POSTNATAL CHLORPYRIFOS EXPOSURE INFLUENCES THE GUT MICROBIOTA AND THE EXPRESSION OF BIOLOGICAL AND NEUROBEHAVIORAL CHARACTERISTICS OF THE APOE GENOTYPE IN AN AGE-DEPENDENT MANNER

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Postnatal chlorpyrifos exposure influences the gut microbiota and the expression of biological and neurobehavioral characteristics of the *APOE* genotype in an age-dependent manner

Doctoral thesis

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I STATE that the present study, entitled "*Postnatal chlorpyrifos exposure influences the gut microbiota and the expression of biological and neurobehavioral characteristics of the APOE genotype in an age-dependent manner*", presented by Laia Guardia Escoté has been performed under my supervision at Universitat Rovira i Virgili, in fulfilment of the requirements for the degree of Doctor, and meets the requirements to qualify for International Mention.

Tarragona, 30 August 2019

Doctoral Thesis Supervisors

Maria Teresa Colomina Fosch Maria Cabré Bargalló Jordi Blanco Pérez



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All the experimental phases of this thesis have been performed within the research group of Neurobehavior and Health (NEUROLAB), the Biochemistry and Biotechnology Department, the Research Center for Behavior Assessment (CRAMC) of the Department of Psychology and the Centre for Environmental, Food and Toxicological Technology (TecnATox), of the Universitat Rovira i Virgili, and were funded by:

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A la meva familia.

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It is imperfection – not perfection – that is the end result of the program written into that formidably complex engine that is the human brain, and the influences exerted upon us by the environment and whoever takes care of us during the long years of our physical, psychological and intellectual development

Rita Levi-Montalcini

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ABSTRACT

Genetic determinants such as the apolipoprotein E *(APOE)* gene can influence the individual's response to environmental factors. For example, the most frequent human *APOE* genetic variants produce different vulnerabilities to toxics such as pesticides. Organophosphate pesticides – especially chlorpyrifos (CPF) – are used extensively worldwide. However, the massive use of these pesticides can trigger detrimental effects in non-target organisms, thus representing a risk to public health. Effectively, the general population is primarily exposed to trace levels of CPF in food. Particular attention has been paid to exposure during development, since higher vulnerabilities have been reported during this period. The main aim of this thesis is to evaluate how the *APOE* genotype contributes to the behavioral and biochemical effects of postnatal exposure to chlorpyrifos at several stages of life. To do so, we used apoE3- and apoE4-TR mice, as well as wild type C57BL/6 mice. These animals were exposed to either 0 or 1 mg/kg/day of CPF from postnatal day (PND) 10 to PND 15. Gut microbiota composition and short-chain fatty acid (SCFA) levels in the brain were studied in males at early ages, while recognition memory was assessed in young adult males and females. We also examined the contribution of the cholinergic and GABAergic system using a pharmacological strategy. Spatial learning and memory were evaluated in middle-aged mice of both sexes, as was the gene expression of elements involved in the cholinergic system. Differences between *APOE* genotypes were observed throughout the study and these differences modulated the response to the toxic effects of CPF. ApoE4 presented differences in core microbial communities, including *Akkermansia muciniphila*. Exposure to CPF induced dysbiosis at early ages, with apoE4 mice being the most affected. Mice treated with CPF and apoE3-TR mice presented the highest levels of certain SCFAs in the brain. In terms of recognition memory, apoE3-TR mice failed to recognize the novel object, while CPF-treated mice had impaired discrimination. A pharmacological challenge revealed differences between groups depending on postnatal exposure, sex and genotype. ApoE4 mice presented worse spatial memory than C57BL/6 mice but this result was

modulated by CPF exposure in a sex-dependent manner. ApoE4 mice presented an altered expression of cholinergic elements in the brain, which can also be influenced by postnatal CPF exposure. Overall, the current results show that postnatal exposure to CPF leads to long-lasting effects in adults. The different characteristics conferred by the *APOE* genotype produce different levels of vulnerability to CPF-elicited effects in a sex-dependent manner, which provides further insight into gene-environment interactions at different stages of life.

Keywords:

Chlorpyrifos, Pesticide, *APOE*, Brain development, Gut microbiota, short-chain fatty acids, Cholinergic system, Learning, Spatial memory, Recognition memory.

