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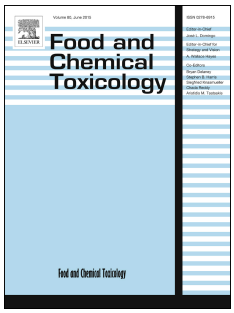

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Postnatal chlorpyrifos exposure and apolipoprotein E *(APOE)* genotype differentially affect cholinergic expression and developmental parameters in transgenic mice

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Abstract

Chlorpyrifos (CPF) is one of the most commonly used organophosphate pesticides in the world. Our previous results described that apolipoprotein E (APOE) polymorphisms are a source of individual differences in susceptibility to CPF. The aim of this study was to assess the physical and biochemical effects of postnatal exposure to CPF in the apoE targeted replacement mouse model. Mice were exposed to CPF at 0 or 1 mg/kg/day from postnatal day 10 to 15. Physical development, plasma and forebrain cholinesterase (ChE) activity and gene expression in liver and forebrain were evaluated. CPF exposure delays physical maturation and decreases the expression of choline acetyltransferase, α4-subunit and the α7 receptor. CPF decreases the expression of vesicular acetylcholine transporter (VAChT) mRNA in the forebrain only in apoE3 mice. The expression of paraoxonase-2 in the forebrain was also influenced by *APOE* genotype and CPF. Differences between genotypes were observed in litter size, ChE activity, expression of butyrylcholinesterase and paraoxonase-1 in liver and variants of acetylcholinesterase, VAChT and the α7 receptor in the forebrain. These results support that there are different vulnerabilities to postnatal CPF exposure according to the APOE polymorphism, which in turn affects the cholinergic system and defenses to oxidative stress.

Keywords: Pesticide, *APOE*, development, cholinergic system, sex differences, paraoxonase

1. Introduction

Organophosphorus (OP) compounds encompass an extensive family of chemicals with several uses such as pesticides, chemical weapons, and medical drugs, among others (Saunders et al., 2012). Chlorpyrifos (CPF) is a potent insecticide and acaricide, being one of the most commonly used OPs in the agricultural, industrial and gardening sectors (Grand View Research, 2014). Its extensive use in the last decades has meant a great challenge for both human health and wildlife. Dietary exposure to CPF – in fruits, vegetables, water and dairy products – appears to be the main source of non-occupational exposure, especially in the child population (Lozowicka et al., 2014; Picó et al., 2018; Saunders et al., 2012). Prenatal and postnatal exposure to CPF occurs via the placenta and breastfeeding, respectively (Saunders et al., 2012). Mansour and Mossa (2010) in rats suggested that CPF transferred through the mother's milk can lead to oxidative stress, as well as biochemical and histopathological alterations in the suckling pups. In fact, data from various studies suggest that early CPF exposures trigger adverse effects on the development period in humans (Sherman, 1999, 1996) and animal models (Moser, 2000; Venerosi et al., 2009).

In the liver, CPF is metabolized to chlorpyrifos-oxon (CPO) by cytochrome P450, this metabolite is responsible for cholinesterase (ChE) inhibition (Casida and Quistad, 2004). CPO is hydrolyzed by the enzyme paraoxonase-1 (PON1) (Costa et al., 1990), which belongs to the PON family, together with the PON2 and PON3 enzymes (Primo-Parmo et al., 1996). The three PONs have antioxidant properties, but only PON1 is involved in the biotransformation of OPs (Furlong et al., 2016). PONs are present in several tissues, while PON2 is that which prevails in the central nervous system (CNS) (Garrick et al., 2016; Giordano et al., 2011). Another crucial step in the detoxification process of CPO is carried out by B-esterases, such as butyrylcholinesterase (BChE), which prevents CPO effects (Chanda et al., 1997). On the other hand, two isoforms of the acetylcholinesterase (AChE) are described in the CNS: the membrane-bound tetramer variant (AChE-S) and the synaptic-soluble monomer (AChE-R)

(Soreq and Seidman, 2001). Likewise, a number of studies have reported that CPF inhibits the AChE-S isoform in adult rodents (Basaure et al., 2017; López-Granero et al., 2013). The inhibitory effect of CPF produces an accumulation of acetylcholine (ACh) in the synaptic cleft that leads to a cholinergic overstimulation, and subsequently, a downregulation of the muscarinic and nicotinic receptors (mAChRs and nAChRs, respectively) (Rhodes et al., 2004; Slotkin et al., 2004; Terry et al., 2007). Moreover, it has been reported that perinatal exposure to CPF has long-term effects on cholinergic signaling elements and might be the cause of several developmental disorders and long lasting neurotoxic effects (Abreu-Villaça and Levin, 2017).

An aspect to bear in mind is that genetic polymorphisms can contribute greatly to individual differences in terms of toxicity. In the case of OPs, research has focused on polymorphic variants of enzymes related to their biotransformation (Adad et al., 2015; Gómez-Martín et al., 2015). Studies on transgenic and knockout animals have shown that each *PON1* polymorphism modulates the effects of CPO in the brain after perinatal exposures (Cole et al., 2011; Marsillach et al., 2016). However, there is much less information about the effects of polymorphic genes, which have been related to differences in cholinergic signaling, as well as in vulnerability to neurodegenerative diseases (Haywood and Mukaetova-Ladinska, 2006; Pomara et al., 2004). For instance, apolipoprotein E (apoE) is a protein involved in lipid transport and distribution, playing a central role in neurobiology and neurodegenerative diseases (Hatters et al., 2006). The *APOE* gene is polymorphic in humans, and the three allelic variants (*ε2*, *ε3*, and *ε4*) result in the most common *APOE* genotypes in the following order: *APOE3>APOE4>APOE2* (Eisenberg et al., 2010). Our previous studies with the apoE targeted replacement (apoE-TR) mice have shown differences between *APOE* genotypes in certain neurochemical systems in adults (Reverte et al., 2016, 2014b), the time of eye opening in the developmental period (Reverte et al., 2014a), and body weight from early ages (Reverte et al., 2013, 2012). Concurrently, prior results have confirmed inter-individual differences in the susceptibility to CPF toxicity in different transgenic animal models, which supports a great variety of gene and environmental interactions (Peris-Sampedro et al., 2015b, 2014; Salazar et al., 2011). We also reported the

influence of *APOE* polymorphisms on the modulation of several CPF effects on the neurobehavioral and metabolic status of adult mice (Peris-Sampedro et al., 2016, 2015a, 2015b). However, currently there is no information available about the contribution that *APOE* genotype makes to the expression of effects induced by early exposure to CPF. Thus, the aim of the current research was to assess the physical and biochemical effects of postnatal exposure to CPF in apoE3 and apoE4 mice of both sexes, from postnatal day (PND) 10 to PND 33. In addition, this study extends our prior findings about the differences between these two *APOE* genotypes during the first month of life.

2. Material and methods

2.1. Animals

Human apoE-TR mice were used for this study. These animals have a C57BL/6 background and express functional human apoE isoforms (Sullivan et al., 1997). Adult homozygous (*ε3* and *ε4* alleles) apoE-TR male and female mice were obtained from Taconic (Taconic Europe, Lille Skensved, Denmark). After a quarantine period of one week, female mice were mated with males of the same genotype during 5 days. Pregnant females were individually housed, being the day of delivery designated as PND 0. The litters were randomly assigned to the control or treated groups. Treatment with CPF was administered from PND 10 to PND 15. The litters were weaned on PND 28 and housed in cages containing 2 to 5 animals of the same sex. All animals were allowed free access to water and food (Panlab rodent chow, Barcelona, Spain). The animal room was kept 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).

2.2. Chemicals and treatment

Chlorpyrifos [O,O-diethyl O-(3,5,6-trichloropyridin-2-yl) phosphorothioate] was supplied by Sigma-Aldrich Co. LLC. (Madrid, Spain). CPF was dissolved in corn oil and adjusted to orally administer 1 mg/kg in 1 µL per g of body weight. The mice were given 0 (vehicle) or 1 mg/kg daily using a micro-pipette. The dose administered and the treatment period were similar to those previously reported (Ricceri et al., 2003; Venerosi et al., 2006). CPF was dispensed in this period because it coincides with the highest peak in brain growth and development in rodents (Watson et al., 2006).

2.3. Experimental groups

A total of 36 litters with 5 to 8 pups of both sexes were monitored. These litters were randomly distributed into four experimental groups as follows: control apoE3 (n = 8), CPF-treated apoE3 (n = 9), control apoE4 (n = 10) and CPF-treated apoE4 (n = 9).

2.4. Litter characteristics, viability, physical and motor development

The number of living and dead pups was recorded on PND 0. Mortality occurring during PND 1 – 4 was recorded, and the viability index was calculated (alive pups PND 4 / litter size). Alive pups from PND 4 – 28 were considered as index of lactation (alive pups PND 28 / alive pups PND 4). The development timeline of the mice was monitored during the postnatal period (PND 4 – 33) using a battery of tests to evaluate physical, motor and sexual development (Table 1). The motor development, tail pull reflex, cling and climb ability were assessed one day after treatment (PND 16) using a metallic grid (24 x 24 cm). The pups were placed on the grid and

gently pulled backwards by the tail and the pup's resistance was evaluated. The metallic grid was inclined at a 45º angle to assess cling and climb abilities (Table 1). The developmental landmarks were adapted from a previous study with apoE transgenic mice (Reverte et al., 2014a). Every pup in each litter carried out all these tests. Then, a median value for males and females in each litter was calculated and used as a litter score to obtain the mean of each group.

2.5. Sacrifice and sampling

Biological samples were obtained at PDN 15 (4 hours after the last dose of CPF) and PND 30. Four animals from different litters, for each age, sex and experimental group were sacrificed by exsanguination under isoflurane anesthesia. Blood was collected and immediately centrifuged to obtain plasma, which was stored at −80ºC until analysis. After exsanguination, mice were rapidly decapitated, and the forebrain and liver were removed, and stored at −80ºC until analysis.

2.6. ChE activity

ChE activity was measured in forebrain and plasma in four animals from different litters, for each age, sex and experimental group. Forebrain samples were weighed, homogenized in cold with 1% Triton X-100 in PBS 0.1 M at pH 7.6 and centrifuged at 15000 g for 10 min at 4ºC (Salazar et al., 2011). The supernatant was removed for analysis. In both cases, enzyme activity was determined spectrophotometrically using an updated version of the Ellman method (Basaure et al., 2017; Peris-Sampedro et al., 2016). Brain ChE activity was calculated relative to protein concentration, which was assessed using the Bradford method (Bradford, 1976). Every absorbance measurement was performed in duplicate. Plasma and brain ChE activity are represented as U/L and U/mg protein, respectively.

