Fig. 1 over here -**
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155 **Spatial learning and memory**
156
157 At 6 months of age, spatial learning and memory were evaluated in a Barnes maze (BM). A total of 8 to 10
158 mice per group were used to test the effects of postnatal and adult exposures to CPF. The maze consisted
159 of a white circular arena (92 cm diameter) elevated 1 m above the floor, with 20 equidistant holes distributed
160 around the edges. Each hole was assigned with a number from 1 to 20, and the arena was divided into four
161 quadrants. A detachable dark box (i.e. escape box) was located under the hole 1 (i.e. target hole). Bright-
162 white light was used to stimulate the animals to enter the escape box. The test took place on 12 consecutive
163 days during the light cycle. Firstly, animals were habituated to the maze for 2 consecutive days, on the first
164 of which general exploratory activity in a new environment was analyzed in terms of the distance traveled
165 in the arena without escape box. Then, the animals entered the acquisition phase, which consisted of 9 daily
166 consecutive sessions of two trials each, with a 120-min inter-trial interval. During each trial, mice were
167 allowed a total of 120 s to find the target hole and the escape box. The starting-trial position was the center
168 of the arena, and the trial finished when the animal entered the escape box. If the animal failed to enter the
169 escape box within 120 s, it was gently guided and placed into the escape box by the experimenter. The
170 mouse remained undisturbed in the escape box for 30 s before being returned to its holding cage. To avoid
171 proximal cues and ensure hippocampus-dependent learning, the arena was rotated between trials but the
172 escape box position was maintained fixed with respect to the external cues. To remove any olfactory cues,

the maze and the escape box were cleaned with 70% ethanol solution between trials. Throughout the acquisition period, the distance traveled in the arena was measured. We also determined the search strategies used to reach the escape box in the first trial (session 1) and the last trial (session 9). The strategies were scored as: *"random"*, when the mouse arbitrarily searched into the maze; *"serial"*, when it traveled around the edge of the maze, crossing at least three adjacent holes; and *"spatial"*, if it traveled directly towards the target hole from the center of the maze (Peris-Sampedro et al. 2015a; Basaure et al. 2017). The retention phase was carried out 24 h after the last acquisition-trial without the escape box. The time spent in the target quadrant searching for the escape box, out of a maximum of 90 s, was measured. The movements and path of the animal were recorded by a video camera (Sony CCD-IRIS), and then computerized through a video-tracking program (Etho-Vision© XT 11.5, Noldus Information Technologies, Wageningen, The Netherlands).

Sacrifice and sampling

Animals were sacrificed by exsanguination under isoflurane anesthesia at the end of the adult treatment period. Blood was obtained by cardiac puncture, being immediately centrifuged to obtain plasma. After exsanguination, mice were rapidly decapitated and brain was quickly removed, dissecting the hippocampus. Plasma and hippocampus samples were stored at −80ºC until subsequent use.

Plasma ChE activity

In order to identify the acute systemic effect of CPF (Peris-Sampedro et al. 2015b, 2016, 2018; Basaure et al. 2018), ChE activity was determined in plasma (n=5/group) and analyzed spectrophotometrically using the Ellman method (Ellman et al. 1961) with a commercially available kit provided by QCA (Química Analítica Clínica S.A., QCA, Amposta, Spain). The absorbance was measured according to the manufacturer's instructions at a constant temperature of 37ºC, in duplicate, with a semiautomatic COBAS MIRA analyzer (Hoffman-La Roche & Co., Basel, Switzerland). Plasma ChE activity was estimated on the basis of the activity value of the control mice and represented as percentages.

Gene expression

Gene expression of ChAT, VAChT, the α4- and α7-subunit nAChRs, AChE-S and AChE-R isoforms in hippocampus (n=4-5/group) was determined with real-time polymerase chain reaction (qPCR) analysis. The full process was performed with RNase-free reagents, tubes and pipette tips, and the surfaces and instruments were cleaned with RNaseZap solution (ThermoFisher Scientific, Waltham, MA, USA). Briefly, total RNA was extracted with the TRIzol™ Plus RNA Purification Kit and Phasemaker™ Tubes (Invitrogen, Carlsbad, CA, USA), and potential contaminating DNA was removed with the DNA-free™ DNA removal kit (Invitrogen, Carlsbad, CA, USA). RNA concentration and purity were determined with a spectrophotometer Nanodrop 2000 (ThermoFisher Scientific, Waltham, MA, USA), and the quality was assessed by microfluidic electrophoresis with the Agilent RNA 6000 Nano kit and the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). To synthesize cDNA from 1 μg RNA samples, High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (ThermoFischer Scientific, Waltham, MA, USA) was used. The cDNA samples were distributed in 384-well plates in triplicate to carry out the qPCR reactions in the 7900HT Fast Real-Time PCR instrument system with the Power SYBR Green PCR Master Mix (ThermoFischer Scientific, Waltham, MA, USA). The primer sequences used have been described elsewhere and were as follows: *Chat* gene of ChAT (García-Gómez et al. 2015); *Slc18a3* gene of VAChT (Yamamuro and Aizawa 2010); *Chrna4* gene of the α4- and *Chrna7* gene of α7-subunit nAChRs (Léna et al. 1999); and *Ache* gene of both AChE-S and AChE-R (Dori et al. 2011). Finally, the comparative cycle threshold (Ct) method was applied to calculate the mRNA expression. *Gapdh* expression (Yao et al. 2016) was used to determine the relative gene expression levels for each sample, and $2^{-\Delta Ct}$ was calculated for analysis purposes.

Statistics

Analyses were performed using the SPSS Statistics 25.0 software (IBM Corp, Chicago, IL, USA) and MATLAB R2017a (The Mathworks Inc., Natwick, MA, USA). Postnatal treatment (P-CPF) and adult treatment (A-CPF) were used as two different factors to address the following three questions: first, *did the postnatal treatment alone cause long-lasting changes in adults*? Second, *did the adult treatment alone lead to short-term effects?* Finally, *did the response to the adult treatment depend on the postnatal treatment?* Likewise, sex and genotype were also evaluated as main factors. To study the BM task, habituation and

acquisition phases were analyzed by means of a repeated measure multivariate analysis of variance (RMANOVA). Search strategies were evaluated by a four-way analysis of variance (ANOVA) and a paired *t*-test. A one-sample *t*-test was used to evaluate the retention of BM. The *post-hoc* Tukey test was used for multiple comparisons between groups. The variance homogeneity was determined using the Levene test. All data were expressed as mean ± SE. Statistical significance was set at $p < 0.05$.