ABBREVIATIONS

5-CSRTT. 5-choice serial reaction time task

Aß. Amyloid beta

Acetyl-CoA. Acetyl-coenzyme A

ACh. Acetylcholine

AChE. Acetylcholinesterase

AD. Alzheimer's disease

ApoE. Apolipoprotein E

BChE. Butyrylcholinesterase

BDE-209. Decabromodiphenyl ether

ChAT. Choline acetyltransferase

ChE. Cholinesterase

CNS. Central nervous system

CPF. Chlorpyrifos

CPO. Chlorpyrifos oxon

DEP. Di-ethyl phosphate

DETP. Di-ethyl thiophosphate

GD. Gestational day

GF. Germ-free

GFAP. Glial fibrillary acidic protein

HDAC. Histone deacetylase

HDL. High-density lipoproteins

LDL. Low-density lipoproteins

mAChRs. Muscarinic receptors

MWM. Morris water maze

nAChRs. Nicotinic receptors

NSE. Neuron-specific enolase

NFTs. Neurofibrillary tangles

OPs. Organophosphorus pesticides

PND. Postnatal day

PON1. Paraoxonase 1

SCFAs. Short-chain fatty acids

TCPy. 3,5,6-trichloro-2-pyridinol

TR. Targeted replacement

VAChT. Vesicular acetylcholine transporter

VLDL. Very low density lipoproteins

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1. INTRODUCTION

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1. INTRODUCTION

Individual differences associated with certain genetic factors lead to different vulnerabilities to environmental insults. This is the case, for example, of the *APOE* gene, whose polymorphisms confer different susceptibilities to cognitive impairments, metabolic alterations and Alzheimer's disease (AD). The *APOE* genotype can influence the response to environmental toxics such as pesticides. Also, individuals during development present higher vulnerability to toxic exposure, as has been demonstrated with the pesticide chlorpyrifos. Much interest has focused on studying the effects of developmental exposure to pesticides and the influence of one's genetic background.

1.1. Apolipoprotein E

Apolipoprotein E (*APOE*) is one of the ten most studied genes of all time (Dolgin, 2017). A member of the family of soluble apolipoproteins, apoE is mainly involved in lipid transport in plasma and the central nervous system (CNS). It is associated with very low density lipoproteins (VLDL), chylomicron remnants and certain high-density lipoproteins (HDL) (Huang and Mahley, 2014; Mahley et al., 1984). Most apoE synthesis takes place in the hepatocytes of the liver, followed by the astrocytes of the brain. However, it is also expressed in a wide range of tissues, including the adrenal glands, spleen, lungs and kidneys (Elshourbagy et al., 1985). Macrophages are also considered important producers of apoE (Basu et al., 1981).

1.1.1. Structure and polymorphisms

ApoE, a polymorphic 34 kDa protein composed by 299 amino acids, is encoded in chromosome 19 (19q13). In humans, it mainly presents three isoforms (apoE2, apoE3 and apoE4), which give rise to three homozygous (E2/E2, E3/E3,

and E4/E4) and three heterozygous (E2/E3, E2/E4, and E3/E4) phenotypes (for a review, see Huang and Mahley, 2014). The three isoforms are differently distributed in the population. Specifically, the allele *ε*3 is the one which is most common in the human population worldwide, with a frequency of 73.3% (SD = 12.1), while the allele *ε*4 is present in 14.5% (SD = 8.5) and the allele *ε*2 is present in only 6.4% (SD = 5.1) (Eisenberg et al., 2010).

The three different isoforms are generated by two single nucleotide polymorphisms (rs429358 and rs7412) in the *APOE* gene (Bekris et al., 2010). Isoforms apoE2 and apoE4 differ from apoE3 by a single aminoacid substitution at position 112 or 158: apoE3 presents a cysteine at position 112 and an arginine at position 158. ApoE4 differs from apoE3 by presenting an arginine at position 112 whereas apoE2 differs from apoE3 by presenting a cysteine at position 158 (Rall et al., 1982; Weisgraber et al., 1981; Zhong and Weisgraber, 2009). These differences in the amino acid sequence lead to changes in structure, that condition its function. ApoE4 is considered the ancestral mammalian isoform because of its resemblance to the apoE in primates. However, the sporadic mutations that produce apoE3 would have been selected during evolution for various reasons, including 'grandmothering' (Finch and Sapolsky, 1999; Mahley and Rall Jr., 1999).

