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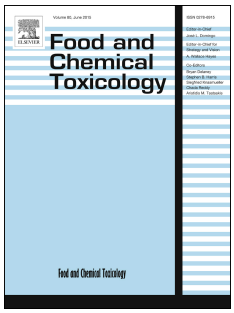

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Postnatal exposure to chlorpyrifos produces long-term effects on spatial memory and the cholinergic system in mice in a sex- and *APOE* genotype-dependent manner

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Abstract

Organophosphorus pesticides – and in particular chlorpyrifos (CPF) – are extensively used worldwide. They mainly exert their toxicity by targeting the cholinergic system. Several studies suggested that the gene coding for apolipoprotein E (apoE), which is a risk factor for several diseases, can also confer different vulnerability to toxic insults. This study was aimed at assessing the long-term effects of postnatal exposure to CPF on learning and memory as well as the expression levels of several genes involved in cholinergic neurotransmission in mice. Both male and female apoE4-TR and C57BL/6 mice were exposed to either 0 or 1 mg/kg/day of CPF by oral gavage using a micropipette on postnatal days 10-15. At 9 months, they were tested in a Morris Water Maze (MWM) and the gene expression in the frontal cortex and hippocampus was evaluated. Our results show that, in males, CPF had an effect on the spatial retention, while in females, it altered the expression levels of nicotinic receptors. Furthermore, apoE4-TR mice performed the worst during the MWM retention and presented low expression levels in a considerable number of cholinergic genes. Taken together, the current results reveal long-term effects in mice nine months after postnatal exposure to CPF, which are modulated by sex and apoE4 genotype.

Keywords:

Chlorpyrifos, Pesticide, APOE, Cholinergic system, Learning and memory, Brain development

1. Introduction

The toxicity of organophosphorus pesticides (OP) is mainly produced by targeting the cholinergic system, leading to an increase of its function. Acetylcholine (ACh) – the key neurotransmitter in cholinergic signaling – plays a pivotal role in the peripheral and central nervous system, being involved in a wide range of functions such as cortical development, arousal and cognitive processes (Ferreira-Vieira et al., 2016; Schliebs and Arendt, 2006). ACh is synthesized from choline and acetyl CoA and stored in synaptic vesicles until it is released into the synaptic cleft. Then, ACh can bind to muscarinic (mAChRs) and nicotinic (nAChRs) receptors. The mAChRs are G protein-coupled receptors located at either pre- or postsynaptic membranes; presynaptic mAChRs act as sensors and regulate ACh neurotransmitter release whereas postsynaptic mAChRs mediate either inhibitory or depolarizing responses depending on the mAChR type. The nAChRs, mostly postsynaptic, are composed of five homologous subunits assembled to form a non-selective cation channel and mediate depolarizing responses. After producing its signal, the ACh in the synaptic cleft is inactivated by the enzyme acetylcholinesterase (AChE), which breaks the neurotransmitter and finalizes its action (Abreu-Villaça et al., 2011; Blake et al., 2014; Gotti et al., 2007). It has been reported that alternative splicing produces different AChE variants, in particular the tetrameric form AChE-S and the monomeric and soluble form AChE-R. In normal conditions, AChE-S is expressed more than AChE-R but this is reversed under stress, which increases the AChE-R variant (Soreq and Seidman, 2001). Another cholinesterase, sharing high homology with the AChE, is the butyrylcholinesterase (BChE). This enzyme can be found throughout the body of mammals and can hydrolyze ACh, albeit less efficiently than AChE. Some studies suggest that BChE protects AChE from esterase inhibitors and, therefore, can be important in the response to exposure to OP pesticides (Darvesh et al., 2003; Hartmann et al., 2007; Soreq and Seidman, 2001).

One of the most common OP pesticides worldwide is chlorpyrifos (CPF), which has been in use since 1965 (Eaton et al., 2008). CPF elicits its toxic effect by persistently inhibiting ChE enzymes such as AChE and BChE, which leads to an overstimulation of the cholinergic system (Eaton et

al., 2008; Flaskos, 2012). The main neurotoxic effect of CPF is mediated by its oxidized form, CPF-Oxon (CPO), which is produced after a biotransformation reaction in the liver (Jokanović, 2001). Exposure to CPF has been related to neurobehavioral and metabolic effects, not only in occupational workers but also in the general population, who are exposed to low doses of the pesticide, mainly through the diet (Rauh et al., 2006; Roldán-Tapia et al., 2005). A number of animal studies have evaluated the effects of CPF exposure during adulthood, and reported effects after both acute (López-Granero et al., 2014; Montes de Oca et al., 2013) and chronic exposures (Basaure et al., 2017; López-Granero et al., 2013; Peris-Sampedro et al., 2015a, 2015b). However, the potential effects of CPF exposure during development have aroused considerable interest because young individuals are more sensitive than adults to cholinergic toxicity (González-Alzaga et al., 2014; Moser and Padilla, 1998; Whitney et al., 1995). For instance, gestational and/or postnatal exposures to CPF have been related with behavioral alterations, including maternal behavior (Venerosi et al., 2008), motor activity and anxiety levels (Ricceri et al., 2006) as well as changes on novelty seeking (Ricceri et al., 2003). Furthermore, it has been reported to trigger persistent deficiencies in cholinergic synaptic functions (Slotkin et al., 2001) as well as long term changes in thyroid status (De Angelis et al., 2009) and normal metabolism function (Buratti et al., 2011; Slotkin et al., 2005).