Results

A moderate 2 mg/kg/day exposure to CPF for 8 weeks decreased plasma ChE activity to 20% with no signs of toxicity

Although A-CPF and A+P-CPF mice showed no signs of cholinergic syndrome during the treatment period, a four-way ANOVA (sex x genotype x P-CPF x A-CPF) indicated that adult-treated groups showed a decrease in plasma ChE activity [$F_{1,88}$=3298.94, p<0.001]. The ChE activity of the A-CPF mice dropped to 19.20% compared to 0-CPF and the activity of the A+P-CPF mice dropped to 20.34% compared to P-CPF (data not shown).

ApoE3 mice explored the BM more than apoE4 mice, and both CPF exposures caused alterations in general activity

General activity, assessed by the distance traveled in the arena without escape box, during the 15 min of habituation to the BM was analyzed by a four-way RMANOVA (sex x genotype x P-CPF x A-CPF), using three time periods of 5-min as the repeated measure factor. A general effect of time [$F_{2,137}$=39.70, p<0.001], an interaction between time and sex [$F_{2,137}$=4.46, p=0.013], and an interaction between time, genotype and P-CPF [$F_{2,137}$=8.15, p<0.001] were found. In addition, an overall effect of genotype [$F_{1,138}$=8.06, p=0.005], and an interaction between P-CPF and A-CPF [$F_{1,138}$=4.34, p=0.039] were also observed. Overall, apoE4 mice explored less than apoE3 mice. Although general effects of both genotype and treatment were observed during habituation, no significant differences between groups were found (Fig. 2).

- Insert Fig. 2 over here -

ApoE4 mice showed worse spatial learning than apoE3 mice in the BM, while adult exposure to CPF improved the performance only in apoE4 females

Spatial learning, assessed by the distance traveled in the arena, during the acquisition period was analyzed by a four-way RMANOVA (sex x genotype x P-CPF x A-CPF), using the sessions as the within-subject factor. We found a general effect of sessions [$F_{8,131}$=28.670, p<0.001], an interaction between sessions and genotype [$F_{8,131}$=2.40, p=0.019] and an interaction between sessions, sex, P-CPF and A-CPF [$F_{8,131}$=2.42, p=0.018] on the total distance traveled. As depicted in Figure 3a, a progressive and significant decrease in the distance traveled over the sessions indicated that all mice learned the task. However, apoE3 mice displayed a better performance than apoE4 mice. Moreover, male and female mice were affected differently by the treatments throughout the sessions (Fig. 3a). An overall effect of genotype [$F_{1,138}$=93.83 , p<0.001], P-CPF [$F_{1,138}$=5.42, p=0.021], an interaction between sex and A-CPF [$F_{1,138}$=5.28, p=0.023], and an interaction between sex, genotype and A-CPF [$F_{1,138}$=4.25, p=0.041] were also observed. Further analysis for each sex and genotype showed that, when exposed to CPF during adulthood, apoE4 female mice improved their performance (Fig. 3b).

- Insert Fig. 3 over here -

The transition to a spatial search strategy was disrupted by postnatal CPF exposure in apoE3 mice whereas both postnatal and adult CPF exposures increased serial and spatial strategies in apoE4 female mice

As a qualitative estimation of proficiency in the task, search strategies were evaluated as the percentage of random, serial or spatial strategies (Fig. 4a). The first trial in session 1 and the last trial in session 9 were evaluated by an ANOVA (sex x genotype x P-CPF x A-CPF). Genotype affected the use of the serial strategy on both days, the first trial [$F_{1,155}$=4.05, p=0.046] and the last trial [$F_{1,155}$=6.26, p=0.013]. Figure 4b shows that apoE3 mice used the serial strategy more frequently while apoE4 mice maintained a significant percentage of random search strategy even during the last session. Moreover, the random strategy in the last trial was influenced by adult CPF exposure [$F_{1,155}$=3.99, p=0.048]. The serial strategy in the first trial was affected by postnatal CPF [$F_{1,155}$=4.65, p=0.033] and an interaction between sex, genotype and A-CPF in the last trial [$F_{1,155}$=13.27, p<0.001]. Finally, spatial strategy in the first trial was

295 altered by postnatal CPF [$F_{1,155}$=4.28, p=0.040], an interaction between sex, genotype and P-CPF in the
296 first trial [$F_{1,155}$=4.28, p=0.040], and an interaction between sex, genotype and A-CPF in the last trial
297 [$F_{1,155}$=7.07, p=0.009].
298
299 In order to understand the transition from one strategy to another, the first and the last acquisition session
300 trials were analyzed with a paired *t*-test in each sex, genotype and treatment groups. Both 0-CPF and A-
301 CPF apoE3 mice significantly changed their strategy from random to serial or spatial (p<0.05; *t*-test full
302 statistical reporting of the statistically significant groups: Supplementary Table 1) (Fig. 4c). Interestingly,
303 this effect was not observed in either male or female P-CPF apoE3 mice. In contrast, most of the apoE4
304 mice did not significantly change their strategy over the sessions (Fig. 4d). However, female P-CPF and A-
305 CPF groups increased their use of serial or spatial strategies (p<0.05; *t*-test full statistical reporting of the
306 statistically significant groups: Table S1 in the Supplementary file).
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308 **- Insert Fig. 4 over here -**
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310 **Postnatal CPF impaired retention in apoE3 mice, while adult CPF improved retention in apoE4**
311 **females**
312
313 Retention was evaluated 24 h after the last acquisition session. The time spent in the target quadrant was
314 compared with chance exploration (i.e., 22.5 s) in each group. As can be seen in Figure 5, all the groups
315 spent more time exploring the target quadrant than the chance level. However, an one-sample *t*-test revealed
316 that most apoE3 groups on the one hand and most apoE4 male groups on the other significantly remembered
317 the previous location of the escape box (p<0.05), but postnatal exposure to CPF disrupted spatial memory
318 in all males and apoE3 females (p>0.05). In the case of apoE4 females, the control group showed poor
319 retention, which was significantly ameliorated by adult CPF exposure (p<0.05) (*t*-test full statistical
320 reporting of the statistically significant groups: Table S2 in the Supplementary file).
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322 **- Insert Fig. 5 over here -**
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324 **Analysis of gene expression**
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Hippocampal ChAT, VAChT, α4- and α7-subunit nAChR, AChE-S and AChE-R mRNA levels were studied at the end of the 8-week dietary adult exposure to CPF and six months after postnatal CPF exposure.