Although apoE was discovered in the early 1970s, it was not until later that its structure was fully elucidated (Chen et al., 2011). ApoE is composed by two different domains united by a hinge region (Figure 1). The N-terminal domain (residues 1-167) is made up of a 4-helix-bundle and contains the LDL receptor binding site, whereas the C-terminal domain (residues 206-299) is made up of three amphipathic α-helices and contains the lipoprotein-binding domain. The hinge region (residues 168-205) regulates interaction between the two domains (Chen et al., 2011; Phillips, 2014).

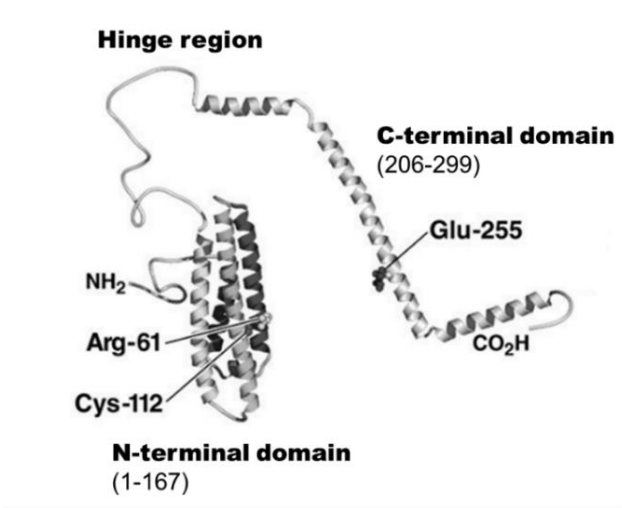


Figure 1. Representation of apolipoprotein E (apoE).
The N-terminal domain (residues 1-167), the C-terminal domain (residues 206-299) and the hinge region (residues 168-205) are represented. Adapted from Mahley (2017).

1.1.2. Functions

The main function of apoE is lipid transfer from circulating lipoproteins to cells and tissues through binding to specific cell surface receptors, which leads to the internalization of these lipoproteins. Interaction between apoE and the receptors favors clearance of the lipoproteins, which maintains lipid homeostasis (Mahley, 1988; Mahley and Huang, 2007). ApoE mainly interacts with the LDL receptor family and with heparin sulfate proteoglycans (Libeu et al., 2001; Morrow et al., 2000a). ApoE has been related to other functions apart from lipid transport. For example, it is reported to play a role in lipid accumulation in adipose tissue and in the differentiation of adipocytes (Lasrich et al., 2015). It may also help to modulate inflammatory and immune responses (Zhang et al., 2011) and the maintenance of the blood brain barrier (Nishitsuji et al., 2011).

ApoE3 and apoE4 bind to LDL receptors with high affinity. However, the cysteine-to-arginine substitution confers apoE2 defective binding, which is associated with type-III hyperlipoproteinemia. This is a genetic disorder characterized by elevated levels of cholesterol and triglycerides, which can lead to the development of premature atherosclerosis (Mahley et al., 1999). However, apoE2 has also been linked to enhanced neuroprotection, unlike apoE4, which has been linked to cognitive decline and AD (Conejero-Goldberg et al., 2014; Corder et al., 1994). In apoE4, the arginine at position 112 alters the orientation of the chain to form a salt bridge with glutamic acid at position 109. The side chain of arginine 61 is then reoriented, which enables its interaction with glutamic acid at position 255 (Dong and Weisgraber, 1996). This N- and C-domain interaction is a unique feature of apoE4 and determines the preference for specific classes of lipoprotein: apoE4 prefers to bind to large lipoprotein particles such as VLDL and LDL, whereas apoE2 and apoE3 prefer the small HDL (Weisgraber, 1990). The isoforms also differ in the stability of their N-terminal domain: apoE4 is the most unstable, while apoE2 is the most stable (Morrow et al., 2000b; Zhong and Weisgraber, 2009). All in all, these differences in apoE4 are considered the basis for its relationship with enhanced vulnerability to metabolic and neurodegenerative diseases (which we discuss further in section 1.1.3.1.).

1.1.3. ApoE in the brain

The *APOE* gene has been linked to neurobiology and has important functions in the CNS. As we mentioned earlier, the astrocytes in the brain are the second major producer of apoE (Pitas et al., 1987), though apoE can also be produced, to a lesser degree, by microglia and neurons under certain stimuli (Xu, 2006). Note that exchange between liver- and brain-produced apoE does not exist because of the presence of the blood-brain barrier (Liu et al., 2012). ApoE is the main lipid transport protein in CSF. Specifically, as the brain is the most cholesterol-rich organ, its regulation by apoE is crucial to correct brain functioning. ApoE helps to redistribute cholesterol and phospholipids

throughout the brain for repair and remodeling (Mahley, 2016). In the central nervous system, it has been related to neuronal plasticity, neurite growth and synaptogenesis, and neuronal repair (Kim et al., 2014).