Over the past few years, we have investigated how the genetic background of individuals can affect the consequences of exposure to CPF, since some genotypes can confer different vulnerability to toxic insults. Specifically, our studies have focused on the main genotypes defined by the gene coding for apolipoprotein E (apoE) (Peris-Sampedro et al., 2015a; Reverte et al., 2016). ApoE is a protein involved in lipid and cholesterol transport and distribution. In humans, it presents three alleles at a single gene locus, resulting in three major isoforms: apoE2, apoE3 and apoE4, being apoE3 the most common one (Mahley, 1988; Mahley and Rall, 2000). Several differences between these isoforms have been described in terms of vulnerability to toxics, neurodegenerative diseases or metabolic disorders. In this sense, a diminished expression of Paraoxonase 1, an enzyme responsible for hydrolyzing organophosphorus pesticides, was found

in apoE4 compared to apoE3 (Boesch-Saadatmandi et al., 2010). Moreover, apoE4 has been related with a greater susceptibility to Alzheimer's Disease (AD) (Roses, 1996) and a diminished cholinergic function (Allen et al., 1997). Various studies link cholinergic alterations with neurological pathologies, including cognitive decline and AD (Hrabovska and Krejci, 2014; Schliebs and Arendt, 2011, 2006). Previous investigations in our laboratory working with targeted replacement (TR) mice that express the different isoforms of the human gene apoE (apoE-TR), showed differences in the toxicity elicited by exposure to CPF in neurobehavioral performances (Peris-Sampedro et al., 2016, 2015a) and metabolic function (Peris-Sampedro et al., 2018, 2015b). Recently, we have demonstrated that postnatal exposure to CPF during development have effects on the cholinergic system of apoE-TR mice. For instance, brain expression levels of genes as VAChT, ChAT, α4 nAChR and α7 nAChR were modified by CPF exposure depending on age, sex and *APOE* genotype (Basaure et al., 2018). Nevertheless, the present study is the first to consider the long-term effects of postnatal exposure to CPF in apoE4-TR mice.

The present study aimed to assess the long-term effects of exposure to CPF on learning and memory and the expression levels of several genes involved in cholinergic neurotransmission nine months after postnatal exposure to CPF. We hypothesized that subclinical exposure to CPF during development could result in neurobehavioral effects and permanent alteration of the cholinergic system in a genotype-dependent manner. Our main goal was to assess and to determine interactions between toxic exposure, apoE4 genotype and sex.

2. Material and methods

2.1. Animals and husbandry

Nine-month-old male and female apoE-TR and C57BL/6 mice were used. Mice homozygous for the *ε4* allele were purchased from Taconic (Taconic Europe, Lille Skensved, Denmark). ApoE-TR animal model, originally created by Sullivan et al. (1997) have a C57BL/6NTac background and their murine apoE gene has been replaced by the human allele apoE4. Hence, they systemically express functional human apoE4 isoform. C57BL/6 were obtained from Charles

River (Charles River, Barcelona, Spain). The animals were housed in plastic cages containing 2-5 individuals of the same genotype. The animal room was maintained at a temperature of 22 ± 2 °C, a relative humidity of 50 ± 10%, and a 12h automatic light/dark cycle. All the mice were allowed free access to food and water and they were fed a normal chow diet (Panlab, Barcelona, Spain). The use of animals and the experimental protocol 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).

2.2. Treatment

Chlorpyrifos [0,0-diethyl O-(3,5,6-trichloropyridin-2-yl) phosphorothioate] (CPF), purity 99,5%, was provided by Sigma-Aldrich Co. LLC. (Madrid, Spain). The compound was dissolved in corn oil as vehicle and adjusted to administer 1 mg/kg in 1 μL/g of body weight. Administration was by oral gavage using a micropipette. The CPF-treated groups received oral CPF during postnatal days 10-15, both included, while animals in the control groups were given the vehicle for the same period. Animals were periodically monitored and maintained under standard conditions for nine months, when the behavioral assessment and biochemical experiments were conducted. A total number of 79 mice were divided into eight experimental groups as shown in Table 1.

2.3. Morris Water Maze

At nine months of age, male and female mice were tested for the long-term effects of CPF on a Morris Water Maze test (MWM) to assess spatial learning and memory. The water maze consisted of a circular pool (1 m diameter, 60 cm high), which was virtually divided into 4 quadrants. An escape platform with a diameter of 10 cm was placed on the center of the target quadrant, submerged 1 cm below the surface of the water. Different shaped black marks were placed on the walls surrounding the maze and used as visual extramaze clues. We predefined four starting positions as well as four positions for an internal rotating wall inside the pool. These positions were changed between trials in order to ensure spatial learning and to prevent internal clues and

trajectory learning. During the acquisition period, mice performed 9 sessions distributed over 9 days. Each session consisted of 3 trials. Each trial lasted until the animal reached the platform or until 90s had elapsed. If the animal did not find the platform, it was guided and placed on it for 30 seconds. The time between trials was 90 min. The collected data were the latency to find the platform and the distance travelled. The retention was evaluated by 3 probe trials: 24 h after the last acquisition trial in sessions 3, 6 and 9. Probe trials consisted of 60-seconds free swimming after the escape platform had been removed. The total time spent on the target quadrant was measured. Experiments were automatically recorded by a video camera (Sony CCD-IRIS model) and analyzed by the video software EthoVision® XT 11.5 (Noldus Information Technologies, Wageningen, The Netherlands).

2.4. Sacrifice and sampling

Immediately after the behavioral test, biological samples were collected. Animals were deeply anesthetized with isoflurane before being euthanized by decapitation. Whole brains were collected and dissected in order to obtain the frontal cortex and the hippocampus. Samples were immediately stored at -80ºC for subsequent analysis.