2.7. Analysis of gene expression

Real-time polymerase chain reaction (qPCR) analysis was used to assess the expression of relevant biomarkers, in four animals from different litters, for each age, sex and experimental group. Gene expression (human *APOE, Bche, pon1, pon2 and pon3*) on liver were assessed according to the sequences described: human *APOE* gene of apoE (Laffitte et al., 2001); *Bche* gene of BChE (García-Gómez et al., 2015); *pon1* gene of PON1 (Ali et al., 2003); *pon2* gene of PON2 (Boesch-Saadatmandi et al., 2009); *pon3* gene of PON3 (Shih et al., 2007). Gene expression (*Bche, pon2, Chat, Slc18a3, Chrm1, Chrm2, Chrm3, Chrm4, Chrm5, Chrna4, Chrna7* and *Ache)* on forebrain were assessed according to the sequences described: *Chat* gene of choline acetyltransferase (ChAT) (García-Gómez et al., 2015); *Slc18a3* gene of vesicular ACh transporter (VAChT) (Yamamuro and Aizawa, 2010); *Chrm1, Chrm2, Chrm3, Chrm4 and Chrm5* genes of M1, M2, M3, M4 and M5 mAChRs, respectively (Laspas et al., 2015); *Chrna4* gene of α4-subunit and *Chrna7* gene of α7 nAChRs (Léna et al., 1999); and *Ache* gene of both AChE-S and AChE-R (Dori et al., 2011). The entire procedure was carried out with RNase-free reagents, tubes and pipette tips and cleaning the surfaces and instruments with RNaseZap solution (ThermoFisher Scientific, Waltham, USA). Total RNA was extracted with the Totally RNA Kit being contaminating DNA removed using a DNA-free kit (Invitrogen, Carlsbad,USA). RNA concentration and purity were measured by a spectrophotometer Nanodrop 2000 (ThermoFisher Scientific, Waltham, USA). The quality of the RNA was assessed by microfluidic electrophoresis using the Agilent RNA 6000 Nano kit and the Bioanalyzer Agilent instrument (Agilent Technologies, Santa Clara, USA). cDNA was synthesized from 1 μg RNA samples using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor Isolation (Thermo Fischer Scientific, Waltham, USA). The qPCR reactions were carried out in 384-well plates, in triplicate, using the Power SYBR Green PCR Master Mix and the 7900HT Fast Real-Time PCR instrument (Thermo Fischer Scientific, Waltham, USA). The mRNA expression for each sample was estimated using the comparative cycle threshold (Ct) method. The relative gene expression levels were determined after normalization to *Gapdh* expression (Yao et al. 2016). $2^{-\Delta Ct}$ was calculated for analysis purposes.

2.8. Statistics

Data were analyzed using the SPSS Statistics 22.0 software (IBM Corp, Chicago, USA). The sex ratio, litter size, viability and lactation index were evaluated by analysis of variance (ANOVA) using the genotype as the main factor. Treatment and genotype were evaluated as the main factors in the rest of the parameters. The litter size was added as a co-variable to analyze the physical, motor and sexual development and the sex was considered as an intra-litter factor. Repeated measure multivariate analysis of variance (RMANOVA) was used to evaluate the effects of genotype and treatment on body weight and eye opening, using the age as the within-subject factor. Age, genotype and sex effects on ChE activity and gene expression were analyzed by a three-way ANOVA, using only the data of the non-treated mice. The effects of CPF were evaluated using the values of all mice at PND 15 and PND 30 separately in order to define short- and long-term effects on these two parameters. The *Post-hoc* Tukey test was used for multiple comparisons. The variance homogeneity was determined using the Levene test. Eta-square ($\eta 2$) was used as an effect size measure. It was calculated by dividing the sum of squares of the effect by the total sum of squares. The magnitude of the effect was determined as small ($\eta 2=0.01 – 0.06$), medium ($\eta 2=0.06 – 0.14$) and large ($\eta 2=0.14 - 1$) (Iacobucci, 1994). The Eta-square was expressed in the text as a percentage of the variance observed. All data were expressed as mean ± SE. Statistical significance was set at $p<0.05$.

3. Results

3.1. Litter characteristics, viability, physical and motor development

The potential effects of the genotype on litter size, sex ratio, and viability and lactation indexes were analyzed by a one-way ANOVA. The genotype only affected the litter size [$F(1,35)=5.224$ $p=0.025$], which was lower in apoE4 mice compared to apoE3 mice (Table 2).

3.2. Body weight

Body weight gain from PND 4 to 10 (Fig. 1) was analyzed by a one-way RMANOVA (genotype), and sex was used as an intra-litter factor. A significant effect of the age [$F(1,33)=55.481$, $p<0.001$] and an interaction between genotype and sex [$F(1,33)=9.453$, $p=0.004$] were observed. In addition, a two-way RMANOVA (genotype x treatment) was used to evaluate body weight from PND 10 to 28. A significant effect of the age [$F(7,25)=5.608$, $p=0.001$], and an interaction between genotype and sex [$F(7,25)=7.676$, $p=0.009$] were found. The results from the two analyses indicated a body weight gain over time in all mice; however, the two sexes showed differences in relation to their genotypes. In order to define the interaction, each PND was studied by a one-way ANOVA for each genotype in males and females. There were only differences between genotypes in males at PND 4 [$F(1,71)=3.302$, $p=0.025$] (Fig. 1). *Post-hoc* analyses revealed that the apoE4 males were heavier than the apoE3 males.

3.3. Eye opening

Eye opening from PND 12 to 16 (Fig. 2) was analyzed by a two-way RMANOVA (genotype x treatment), and sex was used as an intra-litter factor. A general effect of the age [$F(3,30)=947.685$, $p<0.001$], an interaction between age and genotype [$F(5,35)=30.331$, $p<0.001$], and an interaction between age, genotype and treatment [$F(3,30)=4,557$, $p=0,010$] were found (Fig. 2A). To better explore genotype and treatment effects, the mean day of eye opening was calculated and analyzed by a two-way ANOVA. An overall effect of the genotype [$F(1,35)=77.379$, $p<0.001$, $\eta 2=0.669$] (Fig. 2B), and the treatment [$F(1,35)=6.347$, $p=0.017$, $\eta 2=0.054$] (Fig. 2C) was observed. ApoE3 mice showed a delayed eye opening compared to apoE4 mice, and CPF led to a general delay. No sex effects were observed. The eta-square evaluation showed that the genotype had a large size effect (67%) on this variable.

3.4. Motor development

Motor skills, assessed at PND 16, were analyzed by a two-way ANOVA (genotype x treatment), and sex was used as an intra-litter factor. A general effect of the treatment was observed in the three parameters evaluated: tail pull reflex [$F(1,71)=19.133$, $p<0.001$, $\eta 2=0.212$] (Fig. 3A), cling ability [$F(1,71)=8.203$, $p=0.006$, $\eta 2=0.096$] (Fig. 3B), and climb ability [$F(1,71)=11.704$, $p=0.001$, $\eta 2=0.143$] (Fig. 3C). Neither sex nor genotype effects were noted. These results show that CPF impaired the motor functions during development in mice. The eta-square evaluation showed that the treatment had a large size effect on the pull tail reflex (21%) and climb ability (14%).

3.5. Sexual development

Sexual development from PND 28 to 33 was analyzed by a two-way ANOVA (genotype x treatment) in each sex. Neither the genotype nor the treatment affected the vaginal opening and the testes descent.

3.6. Age, genotype and sex effects on plasma and brain ChE activity

The effects of age, genotype and sex were examined in control mice at PND 15 and 30. Plasma ChE activity was analyzed using a three-way ANOVA (age x genotype x sex). An overall effect of age [$F(1,31)=116.298$, $p<0.001$, $\eta 2=0.764$] was observed, indicating a significant increase in plasma ChE activity throughout the postnatal period. An interaction between age and genotype [$F(1,31)=9.125$, $p=0.006$, $\eta 2=0.060$] was also found, showing that plasma ChE activity is different across this period depending on the genotype. Since no effects of sex were observed, the two sexes were combined to assess the effects of genotype at each age with a one-way ANOVA (genotype). Differences between genotypes were noted only at PND 30

[F(1,15)=8.672, p=0.011]. As shown in Table 3, apoE3 mice had higher plasma ChE activity than apoE4 mice. Regarding brain ChE activity, a three-way ANOVA (age x genotype x sex) showed a general effect of age [F(1,31)=12.270, p=0.002, η2=0.256], indicating that all mice increase ChE activity with age. An overall effect of genotype [F(1,31)=5.988, p=0.022, η2=0.125] was also observed, showing that apoE4 mice had higher enzymatic activity in comparison to apoE3 mice (Table 3). No effects of sex or interaction between age, genotype and sex were found.

3.7. Treatment effects on plasma and brain ChE activity

At the end of CPF exposure (PNDs 15 and 30), plasma ChE activity was analyzed using a three-way ANOVA (genotype x sex x treatment). An overall effect of treatment [F(1,31)=114,271, p<0.001, η2=0,809] was observed, indicating the inhibitory effect of CPF (Table 3). Although plasma ChE showed a rise 15 days after CPF exposure, a three-way ANOVA (genotype x sex x treatment) revealed that the effects of the treatment on plasma ChE activity were still present [F(1,31)=9.087, p=0.006, η2=0.168] in exposed groups (Table 3). However, CPF had no effect on brain ChE activity at either 4 hours or 15 days after exposure.

3.8. Effects of age, genotype and sex on liver gene expression

A three-way ANOVA (age x genotype x sex) was used to examine differences in mRNA levels. An overall effect of age was observed in all biomarkers: apoE [F(1,31)=83.176, p<0.001, η2=0.768], BChE [F(1,31)=12.816, p=0.002, η2=0.273], PON1 [F(1,31)=29.418, p<0.001, η2=0.401], PON2 [F(1,31)=7.086, p=0.014, η2=0.173], and PON3 [F(1,31)=7.440, p=0.012, η2=0.213]. A higher expression at PND 15 was observed for all biomarkers (Table 4). Moreover, a general effect of genotype on BChE [F(1,31)=6.923, p=0.015, η2=0.148], and PON1 [F(1,31)=18.864, p<0.001, η2=0.257], indicates that there is a higher expression in apoE3 mice (Table 4). Finally, sex influences on BChE [F(1,31)=5.611, p=0.027, η2=0.120]

showed higher expression in female mice (Table 4). No significant interactions between factors were observed.

3.9. Short- and long-term effects of CPF treatment on liver biomarkers

A three-way ANOVA (genotype x sex x treatment) was carried out to analyze the effects of CPF exposure at PND 15 and 30 in each biomarker. No significant effects or interactions of the treatment were noted in the liver (data not shown).