ChAT and VAChT were more expressed in apoE4 mice, while postnatal CPF increased ChAT expression in apoE4 mice

In the case of ChAT (Fig. 6a), an overall effect of the genotype [$F_{1,73}$=7.12, p=0.010] and an interaction between genotype and P-CPF [$F_{1,73}$=4.07, p=0.048], indicated that apoE4 mice expressed more ChAT than apoE3 mice and pointed to an increased expression in apoE4 mice exposed to postnatal CPF (Fig. 6b). On the other hand, VAChT expression (Fig. 6c) was modulated by the genotype [$F_{1,73}$=7.12, p=0.010]. As shown in Figure 6d, apoE4 mice expressed more VAChT than the apoE3 group.

- Insert Fig. 6 over here -

Adult exposure to CPF increased α4-subunit expression in apoE4 female mice, whereas α7-subunit was more expressed in apoE3 females, and postnatal and adult CPF exposures differentially affected apoE4 males and females

Analysis of the α4-subunit (Fig. 7a) showed an overall effect of the genotype [$F_{1,73}$=4.09, p=0.047] and an interaction between genotype, sex and A-CPF [$F_{1,73}$=13.83, p<0.001]. Although apoE4 mice expressed more the α4-subunit than apoE3, a *post-hoc* test indicated that this increased expression was determined by adult exposure to CPF in apoE4 female mice (Fig. 7b). Regarding the α7-subunit receptor expression (Fig. 7c), a general effect of the genotype [$F_{1,73}$=7.79, p=0.007] and an interaction between genotype and sex [$F_{1,73}$=4.35, p=0.042], indicated that even though apoE3 mice had a higher expression than apoE4, this effect mainly occurred in apoE3 female mice, according to a multiple comparison analysis (Fig. 7d). An interaction between genotype, sex, P-CPF and A-CPF [$F_{1,73}$=4.10, p=0.047] was also found. To define this interaction, the effects of each treatment were evaluated in each genotype and sex. Differences emerged only in apoE4 mice: the α7-subunit receptor expression was decreased by postnatal CPF in male mice [P-CPF overall effect: $F_{1,18}$=9.50, p=0.008] and increased by adult CPF in female mice [A-CPF overall effect: $F_{1,18}$=8.43, p=0.011] (Fig. 7c).

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The effects of CPF exposure on AChE-S expression and AChE-R in apoE4 were modulated by genotype and sex

Expression of AChE-S (Fig. 8a) was affected by the genotype [$F_{1,73}$=7.59, p=0.008], and the interactive effects of genotype, sex and A-CPF [$F_{1,73}$=7.59, p<0.001]. A multiple comparison analysis performed in each genotype and sex revealed that apoE4 female mice showed the highest increase related to adult CPF exposure (Fig. 8b). In the case of AChE-R (Fig. 8c), an overall effect of sex [$F_{1,73}$=4.54, p=0.037], an interaction between genotype and sex [$F_{1,73}$=4.38, p=0.041], and an interaction between genotype, sex and P-CPF [$F_{1,73}$=5.98, p=0.018] were observed. Further analyses of this interaction showed that the expression in postnatal-untreated apoE3 female groups was higher than in the remaining groups (Fig. 8d).

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Discussion

This study was to investigate whether a developmental exposure to CPF could affect spatial learning and memory later in life, and how this previous exposure might alter the response to an adult exposure to the same cholinergic pesticide. ApoE3- and apoE4-TR mice were used because these variants elicit different responses to cholinergic stimulation. We evaluated spatial learning and memory as well as cholinergic related gene expression in the hippocampus of young adult mice exposed to CPF during postnatal and/or adult timeframes. Our results herein show differences in spatial learning and memory associated with *APOE* genotype and sex, and responses to CPF that depend on the age at exposure. Specifically, postnatal CPF exposure disrupted the acquisition of a spatial search strategy and reference memory, mainly in apoE3 mice. Adult CPF exposure by itself ameliorated learning and memory abilities in apoE4 female mice. In turn, differences in gene expression between mice were triggered by the *APOE* genotype, sex and CPF exposure. Postnatal exposure to CPF increased ChAT expression in apoE4 mice, while adult exposure to CPF, especially in apoE4 females, induced several changes, among which were an increase in the α4 receptor and AChE-S and a decrease in the α7-subunit receptor.

The present behavioral results shed light on several commonly described differences between *APOE* variants. Firstly, the exploratory activity of a novel environment, assessed during the habituation of the BM task, was lower in apoE4 than in apoE3 mice. This decrease could be produced by the frightening characteristics of the space, since the BM environment is white and illuminated with no surrounding walls. In this regard, it has been suggested that apoE4 mice identify the potential risks related to open spaces more quickly than apoE3 mice, which may lead to decreased activity levels in some tasks (Hartman et al. 2001). In addition, some studies have described increased anxiety-like behaviors in apoE4-TR mice (Hartman et al. 2001; Reverte et al. 2014; Meng et al. 2017). Thus, the current results are in agreement with an anxious-like phenotype related to apoE4 mice. In turn, a few studies have reported poor learning and memory results on BM task in mouse strains, which had a high anxiety-like behavior and low exploratory behavior (Võikar et al. 2001; Holmes et al. 2002). In the current study, while apoE4 mice displayed a reduced activity during habituation, these subjects traveled a greater distance compared to apoE3 mice over the acquisition sessions. These results suggest that the activity levels did not affect the learning and memory.