1.1.3.1. APOE and neurodegeneration

APOE4 is known to be the main genetic risk factor for the development of AD (Corder et al., 1993; Roses, 1996). AD is one of the most common neurodegenerative diseases. Characterized by progressive deterioration in cognition, function and behavior, it is associated with pathological changes in the brain, including extracellular amyloid-ß (Aß) plaques and the hyperphosphorylated tau protein that forms intracellular neurofibrillary tangles (NFTs) (Reitz and Mayeux, 2014), as well as cholinergic dysfunction (Hampel et al., 2018). ApoE is reported to be related to the metabolism of Aß in an isoform-dependent manner. Indeed, apoE4 binds more efficiently under certain conditions, and decreases the clearance of Aß in mice, thus contributing to the pathology of AD (Castellano et al., 2011). ApoE4 is also linked to enhanced levels of tau in the brain and therefore to the formation of NFTs (Shi et al., 2017). Moreover, apoE4's unique conformation is reported to make it susceptible to proteolytic cleavage, which produces truncated fragments that can produce neurotoxicity. The greater presence of truncated fragments correlates with the most affected brain regions in AD (Brecht, 2004). It has been shown that these fragments are produced by neurons in response to brain injury, as part of the repair response. ApoE4's neurotoxic effect may be related to cytoskeletal disruption and mitochondria dysfunction (Mahley and Huang, 2012), which have also been detected in amyloid plaques and NFT-like inclusions of the brain (Dafnis et al., 2016; Huang et al., 2001).

1.1.3.2. APOE and cholinergic system

Cognitive decline and AD have been linked to alterations in cholinergic function (Hrabovska and Krejci, 2014; Muir, 1997; Schliebs and Arendt, 2011, 2006). ApoE4 has also been related to diminished cholinergic function (Allen et al., 1997). Acetylcholine (ACh) is one of the main neurotransmitters in the brain, where it is involved in functions such as cognition, learning, memory and stress response (Ferreira-Vieira et al., 2016). The cholinergic system is made up of several elements that are involved in the synthesis, storage and release of the system's neurotransmitter to the synaptic cleft, where it can bind to the receptors and transmit the signal, as well as elements implicated in its degradation and re-uptake (Figure 2). The cholinergic system neurotransmitter ACh is synthesized from choline and acetyl-coenzyme A (acetyl-CoA) in the cytoplasm of cholinergic neurons by the enzyme choline acetyltransferase (ChAT) and stored by the vesicular acetylcholine transporter (VAChT) into synaptic vesicles. These vesicles are released into the synaptic cleft when cholinergic neurons are depolarized. ACh then binds to the different receptors (Abreu-Villaça et al., 2011; Ferreira-Vieira et al., 2016; Prado et al., 2013).

There are two main types of acetylcholine receptors: muscarinic (mAChRs) and nicotinic (nAChRs) receptors. More specifically, mAChRs are G-protein-coupled receptors made up of seven transmembrane domains. They account for five different isoforms (M1–M5), which can activate different signaling pathways. M1, M3 and M5, for instance, couple to GTP-binding proteins (Gq) and activate phospholipase C, thus mobilizing intracellular calcium. The binding of M2 and M4 to the protein G (Gi), on the other hand, inhibits adenylyl cyclase activity and reduces intracellular cAMP levels (Jiang et al., 2014; Koch et al., 2005). On the other side, nAChRs are pentameric ion gated receptor channels that are selective for cations generated by the combination of different subunits α (α2–α10) and β (β2–β4). They can be heteropentameric, with α4β2 nAChR being predominant in the mammalian brain, or homopentameric, with α7 nAChR being the most highly expressed (Abreu-Villaça et al., 2011). Depending on the receptor and its

neuronal localization, their activation by ACh can promote either stimulation or inhibition and thus modulate CNS function (Ferreira-Vieira et al., 2016).