2.5. Analysis of gene expression

The expression of the α4-subunit and α7 nAChRs, M1 and M2 mAChRs, AChE-S and AChE-R transcripts, and BChE was assessed by real-time polymerase chain reaction. Total RNA was isolated from the frontal cortex and hippocampus using the Speedtools total RNA extraction kit (Biotools, Madrid, Spain). The concentration and purity of total RNA were detected by spectrophotometry using UV absorbance at 260 and 280 nm. RNA was reversely transcribed from 1 μg of total RNA from each sample, using a Maxima First Strand cDNA Synthesis Kit for RT-qPCR (ThermoFisher Scientific, Waltham, USA). The complementary DNA (cDNA) was subsequently amplified by PCR, using a Maxima SYBR Green/ROX qPCR Master Mix (2X) kit (ThermoFisher Scientific, Waltham, USA) and Rotor-Gene Q Real-Time PCR cycler (Qiagen Inc., Hilden, Germany). The primers used are indicated in Table 2. The cycle threshold (Ct) was

calculated by Rotor-Gene Q 2.0 software to identify significant fluorescence signals. The relative levels of expression of the target genes were measured using *Gapdh* mRNA as an internal control according to the $2^{-\Delta\Delta Ct}$ method.

2.6. Statistical analysis

Data were analyzed with the SPSS 25.0 software (IBM Corp, Chicago, USA). A repeated measure multivariate (ANOVA) with sex, genotype and treatment as main factors was used to analyze MWM acquisition. In the case of no homogeneous data, Greenhouse-Geisser was used to recalculate the F and the significance values. A one-sample t-test was also used to analyze differences in retention. For the gene expression experiment, a two-way analysis of variance (ANOVA) was used, with genotype and treatment as the main factors. Post-hoc Tukey's test of variance was used to analyze differences between groups. The variance homogeneity was assessed by a Levene test. Statistical significance was set at $p<0.05$. Results are reported as mean values ± S.E.M.

3. Results

3.1. Spatial learning and memory in the MWM

3.1.1. Acquisition: sex and genotype influenced the learning of a spatial task.

Learning performance during the acquisition was analyzed by a three-way ANOVA (sex x genotype x treatment) for repeated measures. Session was the within-subject factor while the escape latency and the distance traveled were studied as the dependent variables. An overall improvement in performance was observed throughout the acquisition sessions by the decrease in the distance traveled to the platform [$F(8,64)=149.950$, $p<0.001$] and the escape latency [$F(8,64)=113.304$, $p<0.001$] (data not shown). The distance was also modified by the sex factor [$F(1,71)=23.611$, $p<0.001$] and the following interactions: session x sex [$F(8,64)=10.731$, $p<0.001$], session x genotype [$F(8,64)=3.872$, $p=0.002$] and session x sex x genotype [$F(8,64)=4.004$, $p=0.001$] (Table 3). As shown in Figure 1A, acquisition over the sessions

depends on sex and genotype. Male mice learned faster and obtained better scores than females. ApoE4 females were the worst in this task (Fig. 1B).

3.1.2. Retention: sex and genotype modulated the long-term effects of postnatal CPF exposure on the retrieval of a spatial task

Retention was assessed using three different probe sessions 24 h after acquisition trials in sessions 3, 6 and 9. We first assessed the differences between groups by a three-way ANOVA (sex x genotype x treatment) in each of the three probe sessions. We found a significant effect of the genotype in the first [$F(1,71)=4.481$, $p=0.038$] and the second [$F(1,71)=13.787$, $p<0.001$] probe, with apoE4 mice being the ones with the worst retention in both cases (Fig. 2C and 2D). A tendency towards a significant effect of the treatment was also observed in probe 2 [$F(1,71)=3.465$, $p=0.067$], being CPF-treated groups worse than their control counterparts (Fig. 2E). In the third probe, we obtained a significant effect of the sex [$F(1,71)=6.613$, $p=0.012$] and a tendency of sex x genotype x treatment [$F(1,71)=3.368$, $p=0.071$] (Fig. 2F). Significant effects are described in Table 3. In order to further analyze these differences, we performed a one-way ANOVA (group). Differences between groups [$F(7,71)=3.117$, $p=0.006$] during the second probe were observed. A subsequent *post hoc* analysis showed significant differences between control C57BL/6 and CPF-treated apoE4 mice ($p=0.042$). These results suggest that the treatment had a long-term detrimental effect on males. We then used a one-sample t-test to analyze the time spent in the target quadrant (TQ), where the escape platform was previously located, in comparison with the chance level of 15s. Both males and females of the C57BL/6 genotype showed a significant preference for the TQ in all retention sessions (Fig. 2A and 2B). Male apoE4 control mice showed a progressive preference for the TQ, although it was only significant from probe 2 onwards [$t=4.893$, d.f.6, $p=0.003$; $t=3.675$, d.f.6, $p=0.010$; respectively]. By contrast, the CPF-treated apoE4 male group showed their preference in probe 1 [$t=2.612$; d.f.12; $p=0.023$] and 3 [$t=3.390$; d.f.12; $p=0.005$]. On the other hand, the female apoE4 control group showed no preference for the TQ, whereas the CPF-treated group presented a significant preference for the TQ only in probe 3 [$t=2.713$; d.f.9; $p=0.024$]. These results show an improvement in CPF-treated

apoE4 females in comparison with the control apoE4 female group at the end of the task, although they do not reach the control C57BL/6 levels.

3.2. Gene expression in the frontal cortex and hippocampus

Males and females were analyzed separately as the Ct values were normalized to each corresponding C57BL/6 control. Data from four samples of the frontal cortex and two samples of the hippocampus were excluded from the analysis because of technical issues. Significant effects from gene expression for each gene are summarized in Table 3.