The *APOE4* genotype has been widely reported to have a negative effect on learning and memory abilities in healthy humans (Greenwood et al. 2005; Wisdom et al. 2011; Shine et al. 2015) and young apoE-TR mice (Reverte et al. 2012, 2013; Rodriguez et al. 2013). Likewise, in the present study, apoE4 mice exhibited poorer spatial learning than apoE3 mice. On the other hand, a qualitative assessment of search strategies shows that apoE4 mice persisted in random search strategies after 9 acquisition sessions, which indicates that the shift from random to serial or spatial strategies is disrupted in apoE4. These findings are consistent with previous results that associated a worse performance of apoE4 mice in the BM, with the use of more random strategies (Peris-Sampedro et al. 2015a). Spatial navigation depends on areas such as the hippocampus and entorhinal cortex (Deiana et al., 2011). The acquisition of proficiency in a spatial task requires a shift from non-spatial to spatial strategies (Harrison et al. 2006), in which the entorhinal cortex is involved in distributing the processed information (Witter et al. 2000), with the ventral/intermediate hippocampus playing a key role in this shift (Ruediger et al. 2012). Strikingly, postnatal CPF exposure altered the transition from random to serial or spatial strategies in apoE3 mice, while adult CPF exposure enhanced acquisition and boosted the transition from random to spatial in apoE4 female mice. It is worthwhile noting that the beneficial effects of cholinergic stimulation were only observed in apoE4 mice. However, we cannot discard disruptive effects after cholinergic overstimulation in normal subjects. In this

418 sense, in a recent study, we found that adult C57BL/6 mice exposed to an 8-week exposure to CPF of 5
419 mg/kg/day were unable to change the random strategy after 5 days of training in BM (Basaure et al. 2017).
420
421 Indeed, the limitations on strategy transition recognized in postnatal-CPF apoE3 mice and the absence of
422 spatial strategies in apoE4 female mice are strongly associated with the observed retention scores. The
423 results of the current investigation are in accordance with those of several published studies describing
424 long-term spatial learning and memory impairments in adult rodents, elicited by low-doses of CPF during
425 the postnatal period (Levin et al. 2001; Jett et al. 2001; Turgeman et al. 2011). Remarkably, we only found
426 these deleterious effects in those subjects that were most skilled at this task. In contrast, apoE4 females, the
427 least skilled, seem to benefit somewhat from either postnatal or adult CPF exposures, but not from a
428 combination of both. Similarly, in a previous research, Salazar et al. (2011) observed that spatial memory
429 was ameliorated in a mouse model of AD after acute CPF treatment. Taken together, these results indicate
430 cholinergic imbalances in apoE4 females, which can be redressed by cholinergic stimulation especially
431 during adulthood. However, it must be taken into account that exposure to CPF in adulthood might induce
432 delayed-onset deficits in spatial learning and memory (Terry et al. 2007, 2012; Peris-Sampedro et al. 2014).
433 Therefore, we cannot discard the lack of deleterious effects after adult exposure in apoE4 subjects, because
434 some cognitive alterations can appear long after exposure.
435
436 It is well-established that cholinergic signaling is fundamental when animals need to evaluate novel stimuli
437 in new places and contexts, and most importantly, in formation and consolidation of hippocampus-mediated
438 spatial memory (Deiana et al., 2011; Pepeu and Giovannini, 2004). Our results show that cholinergic
439 expression in the hippocampus of apoE-TR mice is greatly influenced by the *APOE* genotype. The
440 expression of presynaptic components of ACh synthesis such as ChAT and VAChT were lower in apoE3
441 mice than in apoE4. It can be assumed that the cholinergic function of apoE3 mice is normal, being the
442 presynaptic and postsynaptic components in balance. In contrast, apoE4 mice had a higher expression of
443 synthesis-related elements, which may sustain a high release of ACh. In parallel, while the ACh degradation
444 enzyme AChE-S was more expressed in apoE4, the soluble isoform AChE-R was higher in apoE3
445 postnatally untreated females, compared to their apoE4 counterparts. Intriguingly, the α4-subunit pattern
446 expression was rather similar to AChE-S expression in both apoE-TR groups. On the other hand, AChE-R
447 variant expression has been related with a restore mechanism after exposure to organophosphates and under

448 stress conditions (Dori et al. 2011; Härtl et al. 2011; López-Granero et al. 2013a, b). In contrast with the
449 current findings, we have described a diminished expression of VAChT and an increased expression of α7-
450 subunit nAChR and AChE-R in apoE4- TR mice compared to apoE3 mice at 30 days of age (Basaure et al.
451 2018). These rather contradictory results may be due to specific fluctuations in the cholinergic expression
452 during development. Differences in cholinergic signaling may partly explain some intrinsic functional
453 strengths and weaknesses of *APOE3* and *APOE4* carriers, and reflect differences in cholinergic efficiency
454 related to the abnormalities in lipid rafts (Sebastião et al. 2013) and membrane lipid composition described
455 in apoE4-TR mice (Igbavboa et al. 2005).
456
457 Although it cannot be ruled out that muscarinic receptors or other cholinergic elements are involved in
458 maintaining the cholinergic balance, synaptic compensatory mechanisms such as an increase in AChE or a
459 downregulation of nAChRs, could be expected. In the present study, these effects were more evident after
460 adult CPF exposure. An increase in α4 and AChE-S expression and a decrease in α7-subunit were observed
461 in parallel with changes in spatial learning in apoE4 females. Concerning this matter, short-term stimulation
462 of the α7- and α4-subunit with agonist compounds has been associated with improvement in spatial learning
463 and memory (Deiana et al. 2011). Moreover, massive upregulation of α4β2 nAChR has been observed after
464 nicotine exposure (Albuquerque et al. 2009). The increase in the expression of α4 nAChR in apoE4 females
465 after adult exposure to CPF may explain the improvement observed in the spatial task. This increased
466 expression of the α4-subunit in apoE4 mice is in agreement with pharmacological data indicating that
467 nicotine increases the α4-subunit and confers greater benefits to young *ε4* carriers compared to those
468 carrying *ε3* allele (Marchant et al. 2010; Evans et al. 2013). These data support the idea of the cognitive
469 shortfalls in *APOE4* carriers might be triggered by cholinergic dysfunctions. In relation to this, several
470 works have shown deficits in the hippocampus of young apoE4-TR mice, such as accumulation of
471 hyperphosphorylated tau and neuronal Aβ42 (Liraz et al. 2013), and short dendritic length, reduced spine
472 density and impairments in carbachol-induced hippocampal theta oscillations (Sun et al. 2017).
473
474 Data from several studies suggest that perinatal exposure to CPF induces variations in α7-subunit and ChAT
475 levels as well as on other cholinergic elements (Jett et al. 2001; Qiao et al. 2003; Rhodes et al. 2004; Basaure
476 et al. 2018). Some of the alterations were observed up to 60 days after exposure to CPF (Qiao et al. 2004;
477 Rhodes et al. 2004). In our case, 6 months after postnatal treatment with CPF, apoE4 females showed