The enzyme acetylcholinesterase (AChE) hydrolyses and inactivates the ACh neurotransmitter in order to stop the transmission. This releases choline and acetate, which is re-uptaken by the presynaptic neuron. AChE can produce three different isoforms by alternative splicing: synaptic AChE-S, erythrocytic AChE-E and readthrough AChE-R (Soreq and Seidman, 2001). The carboxy-terminal sequences determine their configuration, with AChE-S normally being found as tetramers indirectly attached to the cell surface. AChE-R, on the other hand, is normally found in monomeric soluble form within the synaptic cleft. AChE-S is the most prominent form in the mammalian brain, followed by AChE-R, whose production normally increases in response to stress (Grisaru et al., 1999; Härtl et al., 2011). Another enzyme, butyrylcholinesterase (BChE), can contribute to the hydrolysis and inactivation of ACh, albeit in a less specific manner. BChE, which is also known as serum cholinesterase, can also act as a molecular decoy for anti-AChEs (Soreq and Seidman, 2001).

Studies in human and animal models showed different levels of cholinergic elements depending on the genotype, which supports the hypothesis that cholinergic system alterations exist in apoE4-carriers. Poirier et al., (1995), for example, observed alterations in several cholinergic markers in the postmortem brains of AD patients depending on the apoE4 allele dosage. These markers included a decrease in ChAT activity and nicotinic binding sites in the hippocampus and temporal cortex in apoE4 carriers (Poirier et al., 1995). Differences between genotypes were observed in old mice that express human apoE, which shows that apoE4 targeted replacement (TR) mice presented decreased levels of VAChT, AChE and BChE with age compared with apoE3 mice. ACh release impairments in the hippocampus of old apoE4 mice have also been observed (Dolejší et al., 2016). Another study reported higher distribution

volumes of labelled muscarinic selective agonists in older apoE4 patients, which suggests a lower ACh in the synapse of these subjects (Cohen et al., 2003). However, other studies found no *APOE*-genotype differences in the cholinergic system (Bronfman et al., 2000).

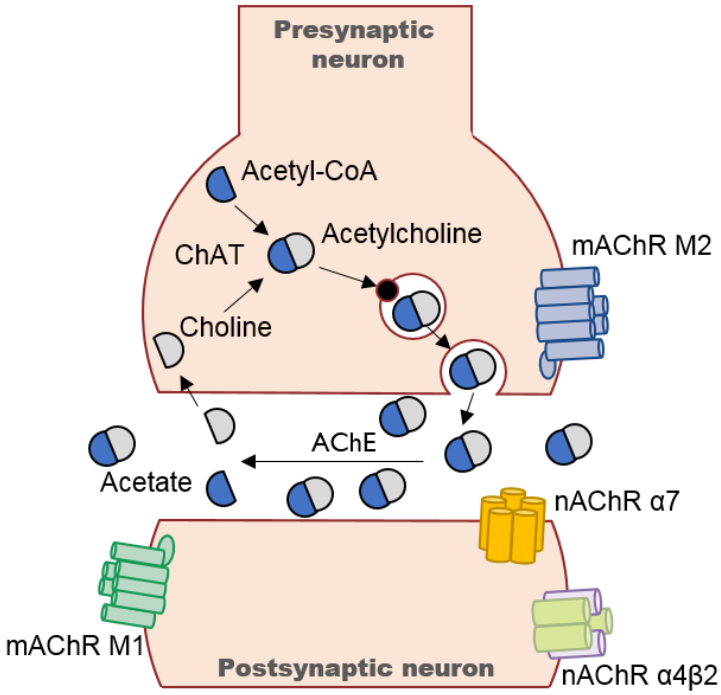


Figure 2. Schematic representation of the cholinergic system.
ACh is synthesized in the presynaptic neuron from choline and acetyl-CoA by the enzyme ChAT. ACh is then stored in the synaptic vesicles by the VAChT until it is released into the synaptic cleft. There, ACh binds to the mAChR and nAChR located either in the presynaptic or postsynaptic neuron. To end the transmission, ACh is degraded by the enzyme AChE. Abbreviations: ACh, acetylcholine; ChAT, choline acetyltransferase; VAChT, vesicular acetylcholine transporter; mAChRs, muscarinic acetylcholine receptors; nAChRs, nicotinic acetylcholine receptors; AChE, acetylcholinesterase.