3.10. Effects of age, genotype and sex effects on brain gene expression

Brain-cholinergic elements, BChE and PON2, were analyzed by a three-way ANOVA (age x genotype x sex). An effect of age was noted in the M2 receptor [$F(1,31)=4.476$, $p=0.045$, $\eta2=0.118$] (Table 5); ChAT [$F(1,31)=34.171$, $p<0.001$, $\eta2=0.550$], VAChT [$F(1,31)=10.317$, $p=0.004$, $\eta2=0.239$], AChE-S [$F(1,31)=28.725$, $p<0.001$, $\eta2=0.441$], and BChE [$F(1,31)=9.090$, $p=0.006$, $\eta2=0.244$] (Table 6). A higher expression of VAChT and AChE-S was observed at PND 15, while ChAT, the M2 receptor and BChE exhibited higher expressions at PND 30. In addition, a general effect of genotype was observed in the α7 receptor [$F(1,31)=7.310$, $p=0.013$, $\eta2=0.180$] (Table 5); VAChT [$F(1,31)=4.639$, $p=0.042$, $\eta2=0.107$]; AChE-S [$F(1,31)=6.839$, $p=0.015$, $\eta2=0.105$]; and AChE-R [$F(1,31)=7.782$, $p=0.010$, $\eta2=0.244$] (Table 6). This indicates that apoE3 mice had a higher expression of VAChT and AChE-S, while apoE4 mice had a higher expression of the α7 receptor and AChE-R. An interaction between age and sex was also found in the M2 receptor [$F(1,31)=7.462$, $p=0.012$, $\eta2=0.196$], α4-subunit [$F(1,31)=4.824$, $p=0.038$, $\eta2=0.137$], α7 receptor [$F(1,31)=7.049$, $p=0.014$, $\eta2=0.174$] (Table 5), and PON2 [$F(1,31)=9.083$, $p=0.006$, $\eta2=0.195$] (Table 6). Interestingly, these four genes displayed a common pattern of maturation: female mice had higher expression than male mice at PND 15, while male mice had higher expression at PND 30. Finally, we found an interaction between

genotype and sex in PON2 expression [F(1,31)=5.750, p=0.025, η2=0.123], indicating that apoE3 male and apoE4 female mice had a higher expression of PON2.

3.11. Short- and long-term effects of CPF treatment on brain biomarkers

Treatment effects on brain-cholinergic elements, BChE and PON2, were analyzed separately at each PND 15 and 30 by a three-way ANOVA (genotype x sex x treatment). In the case of the ChAT expression (Fig. 4A), an overall effect of the treatment at PND 15 [F(1,31)=5.008, p=0,036, η2=0,128], and an interaction between sex and treatment at PND 30 [F(1,31)=4.635, p=0.042, η2=0.153] were noted. Regarding the α7 receptor expression (Fig. 4B), an overall effect of the treatment at PND 15 [F(1,31)=6.976, p=0.015, η2=0.205], and an interaction between sex and treatment at PND 30 [F(1,31)=6.308, p=0.019, η2=0.157] were found. In general, these genes have a lower expression due to CPF exposure at PND 15. Furthermore, CPF-treated male mice showed a lower expression compared to control male mice, while CPF-treated females showed no changes or a higher expression than their counterparts at PND 30. Concerning the α4-subunit expression (Fig. 4C), an overall effect of the treatment at PND 30 [F(1,31)=5.008, p=0.036, η2=0.128] was observed, suggesting that a decrease in the expression is a long-term effect of CPF insult. In addition, interactions between genotype and treatment on VAChT expression [F(1,31)=7.233, p=0.014, η2=0.213] at PND 15, and at PND 30 [F(1,31)=6.322, p=0.019, η2=0.140] were found (Fig. 4D). Interestingly, this biomarker only decreased in male and female apoE3 CPF-treated mice, and remained low 15 days after CPF exposure. This indicates that CPF has a persistent effect on VAChT. Finally, three interactions affected PON2 expression (Fig. 5): a genotype x treatment [F(1,31)=22.286, p<0.001, η2=0.160], a sex x treatment [F(1,31)=23.260, p<0.001, η2=0.166], and a genotype x sex x treatment [F(1,31)=4.488, p=0.045, η2=0.032]. To define these interactions, the treatment was evaluated with a one-way ANOVA for each genotype and sex. Differences were observed in apoE4 male mice [F(1,7)=6.624, p=0.042], and apoE3 female mice [F(1,7)=36.460, p=0.001]. CPF-treated apoE4 male mice showed a lower expression of PON2 than control apoE4 mice,

while CPF-treated apoE3 female mice showed a higher expression of PON2 than the untreated apoE3 mice.

4. Discussion

The present study was designed to determine physical and biochemical changes induced by a limited (PND 10-15) CPF exposure in developing mice carrying different *APOE* polymorphisms. Importantly, CPF treatment affected eye opening pace and the neuromotor development as well as the expression of cholinergic components and PON2 in the brain. Some of these effects were modulated by the genotype and the sex. Furthermore, we observed that the *APOE* genotype influenced the litter size, the physical development, ChE activity and the expression of certain enzymes and cholinergic components. Sex and genotype interactions in the expression of cholinergic components were also observed. The intrinsic differences between animals depending on the genotype and the sex highlight different vulnerabilities to early exposures to the pesticide CPF.

The available literature highlights the potential relevance of exposure to toxic cholinergic agents, such as CPF, on the development of CNS. In our study, the administration of CPF from PND 10 to PND 15 produced a delay in the day of eye opening regardless of the genotype. Since previous studies using a different exposure period (from prenatal day 6 to PND 10), did not find any alteration on this parameter (Mattsson, 2000; Maurissen et al., 2000), we suggest that it could be modulated by a direct effect of CPF during the treatment. Another finding was that CPF also promoted alterations in neuromotor skills. Actually, alterations in motor functions after CPF exposure have been reported. Moser (2000) found that a single postnatal dose of CPF (20 mg/kg) caused a decrease in motor activity, while other authors reported that prenatal CPF exposure produced hyporeflexia at PND 15 (Venerosi et al., 2006) and PND 90 after repeated

low doses of CPF (Abou-Donia et al., 2006). Taken together, these data suggest a long sensitive period where different motor functions can be altered by a broad dose range of CPF.

By the same token, brain expression of VAChT, ChAT, the α4-subunit and α7 receptor, and PON2 were modified by the pesticide. In general, we observed that these genes decreased their expression after the treatment period. In the case of ChAT, various studies confirmed decreased activity after prenatal CPF exposure (Richardson and Chambers, 2004), as well as after postnatal exposure (Slotkin et al., 2001). Intriguingly, 15 days after exposure, ChAT expression remained low in male mice, but it increased in females. Slotkin et al. (2001) found changes in ChAT activity and identified a persistent and sex-selective decrease in the enzyme at PNDs 30 and 60 after postnatal CPF exposure. Likewise, a lasting reduction in the α7 receptor expression has also been reported after CPF treatment (Slotkin et al., 2004; Terry et al., 2007). From a broader perspective, Slotkin et al. (2001) suggested that early exposure to CPF might decrease the tonic activity of cholinergic projections in a sex-dimorphic manner. Taken together, these results provide further support for the hypothesis that low level exposures lead to long-term cholinergic changes, resulting in sex-dependent behavioral differences.

Another important finding was that fifteen days after CPF exposure, the following delayed-onset changes in expression were observed: a decrease in the α4-subunit receptor, an increase in brain-PON2 in apoE3 female mice and a decrease in apoE4 male mice. Our results for the α4-subunit are in accordance with those reported by Slotkin et al. (2004), who found a decrease in the α4β2 subtype receptor in cerebellum 6 days after CPF treatment. Altogether, these findings corroborate the idea that early CPF exposure induces delayed alterations in the cholinergic system. Regarding PON2, it is well known that it has protective effects against oxidative stress and neuroinflammation in brain cells (Furlong et al., 2016). Furthermore, a strong relationship between CPF exposure and ROS formation in several organs and cell lines has been reported (Garcia et al., 2005; Jasna et al., 2014). Garcia et al. (2005) suggested that ROS formation could contribute to the neurotoxic effects elicited by postnatal CPF exposure. Nowadays, only one

study has investigated *in vitro* the defense of cells exposed to CPF in terms of PON2 (Jasna et al., 2014). These authors reported an elevated expression of PON2 after CPF treatment in line with that observed in apoE3 female mice. In contrast, apoE4 male mice did not react to CPF, which could be due to a different antioxidant defense profile between apoE4 and apoE3.

It is interesting to remark that the effect of CPF on VAChT mRNA levels was only decreased in the apoE3 mice after treatment, and remained low 15 days after exposure. Two previous studies have described diminished levels of VAChT, which persisted 30 days after exposure, due to prenatal and postnatal CPF (Richardson and Chambers 2004, 2005). We hypothesize that in the case of *APOE3* carriers their normal cholinergic function is challenged by CPF exposure, while a decrease in the VAChT expression exerts a protective effect against the toxic effect of CPF. The present study, apoE4 mice did not respond to CPF treatment, which suggests that some populations might be more vulnerable to cholinergic stimuli depending on their apoE isoform.

What is surprising is that the effects described on brain cholinergic components occurred in absence of direct cholinergic toxicity in the CNS. Despite no physical signs of intoxication, CPF-treatment produced a 50% drop in plasma ChE activity 4 h after the end of the treatment, while brain ChE activity remained unchanged. Likewise, results from Ricceri et al. (2006, 2003) and Carr et al. (2014) reported plasma ChE inhibition without brain modifications in rodents at low or moderate doses of CPF. Interestingly, both plasma and brain ChE levels were influenced by the genotype. ApoE3 mice showed higher plasma ChE activity than apoE4 mice, while apoE3 mice showed lower ChE activity in the brain than apoE4 mice. There are few studies on the relationship between plasma ChE activity and *APOE* genotype and the results are not always consistent (Rahimi et al 2013, Kálman et al 2004). Regarding brain ChE activity, two studies performed with AD patients found that the enzyme activity was higher in *APOE4* carriers (Eggers et al., 2006; Soininen et al., 1995). Notwithstanding, there are no studies assessing the contribution of *APOE* genotype to the ChE activity in healthy humans or transgenic animal

models. Our findings are probably related to gene expression of BChE in the liver and AChE-R in the brain, as below discussed in more detail.

It is interesting to note that we also observed a maturational pattern in liver gene expression. In fact, the rodent liver is in the process of acquiring the functional ability of an adult from the late prenatal stage to PND 28 (Moscovitz and Aleksunes, 2013). Accordingly, our results showed the highest expression at PND 15 for all the proteins evaluated: human apoE, BChE and PON1, PON2 and PON3. Previous studies reported similar results for apoE (Mangeney et al., 1989), BChE (Lepage et al., 1985), PON1 (Li et al., 1997), PON2 and PON3 expression (Garrick et al., 2016). Moreover, the expression of BChE and PON1 is higher in apoE3 mice compared to apoE4. To date, no prior studies have analyzed the gene expression of BChE in young subjects with different *APOE* genotypes, while only one study has evaluated PON1 expression. As reported by Boesch-Saadatmandi et al. (2010), apoE4-TR mice had a lower PON1 expression in comparison to apoE3 mice at 8 weeks of age. PON1 is the most abundant PON, being the only one that participates in CPF metabolism (Furlong et al., 2016). In the present investigation, the lower ChE activity, and BChE and PON1 expression in apoE4 mice suggest they have higher vulnerability to CPF at an early age. Nevertheless, in this study CPF exposure did not cause modifications in liver gene expression. Therefore, we suggest that the overlap between CPF exposure and the period of maximum gene expression of these enzymes in liver might be the cause of a blunted response to the toxic insult.