differences in ChAT expression while changes in AChE-S were only observed after adult overstimulation with CPF. Studies with transgenic mice over expressing human AChE have found a significant increase in such nAChRs as α4-, β2- and α7-subunit (Svedberg et al. 2002; Mousavi et al. 2004), which highlights the complex interactions involved in expression between AChE and nAChRs. In the current study, α7-subunit nAChR expression was diminished exclusively in postnatal-treated apoE4 males, which did not show significant increases in either VAChT or ChAT. This particular response observed in apoE4 females may indicate a greater undermined cholinergic system in these subjects. With respect to this, *ε4* allele-associated sex differences in cognitive decline have been widely studied. It has been shown that women have a higher risk of MCI and AD and a faster progression from MCI to AD than men (Holland et al. 2013; Lin et al. 2015; Riedel et al. 2016). Despite behavioral effects produced by postnatal exposure to CPF in apoE3 and apoE4 male mice, modifications of cholinergic signaling are not so conclusive.

In summary, the current results support not only the basal differences between apoE3- and apoE4-TR mice in cholinergic signaling but also the conceptual premise that the *APOE* genotype differentially contributes to the effects of CPF. Postnatal CPF deleterious effects were mainly observed in apoE3 mice in the spatial task, while adult CPF exposure had short-term beneficial effects on memory retrieval in apoE4 female mice, which parallels changes in both nAChR and AChE-S expression. The basal cholinergic differences between *APOE3* and *APOE4* carriers together with a differential response after CPF exposure, could support the most controversial issue about the cholinergic contribution to cognitive deficits in *APOE4* population, especially in females. Finally, given that the effects of either perinatal or adult exposures depended largely on the genotype background, it seems evident that genetic factors should be studied as a source of bias in toxicology and pharmacology.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Figures

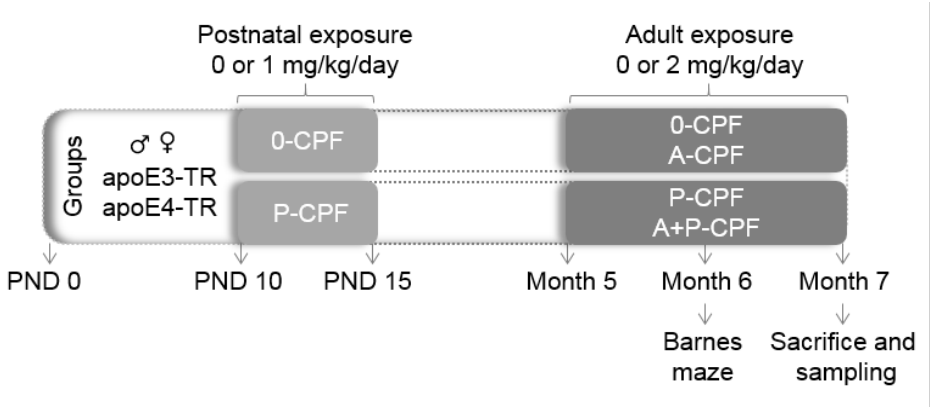

Fig. 1 Schematic diagram of the experimental design showing the ages at CPF exposure, behavioral testing and sacrifice, and the doses used and groups of treatment. Both males and females, apoE3- and apoE4-TR mice were treated with vehicle from PND 10 to 15 (0-CPF), CPF from PND 10 to 15 (P-CPF), CPF at 5-months-of-age for 8 weeks (A-CPF) and CPF from PND 10 to 15 and at 5-months-of-age for 8 weeks (A+P-CPF). Barnes maze task was used to test spatial learning and memory. Biological samples were further analyzed to test cholinesterase activity in plasma and gene expression in the hippocampus.

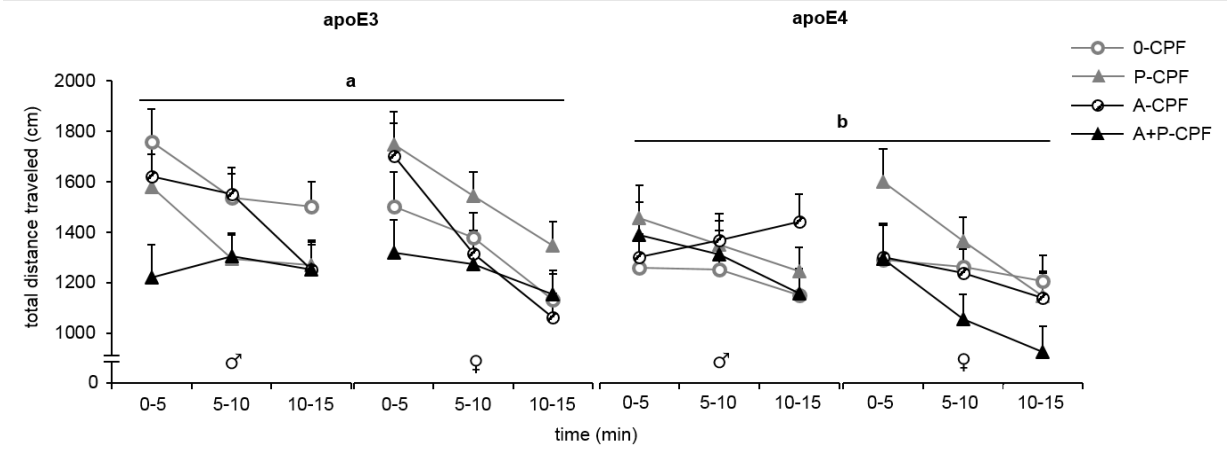

Fig. 2 Habituation to the Barnes maze arena evaluated 30 days after the adulthood exposure to CPF started and 6 months after postnatal CPF exposure. Total distance traveled in the maze over 15 min divided into three 5-min periods evaluated in apoE3- and apoE4-TR mice, males and females. Groups with different letters are significantly different from each other at $p<0.05$.