3.2.1. Expression levels in the frontal cortex were modulated by the genotype and CPF exposure. Genotype was observed to have a significant effect on both male and female α7 nAChR [$F(1,9)=8.000$, $p=0.020$; $F(1,10)=5.714$, $p=0.038$; respectively]. Results showed that apoE4 mice expressed lower levels of α7 nAChR than C57BL/6 mice, which suggests a basal dissimilarity between genotypes at nine month of age (Fig. 3A). Moreover, treatment with CPF modulated the expression of α7 in females [$F(1,10)=12.727$, $p=0.005$]. Although *post-hoc* analysis showed a significant effect only in C57BL/6 females ($p=0.030$), CPF treatment diminished α7 nAChR expression levels in both genotypes. Likewise, α4 nAChR also showed an effect of CPF in females, albeit not significant [$F(1,10)=3.580$, $p=0.088$]. These results suggest that in females both α4 and α7 nAChRs are sensitive to a postnatal exposure to CPF. Interestingly, the female group C57BL/6 seems to be the most affected by the treatment, as its expression decreases to a level that is similar to that of apoE4 female mice. On the other hand, in the case of mAChRs, differences between genotype were observed only in males (M1: [$F(1,9)=5.095$, $p=0.050$] and M2: [$F(1,9)=5.910$, $p=0.038$]). Results reveal that apoE4 mice expressed lower levels of M1 and M2 than C57BL/6 mice, which is in agreement with the differences observed in α7 nAChR levels (Fig. 3B and 3C). Finally, neither of the two isoforms of the AChE or BChE mRNA showed significant effects of genotype or treatment (data not shown).

3.2.2. Gene expression in the hippocampus was modulated by genotype in a sex-dependent manner.

Genotype was found to have an overall effect on α7 nAChR in males [$F(1,15)=7.335$, $p=0.016$]. Indeed, apoE4 mice presented lower expression levels than C57BL/6 mice (Fig. 4A). Genotype was also found to modulate M2 mAChR gene expression in males [$F(1,15)=8.164$, $p=0.012$]. In contrast with the frontal cortex, apoE4 males presented higher levels of M2 in the hippocampus than their C57BL/6 counterparts (Fig. 4B). Post-hoc analysis between groups showed a basal difference between the apoE4 and C57BL/6 control groups ($p=0.016$), suggesting that the *APOE4* genotype leads to an increase in M2 gene expression. Likewise, the expression levels of the isoform AChE-R were also subjected to an overall effect of the genotype in males [$F(1,15)=6.989$, $p=0.018$]. Once again, the apoE4-TR mice expressed higher levels than the C57BL/6 (Fig. 4C). In the case of BChE, the *APOE4* genotype decreased expression only in females [$F(1,15)=10.600$, $p=0.005$] (Fig. 4D). Finally, neither α4 nAChR, M1 mAChR nor the isoform AChE-S showed any significant effects of genotype or treatment (data not shown).

4. Discussion

The present investigation was designed to study the long-term effects of postnatal exposure to CPF. During the acquisition of the MWM task, sex differences were found. Although both sexes improved over acquisition, males performed better than females on this part of the test. Likewise, a number of studies have indicated that in both humans (Piber et al., 2018) and animal models (Monfort et al., 2015; Saucier et al., 2008), males perform spatial navigation tasks better than females

Throughout the retention sessions, genotype had a significant effect on both males and females, with the apoE4 group performing the worst. Indeed, C57BL/6 mice showed a significant preference for the former location of the platform in all the sessions. On the other hand, apoE4 control male mice showed a progressive learning pattern throughout the various trials whereas the CPF-treated group presented irregular outcomes. In contrast, the female apoE4 control group

showed no preference for the TQ in any of the trials although the CPF-treated group showed a significant retention at the end of the task. Previous studies have shown that retention was more impaired in apoE4-TR mice than in apoE3 or C57BL/6, and particularly so in females (Bour et al., 2008; Grootendorst et al., 2005; Reverte et al., 2012). Likewise, the apoE4 link to AD is far more evident in females (Raber et al., 1998; Ungar et al., 2014). Taken together, these findings suggest sexual-dimorphic differences at basal level in the *APOE4* genotype, which provides a distinct response to pesticide toxicity. Therefore, the improvement observed only in apoE4-treated females suggests that CPF administered during development may help redress a possible basal deficiency inherent in this group. This might include a functional improvement due to overstimulation of the cholinergic system. In this regard, the hypothesis that early interventions may ameliorate inherent deficits in female apoE4 mice deserves further investigations.

We observed once more differences of genotype on probe session 1 and 2, and significant effects of sex on probe 3. We also observed tendency towards a treatment influence during the second retention session in males. It suggests a long-term detrimental effect of postnatal exposure to CPF, as treated groups performed the task worse. In fact, CPF has been reported to disrupt the normal execution of spatial learning and memory tasks (Gómez-Giménez et al., 2017; López-Granero et al., 2014; Peris-Sampedro et al., 2014). These differences became more evident in the second retention session. Taking into account that MWM provides remarkably robust learning, we propose that further studies with other spatial tasks may give greater insight into these differences.

The analysis of genes involved in cholinergic signaling in the frontal cortex and hippocampus revealed different patterns of gene expression. In the frontal cortex, we found that the genotype had a significant effect on the expression of three of the ACh receptors studied: α7 nAChR, M1 mAChR and M2 mAChR. This effect was observed mainly in males although for the α7 it was present in both males and females. The current results show that apoE4 mice expressed lower levels of these genes than their C57BL/6 counterparts, which suggests a basal dissimilarity between genotypes. In females the treatment was also observed to have an effect on nicotinic

414 receptors, mainly in α7 nAChR expression levels. Furthermore, C57BL/6 females displayed
415 significantly different levels of α7 nAChR expression between treatment groups. In particular,
416 the CPF-treated group expressed diminished levels of α7 nAChR, suggesting a long-term effect
417 of the postnatal treatment with CPF. Slotkin et al. (2004) also detected a significant decrease in
418 α7 nAChR binding in the forebrain and cerebellum after exposure to 5 mg/kg/day of CPF on
419 postnatal days 11-14. Their findings, observed on postnatal days 15 and 20, indicated that α7
420 nAChR was a specific target for cholinergic neurotoxicants. In our study, the resulting levels of
421 the C57BL/6 CPF-treated female group resemble those of the apoE4 group, which lends further
422 support to the hypothesis of a basal difference in ACh signaling between apoE4-TR and
423 C57BL/6 mice. Taken together, these results highlight the importance of nicotinic receptors and
424 the fact that they may be permanently altered by postnatal exposure to CPF. Considering that
425 these differences in gene expression between treatment groups were still detectable after 9
426 months, they can be an underlying sign of future cognitive impairments. For this reason, it
427 would be interesting to investigate whether they can lead to more evident deficits with age.