Recent studies in our laboratory attempted to shed more light on the link between cholinergic elements and apoE4 allele using apoE3- and apoE4-TR mice. Basaure et al. (2018) reported differences in the gene expression of VAChT, α7 nAChR, AChE-S and AChE-R in the forebrain between genotypes in the developmental period (PND 15 – PND 30). Specifically, apoE3-TR mice showed higher levels of VAChT and AChE-S, whereas apoE4-TR mice presented higher levels of α7 nAChR and AChE-R (Basaure et al., 2018). In another study with apoE-TR mice at 6 months of age, differences were observed in ChAT and VAChT levels (which were higher in apoE4-TR mice than in apoE3-TR mice), which suggests that age strongly influences the regulation of cholinergic elements in apoE-TR mice (Basaure et al., 2019).

1.1.3.3. APOE and behavioral outcomes

Differences in behavior have been described between the various *APOE* allele carriers in terms of learning and memory, stress, attention, impulsivity and compulsivity. Several studies of non-demented elderly patients have observed that memory declined faster in subjects with at least one ε4 allele (Mayeux et al., 2001). Similarly, AD patients with ε4 presented faster cognitive decline, especially at early stages of the disease (Cosentino et al., 2008). Working and recall memory were also influenced by the *APOE* genotype, with ε4 homozygotes exhibiting the worst performance (Reynolds et al., 2006). Also, non-demented healthy elderly non-ε4 carriers outperformed ε4 carriers in object recognition and spatial navigation tests (Berteau-Pavy et al., 2007). A meta-analysis with cognitively healthy adults revealed impaired performance of ε4 in a range of neurocognitive functions, including episodic memory, executive functioning and overall global cognitive ability (Wisdom et al., 2011).

Studies in transgenic mice helped shed more light on the differences observed in humans. Meng et al. (2017), for instance, reported higher anxiety-like behaviors in the open field, light/dark test and elevated plus maze in 3-month-

old GFAP-apoE4 transgenic mice compared with their apoE3 counterparts. Similarly, studies with apoE-TR mice showed that apoE2- and apoE4-TR mice performed less well in acquiring the spatial task Morris water maze (MWM) than apoE3-TR mice. ApoE4-TR mice also showed decreased activity in the open field, while apoE2-TR mice presented hyperactive behaviors at four months of age (Reverte et al., 2012). Differences were also observed in attention and inhibitory control since 8-month-old female apoE4-TR mice presented higher levels of perseverative and premature responses but lower accuracy than apoE2- and apoE3-TR mice in the five-choice serial reaction time task (Reverte et al., 2016).

Differences between genotypes may be influenced by other factors, such as sex and age. Females are known to be more affected by the *APOE4* genotype than males (e.g. they show higher neurocognitive impairments) (Pontifex et al., 2018; Toro et al., 2019). Although enhanced AD risk with apoE4 is observed in both males and females, female apoE4 carriers presented earlier onset and more extended AD pathology than males. Moreover, Corder et al. (2004) observed that women who carried the ε4 allele presented a greater presence of NFT and senile plaques beginning in middle age in a study of more than 5000 brain samples. ApoE4 also presented lower hippocampal volume in female than in male carriers with mild cognitive impairment (Fleisher et al., 2005). Animal studies corroborate these observations obtained in human patients. Grootendorst et al. (2005) observed effects on learning and memory capacities at 4-5 months of age depending on the *APOE* isoform and gender, with apoE4 females being the most affected. When studying the same cognitive capacities at 15-18 months of age, apoE4 also showed the worst performance, as well as age-dependent impairments (Bour et al., 2008). To sum up, age and sex are important factors that can modulate *APOE* genotype-dependent differences.

1.1.4. ApoE and metabolism

Different metabolic profiles have been observed between genotypes. Although studies in humans have presented controversial results, probably due to the potency of the study and possible confounder effects, most agree on a body mass index distribution as follows: E2 > E3 > E4. This observation has been corroborated in studies with adult (Gottlieb et al., 2004; Kulminski et al., 2019; Tejedor et al., 2014; Volcik et al., 2006) and child participants (Ellis et al., 2011). Ellis et al. (2011) observed that 8-year-old apoE4-carriers presented decreased levels of adiposity. To shed more light on this subject, studies with apoE-TR mice were performed, the results of which suggest that *APOE* can determine susceptibility to obesity and insulin resistance. Karagiannides et al. (2008) showed that female apoE3-TR mice were more sensitive to obesity and related metabolic dysfunctions after exposure to a western diet. More specifically, apoE3-TR mice presented a more obese phenotype and developed higher hyperglycemia, hyperinsulinemia, glucose intolerance and insulin resistance than wild type C57BL/6 mice. Interestingly, apoE KO remained resistant to these conditions (Karagiannides et al., 2008). Other studies also obtained similar results, with male apoE3-TR mice presenting higher net body weight than their E4 counterparts after a high-fat western diet (Arbones-Mainar et al., 2008; Huebbe et al., 2015; Segev et al., 2016). Huebbe et al. (2015) attributed these differences to a more efficient use of nutrients by apoE3-TR mice. However, it has also been reported that male apoE4-TR mice are more likely to develop impaired glucose tolerance and are therefore more prone to developing diabetes mellitus-like metabolic features (Arbones-Mainar et al., 2008; Segev et al., 2016).