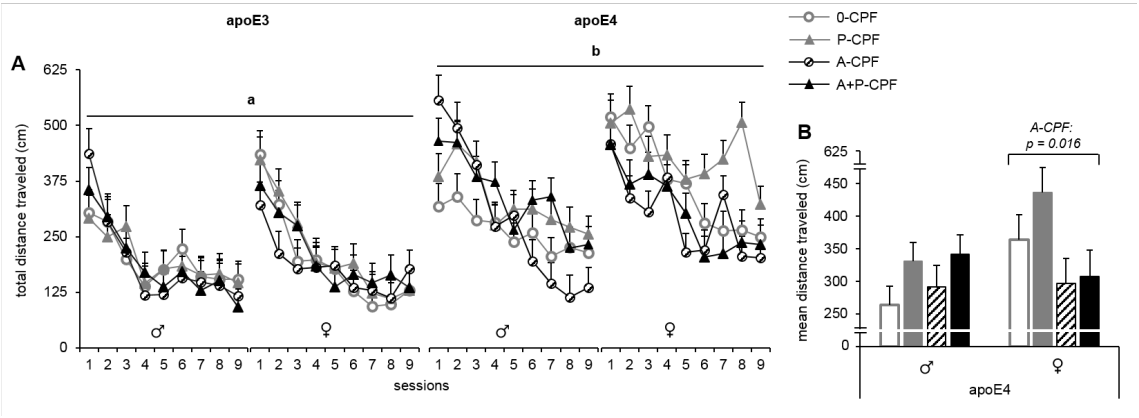

Fig. 3 Acquisition of a 9-day spatial learning task evaluated in a Barnes maze 30 days after the adulthood exposure to CPF started and 6 months after postnatal CPF exposure. a Total distance traveled in the maze searching for the target hole over the 9 sessions evaluated in apoE3- and apoE4-TR mice, males and females. b Mean time of the apoE4 male and female mice. Groups with different letters are significantly different from each other at $p<0.05$. Overall effect (p) of adult exposure to CPF is indicated above the female group.

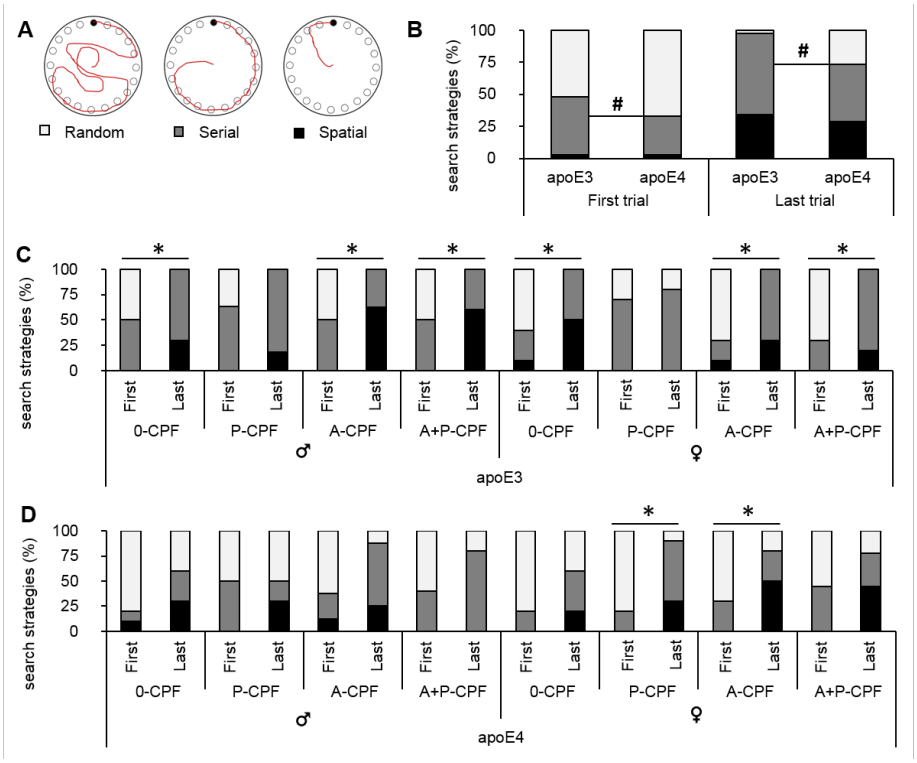

Fig. 4 Percentage of search strategies used to find the escape box of the Barnes maze task during the first trial in session 1 and the last trial in session 9. a Representative images of strategies: random (arbitrary

776 pattern), serial (mice go through consecutive holes), and spatial (mice move directly towards the target
777 hole). b Effects of genotype on search strategies. c Effects of treatment on apoE3 male and female mice,
778 and d on apoE4 male and female mice. The symbol # represents differences between genotypes in their
779 choice of serial strategy in both trials. Differences between the first and last trial in each group are
780 represented with an asterisk at $p<0.05$.
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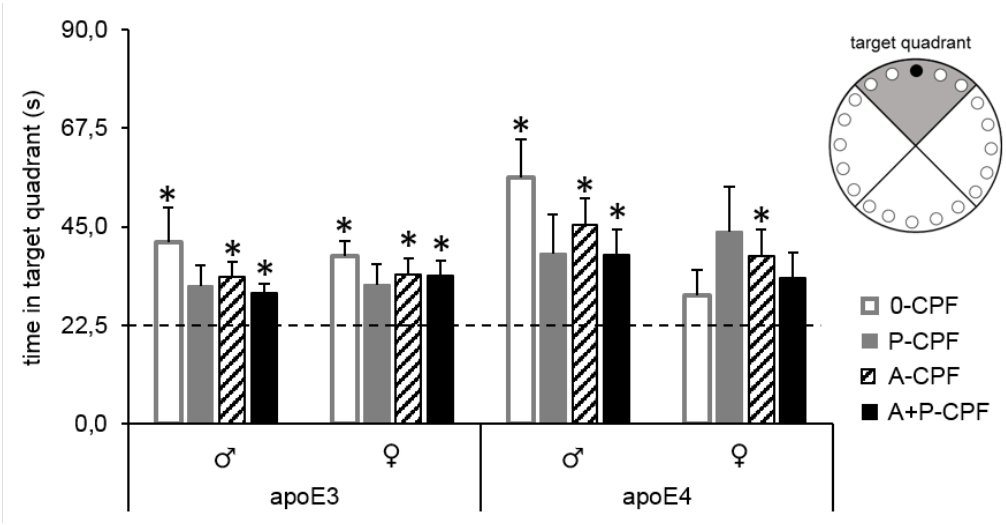