428
429
430 Results from the hippocampus showed again that the genotype was the main factor modulating
431 the expression of α7 nAChR and M2 mAChR in males. In particular, apoE4 mice presented
432 lower levels of α7 nAChR than C57BL/6 mice did, which also occurred in the frontal cortex.
433 The differences observed in females were no longer discernible. The *APOE4* genotype was
434 reported to have an overall effect on M2 mAChR, being their expression levels significantly
435 greater than those in the C57BL/6. This was just the opposite of the expression levels in the
436 frontal cortex. Previous studies described M2 mAChRs as presynaptic autoreceptors, involved
437 in modulating cholinergic neurotransmission in both the hippocampus and the cerebral cortex.
438 In particular, M2 autoreceptors would inhibit the release of ACh by feedback inhibition
439 (Douglas et al., 2001; Kitaichi et al., 1999; Zhang et al., 2002). Increased levels of this receptor,
440 as obtained in the hippocampus of apoE4 mice, can mean an attempt to regulate cholinergic tone
441 through presynaptic mechanisms.

The results of the current study also showed that genotype has an overall effect on hippocampus AChE-R expression levels, with the apoE4 group showing the most significant increase. Expression of AChE-R is normally rare and primarily induced by multiple stress stimuli, including psychological, physical or chemical stress. This includes exposure to cholinesterase inhibitors such as OPs (Grisaru et al., 1999; Perrier et al., 2005). In the present investigation, and taking into account that the determinations were carried out 9 months after CPF exposure, no significant changes in expression levels due to CPF were noticed. It is consistent with the recent results of Basaure et al. (2018), who also found that expression levels of AChE-R in apoE4 mice were higher than those in apoE3 mice during development. These results suggest that the basal differences between genotypes observed in early ages are maintained over time. Furthermore, the greater expression of AChE-R in apoE4 also matches the increased levels observed post-mortem in the hippocampus (Berson et al., 2008) and frontal cortex (Campanari et al., 2016) of AD patients. Finally, genotype modulated the expression of BChE in females, and led to lower expression levels in apoE4 mice than in C57BL/6 animals. Although we only found this significant effect in females, Dolejší et al. (2016) reported differences that depended on the apoE genotype and age in males: at 4 months of age, hippocampal BChE activity was similar in apoE3 and apoE4, but it significantly decreased in apoE4 at 8 months of age. Other studies with AD patients reported that BChE activity in the cerebrospinal fluid was lower in *ε4* allele carriers (Darreh-Shori et al., 2011, 2006). This decrease in BChE levels can mean that apoE4-TR mice are less able to hydrolyze ACh than the C57BL/6 group, which could be an attempt to compensate for the cholinergic deficit by trying to increase the neurotransmitter levels. At the same time, altered levels of BChE may determine the risk of CPF exposure: that is to say, individuals expressing lower levels of BChE are more sensitive to the detrimental effects of the pesticide.

Taken together, these results suggest a different modulation by the treatment and the genotype depending on the brain region. Both the frontal cortex and the hippocampus have been reported to play an important role in spatial navigation although they contribute in a different manner. Moreover, their functional interaction is required for a correct goal-directed navigation (Ito,

2018). The impairments observed in the *APOE4* genotype during the MWM correspond to the differences detected in the expression levels of some of the genes involved in the correct functioning of the cholinergic system, especially in males. In fact, sex differences involving the *APOE4* genotype remain constant in behavioral and biochemical tests. It is well known that altered levels of cholinergic elements are related to defective cognitive processes (Schliebs and Arendt, 2011, 2006). Nonetheless, cholinergic impairments cannot be the only accountable reason for the differences between groups in spatial learning and memory although they may be one of the underlying mechanisms. This is illustrated by the fact that some effects can still be observed 9 months after postnatal treatment in a number of the genes studied. On the other hand, the absence of genes modified by CPF exposure in the *APOE4* genotype suggest some protection to cholinergic overstimulation. Notwithstanding, treatment with CPF had an effect on expression levels of α7 nAChR in C57BL/6 females. Considering that previous studies on development have found short-term changes in the same genes (Basaure et al., 2018), we suggest that most of them do not persist for 9 months after exposure to the toxic. However, it should also be taken into account that any compensating mechanisms can become imbalanced with aging. It is also important to consider that in real life we are exposed to a wide variety of chemical compounds that can interact between them and present cumulative effects. Hereby the importance of designing new protocols considering these multi-chemical exposures (Tsatsakis et al., 2017, 2016), as well as the aforementioned parameters genotype and sex.

In conclusion, the current results show that CPF has effects on spatial memory and on the expression of some cholinergic genes 9 months after postnatal exposure. CPF elicited its toxicity in a sexual-dimorphic manner, which was particularly evident in the apoE4 group during the spatial memory task. The *APOE4* genotype was a determining factor, as apoE4-TR mice showed the worst performance during the MWM retention and presented lower expression levels in a considerable number of cholinergic genes. However, CPF treatment did not affect the expression levels of the apoE4 group. Instead, the altered expression levels of the nicotinic receptors showed that C57BL/6 females were the most sensitive to the effects of CPF. In summary, the results of

the present study suggest that postnatal exposure to the pesticide chlorpyrifos can have long-term effects on spatial learning and memory, and the cholinergic system, being these effects potentially modulated by sex and apoE4 genotype.

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Figure Captions

Fig. 1. Acquisition in the Morris Water Maze. Distance to escape platform traveled (A) by males and females over the nine sessions of training, and their cumulative distance depending on the genotype (B). Different letters (a,b) indicate a significant difference between groups at $p<0.05$.