On the other hand, the cholinergic system plays a pivotal role in the development of the nervous system, being ChAT, VAChT, mAChRs, nAChRs, BChE and AChE expressed early during the embryonic period in cholinergic neurons (Abreu-Villaça et al., 2011). In the current study, different peak expressions were observed; VAChT and AChE-S showed the highest expression at PND 15, while ChAT, the M2 receptor, and BChE had the highest expression at PND 30. Our results match well with those earlier reported on maximum expression periods during the first 3 weeks of life in rodents for VAChT (Sun et al., 1997), AChE-S (Oriel and Kofman, 2015),

ChAT (Sun et al., 1997), and the M2 receptor (Wall et al., 1992). It seems that the maturation pace of cholinergic elements is relatively similar between mice models. However, genotype effects were observed in the VAChT, α7 receptor and AChE isoform mRNA levels, which fuels the much-debated question on whether cognitive impairments in *APOE4* carriers are related to constitutive differences in the cholinergic signaling system. In this sense, VAChT levels regulate the cholinergic tone, playing a fundamental role in certain cognitive processes (Prado et al., 2013). We here observed a higher VAChT expression in apoE3 mice compared to apoE4 mice. Similarly, Dolejší et al. (2016) reported a marked decrease in VAChT expression in adult apoE4 transgenic mice. According to these data, we can infer that *APOE* genotype may influence VAChT fluctuations in the CNS. Another unanticipated finding was that apoE4 mice showed higher α7 receptor expression. Recently, Wang et al. (2017) found a correlation between *APOE4* genotype and a heightened Aβ42-α7 complex, and the progression of cognitive decline in *APOE4* carriers. The genotype effect on α7 receptor expression may reflect a possible link between *ε4* allele and amyloid deposits in *APOE4* carriers from an early age. Moreover, AChE isoform expression was also influenced by *APOE* genotype. In normal conditions, AChE-S is the predominant isoform; however, cortical and hippocampal neurons increase the AChE-R under stress conditions (Härtl et al., 2011; Soreq and Seidman, 2001). A number of studies have linked oxidative stress derived from lipid metabolism and the *ε4* allele, highlighting that *APOE4* carriers have a reduced antioxidant capacity (Boesch-Saadatmandi et al., 2010; Villasana et al., 2016). A possible explanation for the higher AChE-R expression found in apoE4 mice could be related to the inherent oxidative stress of *APOE4* carriers. We cannot rule out the possibility that the differences between our apoE3 and apoE4 mice in the expression of these cholinergic elements are associated with a particular maturation pattern in each genotype. Therefore, further studies at different ages are necessary to confirm the persistence of these differences. Likewise, these findings suggest that there are differences in cholinergic system between *APOE* genotypes from the developmental stage. If this is confirmed, preventive programs would probably need to be initiated in young *APOE4* carriers.

To date, various studies have attempted to shed light on the role played by apoE in neurodevelopment (Reverte et al., 2014a; Wright et al., 2003). The main function of the apoE protein is the intracellular transport of lipids and cholesterol. Concerning this, cholesterol levels increase from the first to the third trimester in humans during pregnancy. Moreover, it has been reported that hypercholesterolemia decreases litter size and increases the number of post-implantations lost in rats during gestation (De Assis et al., 2003). The results from the current study show that the mean number of pups per litter in apoE3 mice was larger than in the apoE4 mice litters. It is consistent with data from a previous study conducted in our laboratory (Reverte et al., 2014a), in which we obtained similar, although statistically non-significant, results. Coupled with this, several published data describe alterations on lipid metabolism in *APOE4* carriers (Arbones-Mainar et al., 2008; Salameh et al., 2016). Indeed, a recent study showed that homozygosity for the *ε4* allele increases the levels of cholesterol compared to *ε3* homozygosity in pregnant women (Tanyanyiwa et al., 2016). Based on it we suggest that the high levels of cholesterol associated with the *APOE4* genotype might be responsible for the small litter size in the apoE4 groups. On the other hand, body weight at PND 4 was higher in apoE4 male mice compared to those in the apoE3 group, while apoE4 pups opened their eyes earlier than apoE3. These differences can be related to the litter size which can influence both body weight at birth and early maturation (Chahoud and Paumgartten, 2009). Nevertheless, although the litter size did not change, differences in body weight disappeared by PND 10, which suggests prenatal rather than postnatal influences. Altogether, these findings underpin the relevance of maternal cholesterol in reproductive outcomes. Further studies are required to elucidate prenatal factors such as maternal metabolism changes, lipid status and inflammation in order to determine the origin and long-term impact of these disparities between genotypes.

The results of the current investigation support the idea that individual differences in age, sex and *APOE* genotype can markedly evoke different responses to a CPF challenge in the human population. These factors are an important source of variability that constrain and bias the interpretation of epidemiological studies. Moreover, dissimilar or opposite conclusions in terms

of protection and vulnerability can be drawn depending on the targeted system. Thus, apoE3 mice seem more resilient to oxidative stress and detoxifying enzymes, while in apoE4 mice an undermined cholinergic system may confer some protection against exogenous overstimulation. Finally, persistent alterations in the cholinergic system, behavioral disturbances and altered responses to oxidative damage may be induced by CPF exposure during the developmental period.

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References

Abou-Donia, M.B., Khan, W.A., Dechkovskaia, A.M., Goldstein, L.B., Bullman, S.L., Abdel-Rahman, A., 2006. In utero exposure to nicotine and chlorpyrifos alone, and in combination produces persistent sensorimotor deficits and Purkinje neuron loss in the cerebellum of adult offspring rats. Arch. Toxicol. 80, 620–631.

Abreu-Villaça, Y., Filgueiras, C.C., Manhães, A.C., 2011. Developmental aspects of the cholinergic system. Behav. Brain Res. 221, 367–378. doi:10.1016/j.bbr.2009.12.049

Abreu-Villaça, Y., Levin, E.D., 2017. Developmental neurotoxicity of succeeding generations of insecticides. Environ. Int. 99, 55–77.

Adad, L.M. de M., Andrade, H.H.R. de, Kvitko, K., Lehmann, M., Cavalcante, A.A. de C.M., Dihl, R.R., 2015. Occupational exposure of workers to pesticides: Toxicogenetics and susceptibility gene polymorphisms. Genet. Mol. Biol. 38, 308–315.

Ali, A. Bin, Zhang, Q., Lim, Y.K., Fang, D., Retnam, L., Lim, S.K., 2003. Expression of major HDL-associated antioxidant PON-1 is gender dependent and regulated during inflammation. Free Radic. Biol. Med. 34, 824–829.

Arbones-Mainar, J.M., Johnson, L.A., Altenburg, M.K., Maeda, N., 2008. Differential modulation of diet-induced obesity and adipocyte functionality by human apolipoprotein E3 and E4 in mice. Int. J. Obes. 32, 1595–1605.

Basaure, P., Peris-Sampedro, F., Cabre, M., Reverte, I., Colomina, M.T., 2017. Two cholinesterase inhibitors trigger dissimilar effects on behavior and body weight in C57BL/6 mice: The case of chlorpyrifos and rivastigmine. Behav. Brain Res. 318, 1–11.

Boesch-Saadatmandi, C., Niering, J., Minihane, A.M., Wiswedel, I., Gardeman, A., Wolffram, S., Rimbach, G., 2010. Impact of apolipoprotein E genotype and dietary quercetin on paraoxonase 1 status in apoE3 and apoE4 transgenic mice. Atherosclerosis 211, 110–113.

Boesch-Saadatmandi, C., Pospissil, R.T., Graeser, A.C., Canali, R., Boomgaarden, I., Doering, F., Wolffram, S., Egert, S., Mueller, M.J., Rimbach, G., 2009. Effect of quercetin on paraoxonase 2 levels in RAW264.7 macrophages and in human monocytes - Role of quercetin metabolism. Int. J. Mol. Sci. 10, 4168–4177.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248–254.

Carr, R.L., Graves, C.A., Mangum, L.C., Nail, C.A., Ross, M.K., 2014. Low level chlorpyrifos exposure increases anandamide accumulation in juvenile rat brain in the absence of brain cholinesterase inhibition. Neurotoxicology 43, 82–89.

Casida, J.E., Quistad, G.B., 2004. Organophosphate Toxicology: Safety Aspects of Nonacetylcholinesterase Secondary Targets. Chem. Res. Toxicol.

Chahoud, I., Paumgartten, F.J.R., 2009. Influence of litter size on the postnatal growth of rat pups: is there a rationale for litter-size standardization in toxicity studies? Environ. Res. 109, 1021–1027.

Chanda, S.M., Mortensen, S.R., Moser, V.C., Padilla, S., 1997. Tissue-specific effects of chlorpyrifos on carboxylesterase and cholinesterase activity in adult rats: an in vitro and in vivo comparison. Fundam. Appl. Toxicol. 38, 148–57.

Cole, T.B., Beyer, R.P., Bammler, T.K., Park, S.S., Farin, F.M., Costa, L.G., Furlong, C.E., 2011. Repeated developmental exposure of mice to chlorpyrifos oxon is associated with

paraoxonase 1 (PON1)-modulated effects on cerebellar gene expression. Toxicol. Sci. 123, 155–169.

Costa, L.G., McDonald, B.E., Murphy, S.D., Omenn, G.S., Richter, R.J., Motulsky, A.G., Furlong, C.E., 1990. Serum paraoxonase and its influence on paraoxon and chlorpyrifos-oxon toxicity in rats. Toxicol. Appl. Pharmacol. 103, 66–76.

De Assis, S.M.A., Seguro, A.C., Helou, C.M.B., S.M.A., de A., A.C., S., C.M.B., H., 2003. Effects of maternal hypercholesterolemia on pregnancy and development of offspring. Pediatr. Nephrol. 18, 328–334.

Dolejší, E., Liraz, O., Rudajev, V., Zimčík, P., Doležal, V., Michaelson, D.M., 2016. Apolipoprotein E4 reduces evoked hippocampal acetylcholine release in adult mice. J. Neurochem. 136, 503–509.

Dori, A., Oriel, S., Livneh, U., Duek, O., Lin, T., Kofman, O., 2011. Acetylcholinesterase inhibitor pretreatment alters stress-induced expression of acetylcholinesterase transcripts in the mouse brain. Neuroscience 183, 90–98.