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784 Fig. 5 Retention of a spatial learning task assessed in a Barnes maze 30 days after the adulthood exposure
785 to CPF started and 6 months after postnatal CPF exposure. Time spent in the target quadrant throughout
786 the 90 s of testing. The discontinuous line represents the time that animals are expected to spend in each
787 quadrant by chance (i.e., 22.5 s). The differences between each group and the chance level are indicated by
788 an asterisk at $p<0.05$.
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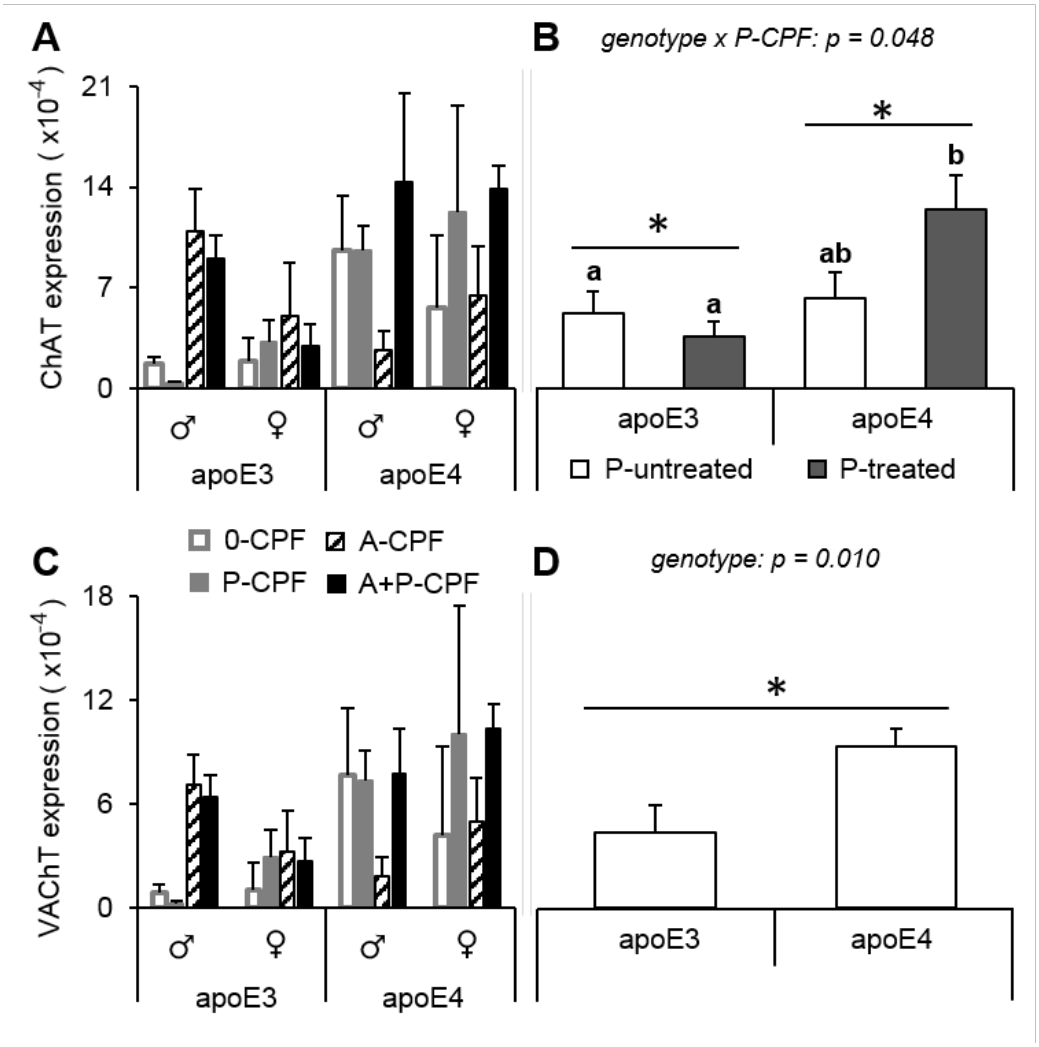

Fig. 6 Gene expression in the hippocampus at the end of the 8-week dietary adult exposure to CPF and six months after postnatal CPF exposure. a ChAT expression in apoE3- and apoE4-TR mice, males and females from the four treatment groups. b Interaction effect between genotype and postnatal exposure to CPF on ChAT expression. c VAChT expression in apoE3- and apoE4-TR mice, males and females from the four treatment groups. d Effect of genotype on VAChT expression. P-untreated include 0-CPF and A-CPF groups, and P-treated include P-CPF- and A+P-CPF groups. Differences between genotypes are indicated with an asterisk, and groups with different letters are significantly different from each other at $p<0.05$.