1.1.5. Experimental models – ApoE-TR

Animal models have become essential for acquiring a more complete understanding of the role of apoE. Several mouse models have been designed over the years. First, *APOE*-deficient mice were produced by gene targeting

(Maeda, 2011; Zhang et al., 1992). Then, mouse models were generated that lacked the murine apoE gene and expressed human isoforms under the regulation of promoters such as the glial fibrillary acidic protein (GFAP) promoter in the astrocytes (Hartman et al., 2001). Another typical promoter is the neuron-specific enolase (NSE) promoter in neurons (Raber et al., 1998). Other groups used these mice models to cross with other mice that carried typical mutations of AD such as mutated forms of the *APP* gene (Graybeal et al., 2015; Holtzman et al., 1999). However, these mice models generally produced mice with varying levels of apoE that could ultimately result in imprecise spatial and temporal expression in the brain and other tissues. To address this issue, transgenic mice that express human apoE isoforms rather than the murine protein, and therefore leave the *APOE* regulatory sequences intact, were designed. ApoE-TR mice that express human apoE at physiological levels in a similar pattern to wild-type mice and humans, emerged as a widely validated apoE mice model (Sullivan et al., 2004, 1997).

1.2. Chlorpyrifos

Chlorpyrifos (CPF) is a widely used pesticide belonging to the OP family. Pesticides are a large, heterogeneous group of compounds used to control and repel pests. However, they also represent a public health concern because of their possible toxicity for non-target organisms. In the last century, new and more potent pesticides increased the worldwide use of these chemicals. First, chlorinated hydrocarbons, and then organophosphorus compounds, methylcarbamates and pyrethroids were introduced into the marketplace. For a period of time known as the golden age of pesticides, a large number of new pesticides were synthesized, thus improving agricultural productivity. During that period, pesticides were generally considered safe. However, their large-scale use began to worry the population. This concern culminated in 1962 with the publication of Rachel Carson's book, the Silent Spring, in which the author argued that a massive release of pesticides into the environment would have a

huge negative impact on the welfare of humans and animals. This had a great impact on the population and led to certain regulations being introduced. In the early 1970s, persistent organochlorine insecticides, including the widely used DDT, were restricted or banned in the US and other countries around the world (Casida and Quistad, 1998). Later, less persistent but highly effective organophosphorus pesticides (OPs) emerged as those most widely chosen. However, since their discovery, OPs have been used not only as insecticides but also as therapeutic agents, plasticizers, lubricants fuel additives and even agents of warfare (Pope, 1999). OPs remain one of the most common pesticides in the world. In fact, statistics reported that in 2015, 2280 tons of organophosphorus pesticides were used for agriculture use in Spain alone (Food and Agriculture Organization – FAO Statistics, 2015).

OPs include a wide range of chemicals obtained from phosphoric, phosphonic, phosphinic and thiophosphoric acids (Balali-Mood and Saber, 2012; Naughton and Terry, 2018). The general structure of OPs is shown in Figure 3, where the X represents the group displaced when the OP phosphorylates the acetylcholinesterase. Groups R1 and R2 are generally alkyl-, alkoxy-, alkylthio- or amid groups (Balali-Mood and Saber, 2012; Costa, 2006).

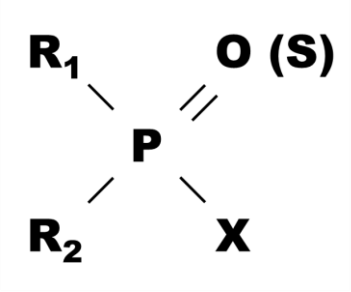


Figure 3. Schematic representation of OP structure.
The OP is composed by a phosphorus atom with a double bond to sulphur.