Eggers, C., Herholz, K., Kalbe, E., Heiss, W.-D., 2006. Cortical acetylcholine esterase activity and ApoE4-genotype in Alzheimer disease. Neurosci. Lett. 408, 46–50.

Eisenberg, D.T.A., Kuzawa, C.W., Hayes, M.G., 2010. Worldwide allele frequencies of the human apolipoprotein E gene: Climate, local adaptations, and evolutionary history. Am. J. Phys. Anthropol. 143, 100–111.

Furlong, C.E., Marsillach, J., Jarvik, G.P., Costa, L.G., 2016. Paraoxonases-1, -2 and -3: What are their functions? Chem. Biol. Interact. 259, 51–62. doi:10.1016/j.cbi.2016.05.036

García-Gómez, B.E., Fernández-Gómez, F.J., Muñoz-Delgado, E., Buée, L., Blum, D., Vidal, C.J., 2015. mRNA Levels of ACh-Related Enzymes in the Hippocampus of THY-Tau22 Mouse: A Model of Human Tauopathy with No Signs of Motor Disturbance. J. Mol. Neurosci. 1–5.

Garcia, S.J., Seidler, F.J., Slotkin, T.A., 2005. Developmental neurotoxicity of chlorpyrifos: Targeting glial cells, in: Environmental Toxicology and Pharmacology. pp. 455–461.

Garrick, J.M., Dao, K., de Laat, R., Elsworth, J., Cole, T.B., Marsillach, J., Furlong, C.E.,

Costa, L.G., 2016. Developmental expression of paraoxonase 2. Chem. Biol. Interact. 259, 168–174.

Giordano, G., Cole, T.B., Furlong, C.E., Costa, L.G., 2011. Paraoxonase 2 (PON2) in the mouse central nervous system: a neuroprotective role? Toxicol. Appl. Pharmacol. 256, 369–78.

Gómez-Martín, A., Hernández, A.F., Martínez-González, L.J., González-Alzaga, B., Rodríguez-Barranco, M., López-Flores, I., Aguilar-Garduno, C., Lacasana, M., 2015. Polymorphisms of pesticide-metabolizing genes in children living in intensive farming communities. Chemosphere 139, 534–540.

Grand View Research, 2014. Chlorpyrifos Market Analysis, Market Size, Application Analysis, Regional Outlook, Competitive Strategies, and Forecasts, 2015 To 2022 http://www.grandviewresearch.com/industry-analysis/chlorpyrifos-market (accessed 13 september 2017).

Härtl, R., Gleinich, A., Zimmermann, M., 2011. Dramatic increase in readthrough acetylcholinesterase in a cellular model of oxidative stress. J. Neurochem. 116, 1088–1096.

Hatters, D.M., Peters-Libeu, C.A., Weisgraber, K.H., 2006. Apolipoprotein E structure: insights into function. Trends Biochem. Sci. 31, 445–454.

Haywood, W.M., Mukaetova-Ladinska, E.B., 2006. Sex influences on cholinesterase inhibitor treatment in elderly individuals with Alzheimer's disease. Am. J. Geriatr. Pharmacother. 4, 273–86.

Iacobucci, D., 1994. Analysis of Experimental Data, in: Bagozzi, R.P. (Ed.), Principles of Marketing Research. Cambridge, MA, pp. 224–278.

Jasna, J.M., Anandbabu, K., Bharathi, S.R., Angayarkanni, N., 2014. Paraoxonase enzyme protects retinal pigment epithelium from chlorpyrifos insult. PLoS One 9, 1–10.

Kálmán, J., Juhász, A., Rakonczay, Z., Ábrahám, G., Zana, M., Boda, K., Farkas, T., Penke, B., Janka, Z., 2004. Increased serum butyrylcholinesterase activity in type IIb hyperlipidaemic patients. Life Sci. 75, 1195–1204.

Laffitte, B.A., Joseph, S.B., Walczak, R., Pei, L., Wilpitz, D.C., Collins, J.O.N.L., Tontonoz, P.,

2001. Autoregulation of the Human Liver X Receptor alfa Promoter. Mol. Cell. Biol. 21, 7558–7568.

Laspas, P., Sniatecki, J.J., Brochhausen, C., Steege, A., Goloborodko, E., Kordasz, M.L., Grus, F.H., Pfeiffer, N., Gericke, A., 2015. Effect of the M1 Muscarinic Acetylcholine Receptor on Retinal Neuron Number Studied with Gene-Targeted Mice. J. Mol. Neurosci. 56, 472–479.

Léna, C., de Kerchove D'Exaerde, A., Cordero-Erausquin, M., Le Novère, N., del Mar Arroyo-Jimenez, M., Changeux, J.P., 1999. Diversity and distribution of nicotinic acetylcholine receptors in the locus ceruleus neurons. Proc. Natl. Acad. Sci. U. S. A. 96, 12126–31.

Lepage, L., Schiele, F., Gueguen, R., Siest, G., 1985. Total cholinesterase in plasma: biological variations and reference limits. Clin. Chem. 31.

Li, W., Matthews, C., Disteche, C., Costa, L., Furlong, C., 1997. Paraoxonase (PON1) gene in mice: sequencing, chromosomal localization and developmental expression. Pharmacogenetics 7, 137–44.

López-Granero, C., Cardona, D., Giménez, E., Lozano, R., Barril, J., Sánchez-Santed, F., Cañadas, F., 2013. Chronic dietary exposure to chlorpyrifos causes behavioral impairments, low activity of brain membrane-bound acetylcholinesterase, and increased brain acetylcholinesterase-R mRNA. Toxicology 308, 41–9.

Lozowicka, B., Kaczynski, P., Paritova, A.E., Kuzembekova, G.B., Abzhalieva, A.B., Sarsembayeva, N.B., Alihan, K., 2014. Pesticide residues in grain from Kazakhstan and potential health risks associated with exposure to detected pesticides. Food Chem. Toxicol. 64, 238–248.

Mangeney, M., Cardot, P., Lyonnet, S., Coupe, C., Benarous, R., Munnich, A., Girard, J., Chambaz, J., Bereziat, G., 1989. Apolipoprotein-E-gene expression in rat liver during development in relation to insulin and glucagon. Eur. J. Biochem. 181, 225–230.

Mansour, S., Mossa, A., 2010. Adverse effects of lactational exposure to chlorpyrifos in suckling rats. Hum. Exp. Toxicol. 29, 77–92.

Marsillach, J., Costa, L.G., Furlong, C.E., 2016. Paraoxonase-1 and Early-Life Environmental

Exposures. Ann. Glob. Heal. 82, 100–10.

Mattsson, J.L., 2000. Lack of Differential Sensitivity to Cholinesterase Inhibition in Fetuses and Neonates Compared to Dams Treated Perinatally with Chlorpyrifos. Toxicol. Sci. 53, 438–446.

Maurissen, J., Hoberman, A., Garman, R., Hanley, T., 2000. Lack of selective developmental neurotoxicity in rat pups from dams treated by gavage with chlorpyrifos. Toxicol. Sci. 57, 250–63.

Moscovitz, J.E., Aleksunes, L.M., 2013. Establishment of Metabolism and Transport Pathways in the Rodent and Human Fetal Liver. Int. J. Mol. Sci. 14, 23801–23827.

Moser, V., 2000. Dose-response and time-course of neurobehavioral changes following oral chlorpyrifos in rats of different ages. Neurotoxicol. Teratol. 22, 713–723.

Oriel, S., Kofman, O., 2015. Strain dependent effects of conditioned fear in adult C57Bl/6 and Balb/C mice following postnatal exposure to chlorpyrifos: relation to expression of brain acetylcholinesterase mRNA. Front. Behav. Neurosci. 9, 110.

Peris-Sampedro, F., Basaure, P., Reverte, I., Cabré, M., Domingo, J.L., Colomina, M.T., 2015a. Chronic exposure to chlorpyrifos triggered body weight increase and memory impairment depending on human apoE polymorphisms in a targeted replacement mouse model. Physiol. Behav. 144, 37–45.

Peris-Sampedro, F., Cabré, M., Basaure, P., Reverte, I., Domingo, J.L., Teresa Colomina, M., 2015b. Adulthood dietary exposure to a common pesticide leads to an obese-like phenotype and a diabetic profile in apoE3 mice. Environ. Res. 142, 169–176.

Peris-Sampedro, F., Reverte, I., Basaure, P., Cabré, M., Domingo, J.L., Colomina, M.T., 2016. Apolipoprotein E (APOE) genotype and the pesticide chlorpyrifos modulate attention, motivation and impulsivity in female mice in the 5-choice serial reaction time task. Food Chem. Toxicol. 92, 224–235.

Peris-Sampedro, F., Salazar, J.G., Cabré, M., Reverte, I., Domingo, J.L., Sánchez-Santed, F., Colomina, M.T., 2014. Impaired retention in AβPP Swedish mice six months after oral exposure to chlorpyrifos. Food Chem. Toxicol. 72C, 289–294.

Picó, Y., El-Sheikh, M.A., Alfarhan, A.H., Barceló, D., 2018. Target vs non-target analysis to determine pesticide residues in fruits from Saudi Arabia and influence in potential risk associated with exposure. Food Chem. Toxicol. 111, 53–63.

Pomara, N., Willoughby, L.M., Wesnes, K., Sidtis, J.J., 2004. Increased Anticholinergic Challenge-Induced Memory Impairment Associated with the APOE-ε4 Allele in the Elderly: A Controlled Pilot Study. Neuropsychopharmacology 29, 403–409.

Prado, V.F., Roy, A., Kolisnyk, B., Gros, R., Prado, M.A.M., 2013. Regulation of cholinergic activity by the vesicular acetylcholine transporter. Biochem. J. 450, 265 LP-274.

Primo-Parmo, S.L., Sorenson, R.C., Teiber, J., LaDuab, B.N., 1996. The Human Serum Paraoxonase/Arylesterase Gene (PON1) Is One Member of a Multigene Family. Genomics 33, 498–507.

Rahimi, Z., Ahmadi, R., Vaisi-Raygani, A., Rahimi, Z., Bahrehmand, F., Parsian, A., 2013. Butyrylcholinesterase (BChE) activity is associated with the risk of preeclampsia: influence on lipid and lipoprotein metabolism and oxidative stress. J. Matern. Neonatal Med. 26, 1590–1594.

Reverte, I., Domingo, J.L., Colomina, M.T., 2014a. Neurodevelopmental effects of decabromodiphenyl ether (BDE-209) in APOE transgenic mice. Neurotoxicol. Teratol. 46, 10–17.

Reverte, I., Klein, A.B., Domingo, J.L., Colomina, M.T., 2013. Long term effects of murine postnatal exposure to decabromodiphenyl ether (BDE-209) on learning and memory are dependent upon APOE polymorphism and age. Neurotoxicol. Teratol. 40, 17–27.