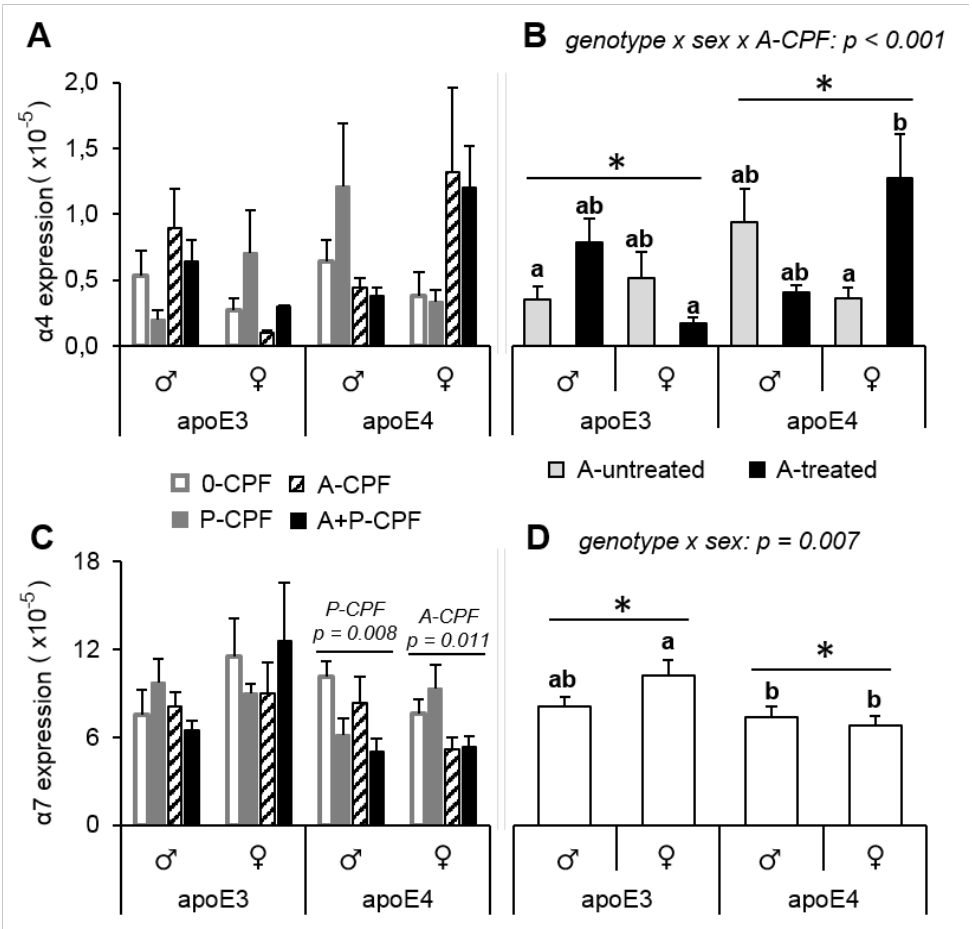

Fig. 7 Gene expression in the hippocampus at the end of the 8-week dietary adult exposure to CPF and six months after postnatal CPF exposure. a α4-subunit nAChR expression in apoE3- and apoE4-TR mice, males and females from the four treatment groups. b Interaction effect between genotype, sex and adult exposure to CPF on α4-subunit nAChR expression. c α7-subunit nAChR expression in apoE3- and apoE4-TR mice, males and females from the four treatment groups. d Interaction effect between genotype and sex on α7-subunit nAChR expression. A-untreated include 0-CPF and P-CPF groups, and A-treated include A-CPF and A+P-CPF groups. Differences between genotypes are indicated with an asterisk, and groups with different letters are significantly different from each other at $p<0.05$. Overall effects (p) of postnatal exposure to CPF and adult exposure to CPF are specified above the apoE4 male and female mice, respectively.

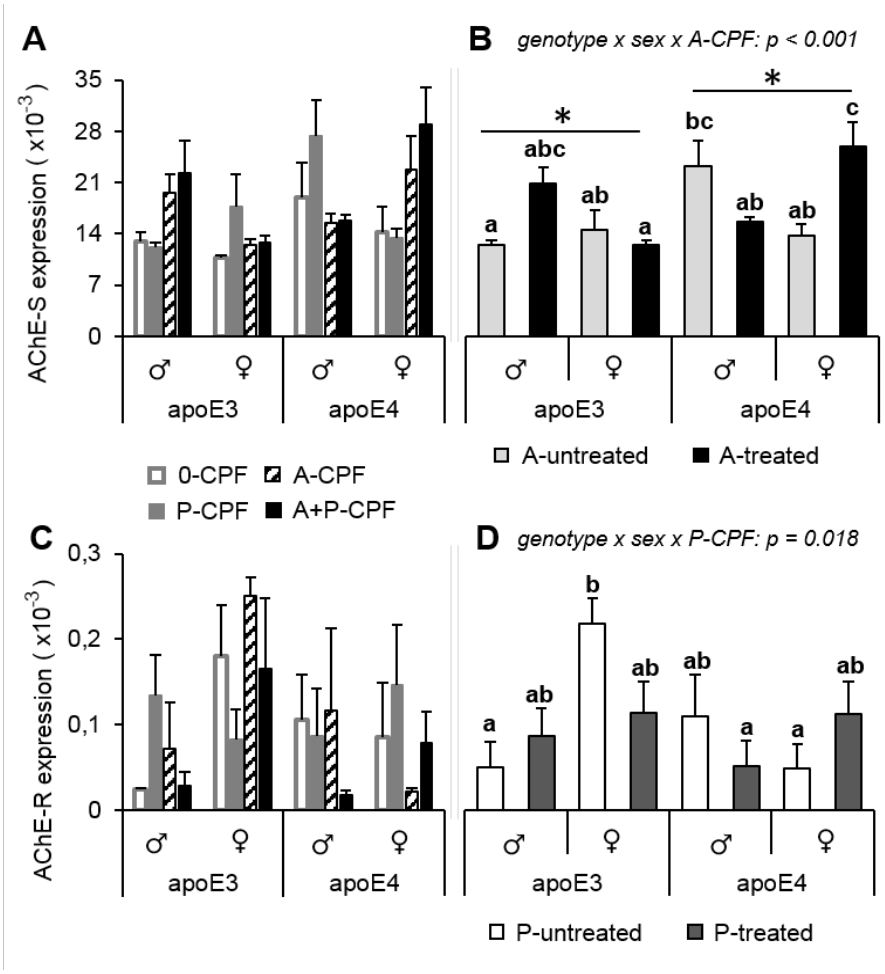

Fig. 8 Gene expression in the hippocampus at the end of the 8-week dietary adult exposure to CPF and six months after postnatal CPF exposure. a AChE-S expression in apoE3- and apoE4-TR mice, males and females from the four treatment groups. b Interaction effect between genotype, sex and adult exposure to CPF on AChE-S. c AChE-R expression in apoE3- and apoE4-TR mice, males and females from the four treatment groups. d Interaction effect between genotype, sex and postnatal exposure to CPF on AChE-R. A-untreated include 0-CPF and P-CPF groups, and A-treated include A-CPF and A+P-CPF groups P-untreated include 0-CPF and A-CPF groups, and P-treated include P-CPF- and A+P-CPF groups. Differences between genotypes are indicated with an asterisk, and groups with different letters are significantly different from each other at $p<0.05$.