Fig. 2. Retention in the Morris Water Maze. Time in the target quadrant in males (A) and females (B) over the 3 retention sessions performed 24 hours after the third (Probe 1), the sixth (Probe 2) and the ninth (Probe 3) acquisition days. General effects of the genotype in the first (C) and the second probe (D). General effects of treatment in the second probe (E) and general effects of sex in the third probe (F). An asterisk indicates a performance significantly different from the chance level of 15s at $p<0.05$. Different letters (a,b) indicate significant differences between groups ($p<0.05$).

Fig. 3. Relative gene expression in the frontal cortex of α7 nAChR (A), α4 nAChR (B), M1 mAChR (C) and M2 mAChR (D). An asterisk indicates significant genotype differences at $p<0.05$. Different letters (a,b) indicate a significant difference between groups ($p<0.05$).

Fig. 4. Relative gene expression in the hippocampus of α7 nAChR (A), M2 mAChR (B), AChE-R (C) and BChE (D). An asterisk indicates significant genotype differences at $p<0.05$. Different letters (a,b) indicate a significant difference between groups ($p<0.05$).

879 **Table 1.** Total number of animals per group in the Morris Water Maze (MWM) and gene expression
880 on the frontal cortex (FC) and hippocampus (HC).

	MWM		Gene Expression FC		Gene Expression HC	
Age	9 months		10 months		10 months	
Group	Males	Females	Males	Females	Males	Females
Control C57BL/6	9	10	4	4	5	5
CPF-treated C57BL/6	9	10	4	4	5	5
Control apoE4	7	11	3	4	5	5
CPF-treated apoE4	13	10	4	4	5	5

881
882 **Table 2.** Primers sequence used for the gene expression analysis.

Gene	Protein	Forward primer	Reverse primer	Reference
Bche	BChE	TAGCACAATGTGGCC TGTCT	ATTGCTCCAGCGATG AAATC	(García-Gómez et al., 2016)
Chrm1	M1 mAChR	TGACAGGCAACCTGC TGGTGCT	AATCATCAGAGCTGC CCTGCGG	(Laspas et al., 2015)
Chrm2	M2 mAChR	CGGACCACAAAAATG GCAGGCAT	CCATCACCACCAGGC ATGTTGTTGT	(Laspas et al., 2015)
Chrna4	α4 nAChR	GTTCTATGACGGAAG GGTGCAGTGGACA	GGGATGACCAGCGAG GTGGACGGGATGAT	(Léna et al., 1999)
Chrna7	α7 nAChR	GTGGAACATGTCTGA GTACCCCGGAGTGAA	GAGTCTGCAGGCAGC AAGAATACCAGCA	(Léna et al., 1999)
Ache	AChE-S	CTGAACCTGAAGCCC TTAGAG	CCGCCTCGTCCAGAG TAT	(Dori et al., 2011)
Ache	AChE-R	GAGCAGGGAATGCAC AAG	GGGGAGGTGGAGAA GAGAG	(Dori et al., 2011)
Gapdh	GAPDH	ACAACTTTGGCATTG TGGAA	GATGCAGGGATGATG TTCTG	(Yao et al., 2016)

883
884
885 Abbreviations: butyrylcholinesterase (BChE); M1 and M2 muscarinic acetylcholine receptor (M1 and
886 M2 mAChR); α4 and α7 muscarinic acetylcholine receptor (α4 and α7 nAChR); acetylcholinesterase-
887 S and -R (AChE-S and AChE-R) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

888 **Table 3.** Summary table of results in the Morris Water Maze (MWM) and gene expression on
889 the frontal cortex (FC) and hippocampus (HC).

		Statistics	Results
MWM	Acquisition (Distance traveled)	Session: $p<0.001$ Session x sex: $p<0.001$ Session x sex x genotype: $p=0.001$	All animals learned over sessions Males learned faster than females ApoE4 females performed worse than C57BL/6
	Retention (Time TQ)	Probe 1. Genotype: $p=0.038$ Probe 2. Genotype: $p<0.001$ Treatment: $p=0.067$ Probe 3. Sex: $p=0.012$ Sex x genotype x treatment: $p=0.071$	C57BL/6 > apoE4 CPF treatment impaired retention, mainly in males, from probe 2 onwards Males > females
Gene Expression (FC)	α7 nAChR	Genotype males: $p=0.020$ Genotype females: $p=0.038$ Treatment females: $p=0.005$	ApoE4 < C57BL/6 CPF treatment ↓ expression
	α4 nAChR	Treatment females: $p=0.088$	Sensible to CPF exposure
	M1 mAChR	Genotype males: $p=0.050$	apoE4 < C57BL/6
	M2 mAChR	Genotype males: $p=0.038$	apoE4 < C57BL/6
Gene Expression (HC)	α7 nAChR	Genotype males: $p=0.016$	apoE4 < C57BL/6
	M2 mAChR	Genotype males: $p=0.012$	apoE4 > C57BL/6
	AChE-R	Genotype males: $p=0.018$	apoE4 > C57BL/6
	BChE	Genotype females: $p=0.012$	apoE4 < C57BL/6