Reverte, I., Klein, A.B., Ratner, C., Domingo, J.L., Colomina, M.T., 2012. Behavioral phenotype and BDNF differences related to apoE isoforms and sex in young transgenic mice. Exp. Neurol. 237, 116–25.

Reverte, I., Peris-Sampedro, F., Basaure, P., Campa, L., Suñol, C., Moreno, M., Domingo, J.L., Colomina, M.T., 2016. Attentional performance, impulsivity, and related neurotransmitter systems in apoE2, apoE3, and apoE4 female transgenic mice. Psychopharmacology (Berl). 233, 295–308.

Reverte, I., Pujol, A., Domingo, J.L., Colomina, M.T., 2014b. Thyroid hormones and fear learning but not anxiety are affected in adult apoE transgenic mice exposed postnatally to decabromodiphenyl ether (BDE-209). Physiol. Behav. 133, 81–91.

Rhodes, M.C., Seidler, F.J., Qiao, D., Tate, C.A., Cousins, M.M., Slotkin, T.A., 2004. Does pharmacotherapy for preterm labor sensitize the developing brain to environmental neurotoxicants? Cellular and synaptic effects of sequential exposure to terbutaline and chlorpyrifos in neonatal rats. Toxicol. Appl. Pharmacol. 195, 203–17.

Ricceri, L., Markina, N., Valanzano, A., Fortuna, S., Cometa, M.F., Meneguz, A., Calamandrei, G., 2003. Developmental exposure to chlorpyrifos alters reactivity to environmental and social cues in adolescent mice. Toxicol. Appl. Pharmacol. 191, 189–201.

Ricceri, L., Venerosi, A., Capone, F., Cometa, M.F., Lorenzini, P., Fortuna, S., Calamandrei, G., 2006. Developmental Neurotoxicity of Organophosphorous Pesticides: Fetal and Neonatal Exposure to Chlorpyrifos Alters Sex-Specific Behaviors at Adulthood in Mice. Toxicol. Sci. 93, 105–113.

Richardson, J.R., Chambers, J.E., 2005. Effects of Repeated Oral Postnatal Exposure to Chlorpyrifos on Cholinergic Neurochemistry in Developing Rats. Toxicol. Sci. 84, 352–359.

Richardson, J.R., Chambers, J.E., 2004. Neurochemical effects of repeated gestational exposure to chlorpyrifos in developing rats. Toxicol. Sci. 77, 83–90.

Salameh, T.S., Rhea, E.M., Banks, W.A., Hanson, A.J., 2016. Insulin resistance, dyslipidemia, and apolipoprotein E interactions as mechanisms in cognitive impairment and Alzheimer's disease. Exp. Biol. Med. 241, 1676–1683.

Salazar, J.G., Ribes, D., Cabré, M., Domingo, J.L., Sanchez-Santed, F., Colomina, M.T., 2011. Amyloid β peptide levels increase in brain of AβPP Swedish mice after exposure to chlorpyrifos. Curr. Alzheimer Res. 8, 732–40.

Saunders, M., Magnanti, B.L., Correia Carreira, S., Yang, A., Alamo-Hernández, U., Riojas-Rodriguez, H., Calamandrei, G., Koppe, J.G., Krayer von Krauss, M., Keune, H., Bartonova, A., 2012. Chlorpyrifos and neurodevelopmental effects: a literature review and

expert elicitation on research and policy. Environ. Health 11 Suppl 1, S5.

Sherman, J., 1999. Chlorpyrifos (Dursban) exposure and birth defects: report of 15 incidents, evaluation of 8 cases, theory of action, and medical and social aspects. Eur. J. Oncol. 4, 653–659.

Sherman, J.D., 1996. Chlorpyrifos (Dursban) -Associated Birth Defects: Report of Four Cases. Arch. Environ. Heal. An Int. J. 51, 5–8.

Shih, D.M., Xia, Y.-R., Wang, X.-P., Wang, S.S., Bourquard, N., Fogelman, A.M., Lusis, A.J., Reddy, S.T., 2007. Decreased Obesity and Atherosclerosis in Human Paraoxonase 3 Transgenic Mice. Circ. Res. 100, 1200–1207.

Slotkin, T.A., Cousins, M.M., Tate, C. a, Seidler, F.J., 2001. Persistent cholinergic presynaptic deficits after neonatal chlorpyrifos exposure. Brain Res. 902, 229–243.

Slotkin, T.A., Southard, M.C., Adam, S.J., Cousins, M.M., Seidler, F.J., 2004. alpha7 Nicotinic acetylcholine receptors targeted by cholinergic developmental neurotoxicants: Nicotine and chlorpyrifos. Brain Res. Bull. 64, 227–235.

Soininen, H., Lehtovirta, M., Helisalmi, S., Linnaranta, K., Heinonen, O., Riekkinen, P., 1995. Increased acetylcholinesterase activity in the CSF of Alzheimer patients carrying apolipoprotein epsilon4 allele. Neuroreport 6, 2518–20.

Soreq, H., Seidman, S., 2001. Acetylcholinesterase - new roles for an old actor. Nat Rev Neurosci 2, 294–302.

Sullivan, P.M., Knouff, C., Najib, J., Reddick, R.L., Quarfordt, S.H., 1997. Targeted Replacement of the Mouse Apolipoprotein E Gene with the Common Human. J. Biol. Chem. 272, 17972–17980.

Sun, X., Tian, X., Suszkiw, J.B., 1997. Reduction of vesicular acetylcholine transporter mRNA in the rat septum following lead exposure. Neuroreport 8, 891–894.

Tanyanyiwa, D.M., Marais, A.D., Byrnes, P., Jones, S., 2016. The influence of ApoE genotype on the lipid profile and lipoproteins during normal pregnancy in a Southern African population. Afr. Health Sci. 16, 853.

Terry, A. V, Gearhart, D.A., Beck, W.D., Truan, J.N., Middlemore, M., Williamson, L.N.,

Bartlett, M.G., Prendergast, M.A., Sickles, D.W., Buccafusco, J.J., 2007. Chronic , Intermittent Exposure to Chlorpyrifos in Rats : Protracted Effects on Axonal Transport , Neurotrophin Receptors , Cholinergic Markers , and Information Processing 322, 1117–1128.

Venerosi, A., Calamandrei, G., Ricceri, L., 2006. A social recognition test for female mice reveals behavioral effects of developmental chlorpyrifos exposure. Neurotoxicol. Teratol. 28, 466–71.

Venerosi, A., Ricceri, L., Scattoni, M.L., Calamandrei, G., 2009. Prenatal chlorpyrifos exposure alters motor behavior and ultrasonic vocalization in CD-1 mouse pups. Environ. Heal. 8, 12.

Villasana, L.E., Weber, S., Akinyeke, T., Raber, J., 2016. Genotype differences in anxiety and fear learning and memory of WT and ApoE4 mice associated with enhanced generation of hippocampal reactive oxygen species. J. Neurochem. 138, 896–908.

Wall, S., Yasuda, R., Li, M., Ciesla, W., Wolfe, B., 1992. The ontogeny of ml-m5 muscarinic receptor subtypes in rat forebrain. Dev. Brain Res. 66, 181–185.

Wang, H.-Y., Trocmé-Thibierge, C., Stucky, A., Shah, S.M., Kvasic, J., Khan, A., Morain, P., Guignot, I., Bouguen, E., Deschet, K., Pueyo, M., Mocaer, E., Ousset, P.-J., Vellas, B., Kiyasova, V., 2017. Increased Aβ42-α7-like nicotinic acetylcholine receptor complex level in lymphocytes is associated with apolipoprotein E4-driven Alzheimer's disease pathogenesis. Alzheimers. Res. Ther. 9, 54.

Watson, R.E., Desesso, J.M., Hurtt, M.E., Cappon, G.D., 2006. Postnatal growth and morphological development of the brain: a species comparison. Birth Defects Res B Dev Reprod Toxicol 77, 471–484.

Wright, R.O., Hu, H., Silverman, E.K., Tsaih, S.W., Schwartz, J., Bellinger, D., Palazuelos, E., Weiss, S.T., Hernandez-Avila, M., 2003. Apolipoprotein E Genotype Predicts 24-Month Bayley Scales Infant Development Score. Pediatr. Res. 54, 819–825.

Yamamuro, Y., Aizawa, S., 2010. Asymmetric regulation by estrogen at the cholinergic gene locus in differentiated NG108-15 neuronal cells. Life Sci. 86, 839–843.

Yao, Q., Chen, L., Liang, Y., Sui, L., Guo, L., Zhou, J., Fan, K., Jing, J., Zhang, Y., Yao, B., 2016. Blastomere removal from cleavage-stage mouse embryos alters placental function, which is associated with placental oxidative stress and inflammation. Sci. Rep. 6, 25023.

Figure captions

Fig. 1. Body weight of pups (males and females) from PND 4 to 28 in pre-treatment (pre-T), treatment (T) and post-treatment (post-T) period. The litter was used as the statistic unit. The asterisk indicates differences between apoE4 and apoE3 male mice at $p<0.05$.

Fig. 2. Eye opening from PND 12 to 16. (A) Percentage of individuals per litter with their eyes open from PND 12 to 16. (B) Mean day of eye opening according to genotype (C) and treatment (C). The litter was used as the statistic unit. The asterisk indicates differences at $p<0.05$.

Fig. 3 Motor development of the litter at PND 16. (A) Tail pull reflex, (B) cling and (C) climb ability. The litter was used as the statistic unit. The asterisk indicates differences between treatments at $p<0.05$.

Fig. 4. Gene expression on brain of apoE3- and apoE4-TR mice exposed to CPF during PND 10 to 15. (A) Treatment effects on expression of the (A) α7 receptor, (B) ChAT, (C) VAChT and (D) α4-subunit receptor mRNA at PND 15 and PND 30 in male and female mice. Overall

effects and interactions between factors are indicated at each PND. Data for the control groups are also shown in Table 5 and 6.

Fig. 5. Gene expression on brain of apoE3- and apoE4-TR mice exposed to CPF during PND 10 to 15. Treatment effects on expression of PON2 mRNA at PND 15 and PND 30 in male and female mice. Overall effects and interactions between factors are indicated at each PND. The α symbol represents differences between control and CPF-treated mice at $p<0.05$. Data for the control groups are also shown in Table 6.

Table 1. Assessment of the physical development.

Test	Day of evaluation (PND)	Measure/score
Body weight	4, 10-16, 28	Weight (g)
Eye opening	12-16	0 = both eyes closed; 1 = first eye opened
Tail pull reflex	16	0 = the pup offers no resistance; 1 = the pup grasps the grid and offers some resistance to the pull; 2 = the pup offers strong resistance during the whole pull
Cling ability	16	0 = the pup falls off immediately; 1 = the pup falls off within 15 s; 2 = the pup holds on for 15 s at the same position
Climb ability	16	0 = the pup falls off immediately; 1 = the pup climbs half of the grid; 2 = the pup climbs to the top of the grid
Testes descent	28-33	0 = no presences of testes; 1 = testes detected when pressing the abdomen or identified at first sight
Vaginal opening	28-33	0 = no vaginal opening; 1 = vaginal opening

Table 2. Litter characteristics in apoE-TR mice.

Genotype	N	Litter size	Sex ratio	Viability index	Lactation index
apoE3	17	**8.71 ± 0.40***	0.52 ± 0.05	0.89 ± 0.04	0.96 ± 0.03
apoE4	19	**7.58 ± 0.30***	0.49 ± 0.05	0.90 ± 0.03	1.00 ± 0.00

Litter size (alive pups at PND 0), sex ratio (alive male pups / alive female pups), viability index (alive pups PND 4 / litter size) and lactation index (alive pups PND 28 / alive pups PND 4).
The asterisk indicates significant differences between genotypes at $p<0.05$.

Table 3. Cholinesterase (ChE) activity in plasma and brain of apoE-TR mice.

		PND 15		PND 30	
		apoE3	apoE4	apoE3	apoE4
Plasma	Control	1785.0 ± 62.0	1939.5 ± 77.5	2744.2 ± 72.9$^{\alpha}$	2478.9 ± 53.0$^{\alpha}$
(U/L)	CPF-treated	**904.6 ± 117.6†**	**926.4 ± 68.7†**	**2510.8 ± 40.1†**	**2388.1 ± 52.3†**
ANOVA		*age: p<0.001; age x genotype: p=0.006*			
Brain	Control	0.061 ± 0.004	0.086 ± 0.004	0.091 ± 0.005	0.093 ± 0.005
(U/mg prot)	CPF-treated	0.071 ± 0.004	0.073 ± 0.004	0.088 ± 0.005	0.091 ± 0.005
ANOVA		*age: p=0.002; genotype: p=0.022*			

Age, sex and genotype effects in control mice were assessed by an ANOVA. Since no sex effects were detected both sexes were combined for analysis. Mean values and S.E. for each group presented. The α symbol represents differences between apoE3 and apoE4 mice at PND 30, and the † symbol indicates differences between control and CPF-treated mice within each age group at $p<0.05$.

Table 4. Gene expression in liver of apoE3- and apoE4-TR mice. Mean values of human apolipoprotein E (apoE), butyrylcholinesterase (BChE) and paraoxonase-1, -2 and -3 (PON1, PON2 and PON3) mRNA.

mRNA	Sex	PND 15		PND 30	
		apoE3	apoE4	apoE3	apoE4
apoE	♂	10.04 ± 1.12	9.20 ± 1.67	4.10 ± 0.46	3.83 ± 0.60
	♀	10.87 ± 0.83	10.93 ± 1.40	4.97 ± 0.85	3.56 ± 0.41
ANOVA		*age: p<0.001*			
BChE	♂	0.34 ± 0.05	0.29 ± 0.04	0.29 ± 0.03	0.23 ± 0.03
	♀	0.42 ± 0.05	0.36 ± 0.01	0.32 ± 0.04	0.29 ± 0.01
ANOVA		*age: p=0.002; genotype: p=0.015; sex: p=0.027*			
PON1	♂	1.50 ± 0.07	1.00 ± 0.09	1.01 ± 0.10	0.74 ± 0.17
	♀	1.76 ± 0.25	1.15 ± 0.06	0.94 ± 0.15	0.73 ± 0.11
ANOVA		*age: p<0.001; genotype: p<0.001*			
PON2	♂	0.18 ± 0.02	0.33 ± 0.02	0.26 ± 0.03	0.20 ± 0.05
	♀	0.35 ± 0.09	0.37 ± 0.06	0.23 ± 0.01	0.21 ± 0.02
ANOVA		*age: p<0.001*			
PON3	♂	0.16 ± 0.02	0.17 ± 0.01	0.16 ± 0.01	0.13 ± 0.02
	♀	0.17 ± 0.02	0.21 ± 0.04	0.15 ± 0.01	0.12 ± 0.01
ANOVA		*age: p=0.012*			

Table 5. Gene expression in brain of apoE3- and apoE4-TR mice. Mean values of muscarinic and nicotinic receptors (M1-M5; α4 and α7 subunits) mRNA.

mRNA	Sex	PND 15		PND 30	
		apoE3	apoE4	apoE3	apoE4
M1	♂	$57.0 \cdot 10^{-3} \pm 16.0 \cdot 10^{-3}$	$57.0 \cdot 10^{-3} \pm 1.8 \cdot 10^{-3}$	$63.9 \cdot 10^{-3} \pm 6.5 \cdot 10^{-3}$	$68.0 \cdot 10^{-3} \pm 3.1 \cdot 10^{-3}$
	♀	$45.8 \cdot 10^{-3} \pm 2.4 \cdot 10^{-3}$	$51.9 \cdot 10^{-3} \pm 4.2 \cdot 10^{-3}$	$42.2 \cdot 10^{-3} \pm 6.0 \cdot 10^{-3}$	$70.3 \cdot 10^{-3} \pm 10.7 \cdot 10^{-3}$
ANOVA		*NS*			
M2	♂	$7.4 \cdot 10^{-3} \pm 0.6 \cdot 10^{-3}$	$6.7 \cdot 10^{-3} \pm 0.5 \cdot 10^{-3}$	$10.8 \cdot 10^{-3} \pm 0.8 \cdot 10^{-3}$	$9.7 \cdot 10^{-3} \pm 1.1 \cdot 10^{-3}$
	♀	$8.1 \cdot 10^{-3} \pm 1.8 \cdot 10^{-3}$	$8.3 \cdot 10^{-3} \pm 0.4 \cdot 10^{-3}$	$7.4 \cdot 10^{-3} \pm 0.6 \cdot 10^{-3}$	$8.2 \cdot 10^{-3} \pm 0.6 \cdot 10^{-3}$
ANOVA		*age: p=0.045; age x sex: p=0.012*			
M3	♂	$24.5 \cdot 10^{-3} \pm 2.5 \cdot 10^{-3}$	$34.9 \cdot 10^{-3} \pm 5.3 \cdot 10^{-3}$	$34.3 \cdot 10^{-3} \pm 0.8 \cdot 10^{-3}$	$33.8 \cdot 10^{-3} \pm 3.1 \cdot 10^{-3}$
	♀	$28.4 \cdot 10^{-3} \pm 1.7 \cdot 10^{-3}$	$29.2 \cdot 10^{-3} \pm 1.8 \cdot 10^{-3}$	$32.2 \cdot 10^{-3} \pm 5.8 \cdot 10^{-3}$	$36.2 \cdot 10^{-3} \pm 2.3 \cdot 10^{-3}$
ANOVA		*NS*			
M4	♂	$16.3 \cdot 10^{-3} \pm 6.2 \cdot 10^{-3}$	$13.7 \cdot 10^{-3} \pm 2.1 \cdot 10^{-3}$	$20.8 \cdot 10^{-3} \pm 4.0 \cdot 10^{-3}$	$19.2 \cdot 10^{-3} \pm 3.3 \cdot 10^{-3}$
	♀	$10.4 \cdot 10^{-3} \pm 1.7 \cdot 10^{-3}$	$16.2 \cdot 10^{-3} \pm 2.1 \cdot 10^{-3}$	$9.8 \cdot 10^{-3} \pm 2.2 \cdot 10^{-3}$	$19.0 \cdot 10^{-3} \pm 1.8 \cdot 10^{-3}$
ANOVA		*NS*			
M5	♂	$1.3 \cdot 10^{-3} \pm 0.2 \cdot 10^{-3}$	$1.0 \cdot 10^{-3} \pm 0.3 \cdot 10^{-3}$	$1.0 \cdot 10^{-3} \pm 0.1 \cdot 10^{-3}$	$1.2 \cdot 10^{-3} \pm 0.1 \cdot 10^{-3}$
	♀	$1.2 \cdot 10^{-3} \pm 0.4 \cdot 10^{-3}$	$0.9 \cdot 10^{-3} \pm 0.1 \cdot 10^{-3}$	$0.9 \cdot 10^{-3} \pm 0.1 \cdot 10^{-3}$	$1.1 \cdot 10^{-3} \pm 0.3 \cdot 10^{-3}$
ANOVA		*NS*			
α4	♂	$4.1 \cdot 10^{-5} \pm 0.7 \cdot 10^{-5}$	$3.4 \cdot 10^{-5} \pm 0.4 \cdot 10^{-5}$	$3.8 \cdot 10^{-5} \pm 0.2 \cdot 10^{-5}$	$4.0 \cdot 10^{-5} \pm 0.5 \cdot 10^{-5}$
	♀	$5.6 \cdot 10^{-5} \pm 1.1 \cdot 10^{-5}$	$5.9 \cdot 10^{-5} \pm 1.1 \cdot 10^{-5}$	$3.5 \cdot 10^{-5} \pm 0.5 \cdot 10^{-5}$	$4.0 \cdot 10^{-5} \pm 0.5 \cdot 10^{-5}$
ANOVA		*age x sex: p=0.038*			
α7	♂	$4.3 \cdot 10^{-4} \pm 1.0 \cdot 10^{-4}$	$4.8 \cdot 10^{-4} \pm 0.7 \cdot 10^{-4}$	$5.7 \cdot 10^{-4} \pm 0.7 \cdot 10^{-4}$	$6.4 \cdot 10^{-4} \pm 0.6 \cdot 10^{-4}$
	♀	$4.1 \cdot 10^{-4} \pm 0.3 \cdot 10^{-4}$	$6.7 \cdot 10^{-4} \pm 1.8 \cdot 10^{-4}$	$3.1 \cdot 10^{-4} \pm 0.4 \cdot 10^{-4}$	$5.1 \cdot 10^{-4} \pm 0.4 \cdot 10^{-4}$
ANOVA		*genotype: p=0.013; age x sex: p=0.014*			

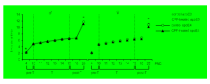
NS: Not significant differences

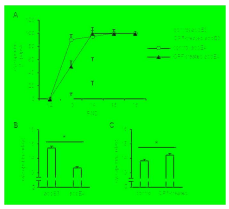
Table 6. Gene expression in brain of apoE3- and apoE4-TR mice. Mean values of choline acetyltransferase (ChAT), vesicular acetylcholine transporter (VAChT), acetylcholinesterase-S and -R (AChE-S, AChE-R), butyrylcholinesterase (BChE) and paraoxonase-2 (PON2) mRNA.