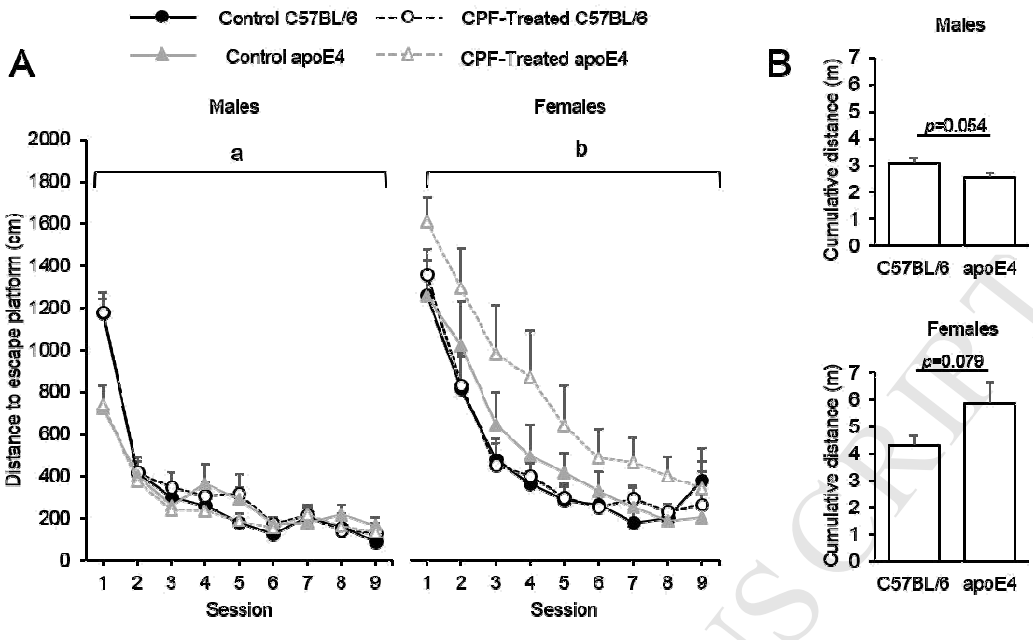

Fig. 1. Acquisition in the Morris Water Maze. Distance to escape platform traveled (A) by males and females over the nine sessions of training, and their cumulative distance depending on the genotype (B). Different letters (a,b) indicate a significant difference between groups at $p<0.05$.

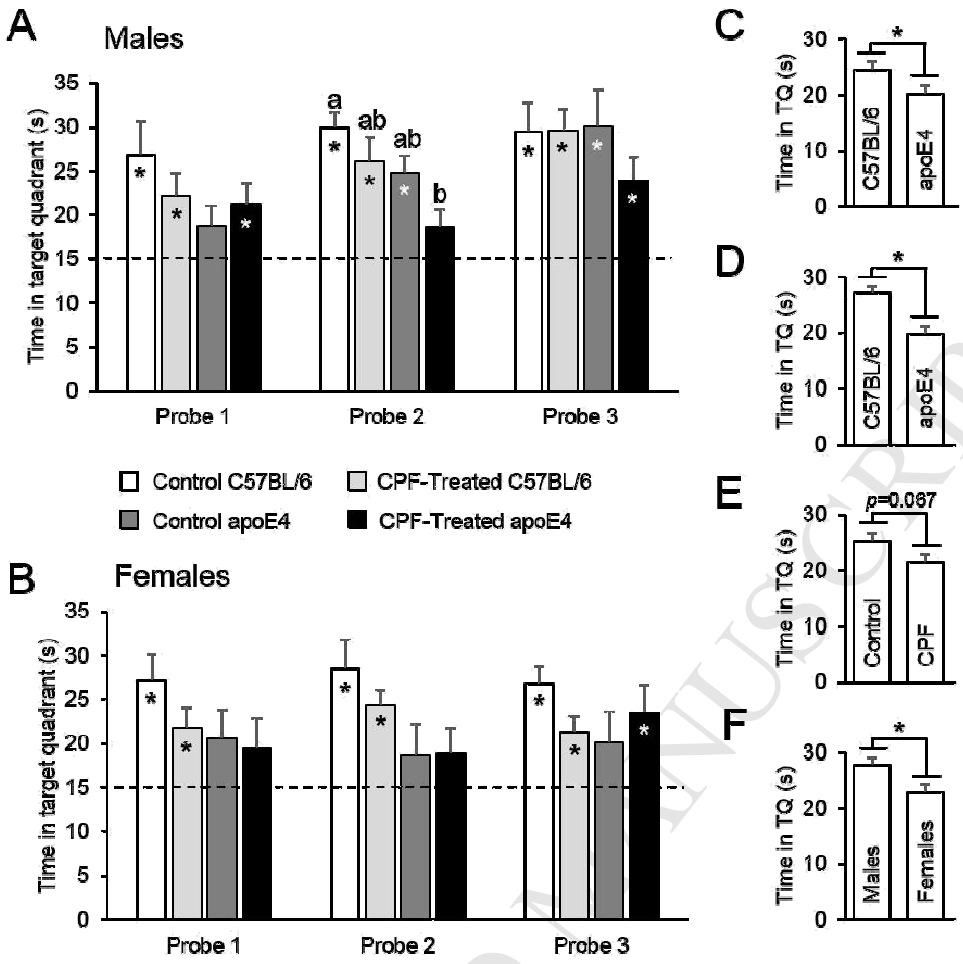

Fig. 2. Retention in the Morris Water Maze. Time in the target quadrant in males (A) and females (B) over the 3 retention sessions performed 24 hours after the third (Probe 1), the sixth (Probe 2) and the ninth (Probe 3) acquisition days. General effects of the genotype in the first (C) and the second probe (D). General effects of treatment in the second probe (E) and general effects of sex in the third probe (F). An asterisk indicates a performance significantly different from the chance level of 15s at $p<0.05$. Different letters (a,b) indicate significant differences between groups ($p<0.05$).

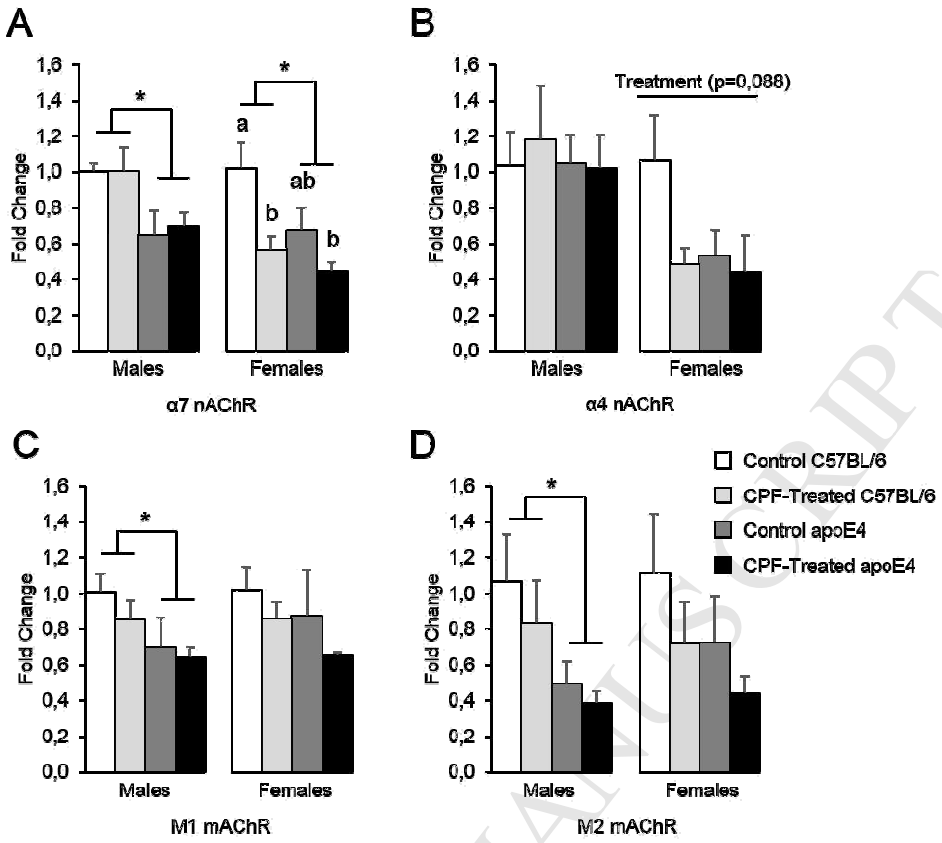

Fig. 3. Relative gene expression in the frontal cortex of α7 nAChR (A), α4 nAChR (B), M1 mAChR (C) and M2 mAChR (D). An asterisk indicates significant genotype differences at $p<0.05$. Different letters (a,b) indicate a significant difference between groups ($p<0.05$).

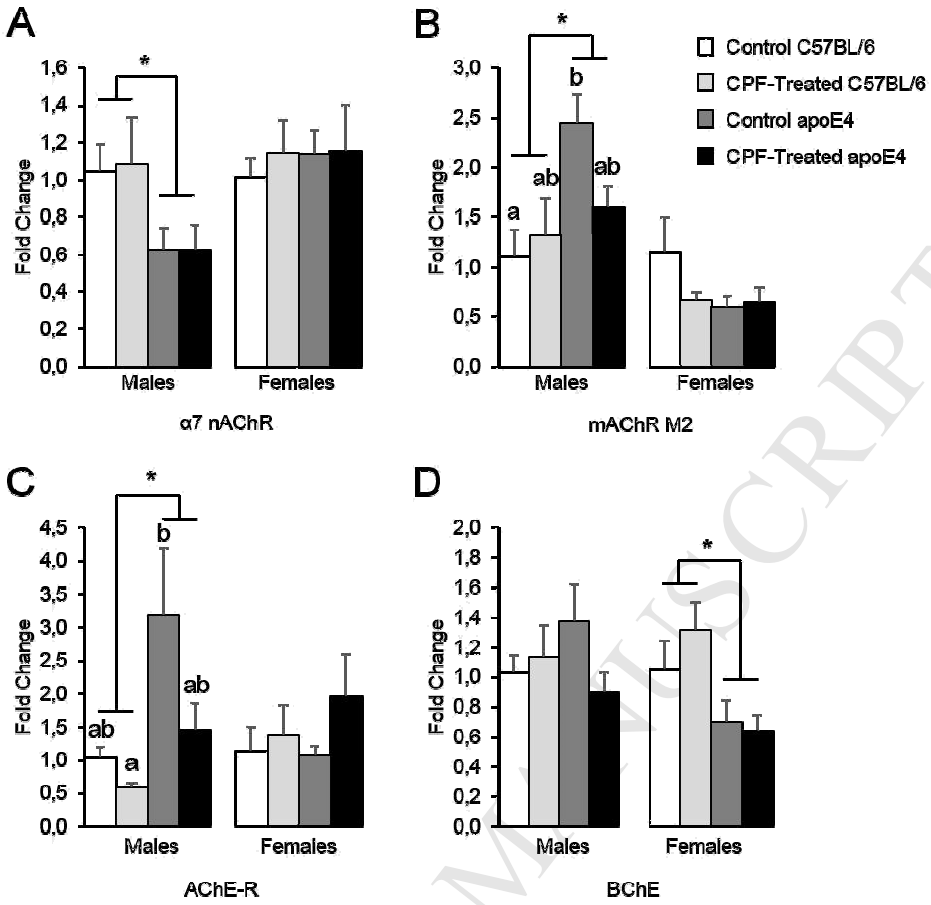

Fig. 4. Relative gene expression in the hippocampus of α7 nAChR (A), M2 mAChR (B), AChE-R (C) and BChE (D). An asterisk indicates significant genotype differences at $p<0.05$. Different letters (a,b) indicate a significant difference between groups ($p<0.05$).

Highlights

**Postnatal CPF exposure impaired the spatial retention in apoE4 male mice.

**ApoE4 transgenic mice performed worse than C57BL/6 mice during the retention of a spatial task.

**ApoE4 transgenic mice and C57BL/6 mice differed in the expression levels of a considerable number of cholinergic genes.

**C57BL/6 females were the most sensitive to the effects of CPF on the expression levels of cholinergic elements.