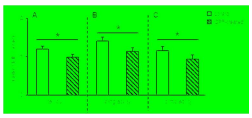
mRNA	Sex	PND 15		PND 30	
		apoE3	apoE4	apoE3	apoE4
ChAT	♂	$3.5 \cdot 10^{-3} \pm 0.6 \cdot 10^{-3}$	$4.1 \cdot 10^{-3} \pm 0.3 \cdot 10^{-3}$	$6.4 \cdot 10^{-3} \pm 0.5 \cdot 10^{-3}$	$6.2 \cdot 10^{-3} \pm 0.2 \cdot 10^{-3}$
	♀	$3.7 \cdot 10^{-3} \pm 0.3 \cdot 10^{-3}$	$4.4 \cdot 10^{-3} \pm 1.0 \cdot 10^{-3}$	$5.7 \cdot 10^{-3} \pm 0.7 \cdot 10^{-3}$	$5.6 \cdot 10^{-3} \pm 0.4 \cdot 10^{-3}$
ANOVA		*age: p<0.001*			
VAChT	♂	$5.4 \cdot 10^{-3} \pm 0.6 \cdot 10^{-3}$	$4.6 \cdot 10^{-3} \pm 0.3 \cdot 10^{-3}$	$3.6 \cdot 10^{-3} \pm 0.3 \cdot 10^{-3}$	$3.5 \cdot 10^{-3} \pm 0.2 \cdot 10^{-3}$
	♀	$5.4 \cdot 10^{-3} \pm 0.4 \cdot 10^{-3}$	$4.7 \cdot 10^{-3} \pm 1.0 \cdot 10^{-3}$	$4.9 \cdot 10^{-3} \pm 0.4 \cdot 10^{-3}$	$3.7 \cdot 10^{-3} \pm 0.5 \cdot 10^{-3}$
ANOVA		*age: p=0.004; genotype: p=0.042*			
AChE-S	♂	$8.9 \cdot 10^{-2} \pm 1.4 \cdot 10^{-2}$	$8.3 \cdot 10^{-2} \pm 0.8 \cdot 10^{-2}$	$5.2 \cdot 10^{-2} \pm 0.3 \cdot 10^{-2}$	$4.5 \cdot 10^{-2} \pm 0.1 \cdot 10^{-2}$
	♀	$9.2 \cdot 10^{-2} \pm 0.9 \cdot 10^{-2}$	$7.6 \cdot 10^{-2} \pm 1.3 \cdot 10^{-2}$	$7.5 \cdot 10^{-2} \pm 0.7 \cdot 10^{-2}$	$4.4 \cdot 10^{-2} \pm 0.3 \cdot 10^{-2}$
ANOVA		*age: p<0.001; genotype: p=0.015*			
AChE-R	♂	$3.9 \cdot 10^{-5} \pm 1.0 \cdot 10^{-5}$	$6.5 \cdot 10^{-5} \pm 0.6 \cdot 10^{-5}$	$4.3 \cdot 10^{-5} \pm 1.0 \cdot 10^{-5}$	$7.1 \cdot 10^{-5} \pm 1.4 \cdot 10^{-5}$
	♀	$2.6 \cdot 10^{-5} \pm 1.0 \cdot 10^{-5}$	$8.5 \cdot 10^{-5} \pm 5.7 \cdot 10^{-5}$	$3.5 \cdot 10^{-5} \pm 1.0 \cdot 10^{-5}$	$6.2 \cdot 10^{-5} \pm 1.0 \cdot 10^{-5}$
ANOVA		*genotype: p=0.010*			
BChE	♂	$10.1 \cdot 10^{-3} \pm 0.6 \cdot 10^{-3}$	$9.4 \cdot 10^{-3} \pm 3.5 \cdot 10^{-3}$	$18.5 \cdot 10^{-3} \pm 3.3 \cdot 10^{-3}$	$16.2 \cdot 10^{-3} \pm 2.6 \cdot 10^{-3}$
	♀	$9.4 \cdot 10^{-3} \pm 1.9 \cdot 10^{-3}$	$15.8 \cdot 10^{-3} \pm 0.9 \cdot 10^{-3}$	$17.4 \cdot 10^{-3} \pm 2.6 \cdot 10^{-3}$	$15.5 \cdot 10^{-3} \pm 3.5 \cdot 10^{-3}$
ANOVA		*age: p=0.006*			
PON2	♂	$10.9 \cdot 10^{-2} \pm 1.4 \cdot 10^{-2}$	$10.0 \cdot 10^{-2} \pm 0.7 \cdot 10^{-2}$	$15.5 \cdot 10^{-2} \pm 1.4 \cdot 10^{-2}$	$11.1 \cdot 10^{-2} \pm 1.2 \cdot 10^{-2}$
	♀	$10.5 \cdot 10^{-2} \pm 0.9 \cdot 10^{-2}$	$12.0 \cdot 10^{-2} \pm 1.5 \cdot 10^{-2}$	$9.2 \cdot 10^{-2} \pm 0.8 \cdot 10^{-2}$	$9.9 \cdot 10^{-2} \pm 0.5 \cdot 10^{-2}$
ANOVA		*age x sex: p=0.006; genotype x sex: p=0.025*			

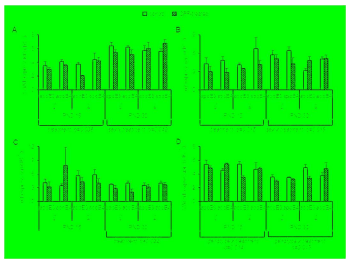
Table 6. Gene expression in brain of apoE3- and apoE4-TR mice. Mean values of choline acetyltransferase (ChAT), vesicular acetylcholine transporter (VAChT), acetylcholinesterase-S and -R (AChE-S, AChE-R), butyrylcholinesterase (BChE) and paraoxonase-2 (PON2) mRNA.

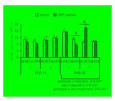
mRNA	Sex	PND 15		PND 30	
		apoE3	apoE4	apoE3	apoE4
ChAT	♂	$3.5 \cdot 10^{-3} \pm 0.6 \cdot 10^{-3}$	$4.1 \cdot 10^{-3} \pm 0.3 \cdot 10^{-3}$	$6.4 \cdot 10^{-3} \pm 0.5 \cdot 10^{-3}$	$6.2 \cdot 10^{-3} \pm 0.2 \cdot 10^{-3}$
	♀	$3.7 \cdot 10^{-3} \pm 0.3 \cdot 10^{-3}$	$4.4 \cdot 10^{-3} \pm 1.0 \cdot 10^{-3}$	$5.7 \cdot 10^{-3} \pm 0.7 \cdot 10^{-3}$	$5.6 \cdot 10^{-3} \pm 0.4 \cdot 10^{-3}$
ANOVA		*age: p<0.001*			
VAChT	♂	$5.4 \cdot 10^{-3} \pm 0.6 \cdot 10^{-3}$	$4.6 \cdot 10^{-3} \pm 0.3 \cdot 10^{-3}$	$3.6 \cdot 10^{-3} \pm 0.3 \cdot 10^{-3}$	$3.5 \cdot 10^{-3} \pm 0.2 \cdot 10^{-3}$
	♀	$5.4 \cdot 10^{-3} \pm 0.4 \cdot 10^{-3}$	$4.7 \cdot 10^{-3} \pm 1.0 \cdot 10^{-3}$	$4.9 \cdot 10^{-3} \pm 0.4 \cdot 10^{-3}$	$3.7 \cdot 10^{-3} \pm 0.5 \cdot 10^{-3}$
ANOVA		*age: p=0.004; genotype: p=0.042*			
AChE-S	♂	$8.9 \cdot 10^{-2} \pm 1.4 \cdot 10^{-2}$	$8.3 \cdot 10^{-2} \pm 0.8 \cdot 10^{-2}$	$5.2 \cdot 10^{-2} \pm 0.3 \cdot 10^{-2}$	$4.5 \cdot 10^{-2} \pm 0.1 \cdot 10^{-2}$
	♀	$9.2 \cdot 10^{-2} \pm 0.9 \cdot 10^{-2}$	$7.6 \cdot 10^{-2} \pm 1.3 \cdot 10^{-2}$	$7.5 \cdot 10^{-2} \pm 0.7 \cdot 10^{-2}$	$4.4 \cdot 10^{-2} \pm 0.3 \cdot 10^{-2}$
ANOVA		*age: p<0.001; genotype: p=0.015*			
AChE-R	♂	$3.9 \cdot 10^{-5} \pm 1.0 \cdot 10^{-5}$	$6.5 \cdot 10^{-5} \pm 0.6 \cdot 10^{-5}$	$4.3 \cdot 10^{-5} \pm 1.0 \cdot 10^{-5}$	$7.1 \cdot 10^{-5} \pm 1.4 \cdot 10^{-5}$
	♀	$2.6 \cdot 10^{-5} \pm 1.0 \cdot 10^{-5}$	$8.5 \cdot 10^{-5} \pm 5.7 \cdot 10^{-5}$	$3.5 \cdot 10^{-5} \pm 1.0 \cdot 10^{-5}$	$6.2 \cdot 10^{-5} \pm 1.0 \cdot 10^{-5}$
ANOVA		*genotype: p=0.010*			
BChE	♂	$10.1 \cdot 10^{-3} \pm 0.6 \cdot 10^{-3}$	$9.4 \cdot 10^{-3} \pm 3.5 \cdot 10^{-3}$	$18.5 \cdot 10^{-3} \pm 3.3 \cdot 10^{-3}$	$16.2 \cdot 10^{-3} \pm 2.6 \cdot 10^{-3}$
	♀	$9.4 \cdot 10^{-3} \pm 1.9 \cdot 10^{-3}$	$15.8 \cdot 10^{-3} \pm 0.9 \cdot 10^{-3}$	$17.4 \cdot 10^{-3} \pm 2.6 \cdot 10^{-3}$	$15.5 \cdot 10^{-3} \pm 3.5 \cdot 10^{-3}$
ANOVA		*age: p=0.006*			
PON2	♂	$10.9 \cdot 10^{-2} \pm 1.4 \cdot 10^{-2}$	$10.0 \cdot 10^{-2} \pm 0.7 \cdot 10^{-2}$	$15.5 \cdot 10^{-2} \pm 1.4 \cdot 10^{-2}$	$11.1 \cdot 10^{-2} \pm 1.2 \cdot 10^{-2}$
	♀	$10.5 \cdot 10^{-2} \pm 0.9 \cdot 10^{-2}$	$12.0 \cdot 10^{-2} \pm 1.5 \cdot 10^{-2}$	$9.2 \cdot 10^{-2} \pm 0.8 \cdot 10^{-2}$	$9.9 \cdot 10^{-2} \pm 0.5 \cdot 10^{-2}$
ANOVA		*age x sex: p=0.006; genotype x sex: p=0.025*			











Highlights

Moderate postnatal CPF exposure delayed physical and motor maturation.

Postnatal CPF exposure decreased ChAT, α4-subunit and α7 receptor expression in forebrain regardless of *APOE* genotype.

Postnatal CPF exposure decreased VAChT expression in apoE3-TR mice.

ApoE4-TR mice gave birth to smaller litters than apoE3-TR mice.

APOE genotype affects the expression of enzymes related to CPF detoxification in liver, and cholinergic elements in brain.