OPs all share the same main toxicity mechanism: they target the enzyme AChE and inhibit its catalytic activity in the central and peripheral nervous system. This inhibition results in an increase in the synaptic levels of ACh, which in turn leads to excessive stimulation of both nicotinic and muscarinic receptors (Flaskos, 2012). Other non-acetylcholinesterase targets have been described for OPs (Naughton and Terry, 2018) that trigger alterations in, for example, oxidative stress (Abolaji et al., 2017; Eftekhari et al., 2018), inflammation (Mohammadzadeh et al., 2018) and axonal transport (Terry et al., 2007). Cases of occupational, accidental or intentional acute OP poisoning have been reported worldwide, with effects ranging from mild cholinergic clinical manifestations (e.g. nausea, blurred vision, salivation, lacrimation and respiratory dysfunctions) to respiratory paralysis and even death (Balali-Mood and Saber, 2012). Notably, chronic exposure to doses of OPs below the threshold for toxicity has been linked to cognitive impairments, depression, metabolism dysfunctions and neurotoxicity. Similarly, a greater sensitivity of younger organisms to OP toxicity has been observed in numerous studies, which underlies the danger of exposure to OPs during development (Burke et al., 2017; Eaton et al., 2008).

1.2.1. CPF use

CPF is an insecticide and acaricide used worldwide, that was first introduced to the marketplace in 1965. Until recently, it was used for both agricultural and non-agricultural purposes. In agriculture it is mainly used to protect corn, tree nuts and soybeans, though it is also used to protect fruit trees. Residential uses formerly included the control of pests such as cockroaches and fleas, and the manufacture of pet collars against tick and fleas. The main manufacturer of CPF in the US is Dow AgroSciences, where it is frequently commercialized under the names Dursban and Lorsban. However, regulations implemented in the US in 2001 banned the residential use of CPF and its use for this purpose dropped to less than 3% of the total (Eaton et al., 2008). Debate on whether to ban CPF in Europe has been continuing for years. EU approval for CPF will continue at least

until 2020. Remarkably, the Spanish Ministry of Agriculture, Fisheries and Food allows products whose active substance is CPF to be used for several purposes in Spain, including domestic outdoor gardening (Ministerio de Agricultura, Pesca y Alimentación, 2019, Saunders et al., 2012).

1.2.2. Structure and chemical properties of CPF

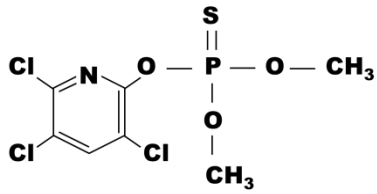
CPF [O,O-diethyl O-(3,5,6-trichloropyridin-2-yl) phosphorothioate] is a colorless-to-white crystalline solid with a faint mecaptan-type odor. It is barely soluble in water but is soluble in most organic solvents such as acetone, xylene and methylene chloride. CPF is stable in neutral and acidic aqueous solutions, and its stability decreases as pH increases. It is moderately volatile with a vapor pressure of $1.87x10^{-5}$ mmHg at 25ºC (Eaton et al., 2008). The physical and chemical properties of CPF are shown in Table 1. CPF is composed by a pentavalent phosphorus atom with a double bond to sulphur. The phosphorus atom presents two more stable unions with ethyl groups and an unstable union to an aromatic structure that is released during the biotransformation process (Karalliedde et al., 2003).

1.2.3. Exposure pathways

After the introduction of the regulations in 2001, non-agricultural exposure to CPF was greatly reduced. Once CPF is applied to crops, it can bind soil and plants and undergoes rapid degradation. However, residual levels may remain for longer periods of time. CPF in the environment is subjected to a number of degradation pathways, e.g. photodegradation, volatilization, hydrolysis and microbial degradation. CPF half-life in the environment is approximately three days, though a range of two to 120 days has been reported under field conditions. Half-life may increase or decrease depending on soil carbon content, moisture, application rate and microbial activity (Eaton et al., 2008; Solomon et al., 2014). CPF has also been detected in locations far removed from sites where

it has been applied to agriculture, which demonstrates that it is potentially subject to long-range transport (Mackay et al., 2014).

Table 1. CPF properties

Molecular structure	
Molecular formula	$C_9H_{11}Cl_3NO_3PS$
Chemical name (IUPAC)	O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl)phosphorothioate
Cas number	2921-88-2
Molecular weight	350.6 g/mol
Density (20ºC)	1.44 g/cm^3
Vapor pressure (25ºC)	1.87x10^{-5} mmHg
Boiling point	No boiling point at normal pressure, decomposes at 160ºC
Melting point	41 – 42ºC
Solubility in water (25ºC)	1.4 mg/L

Adapted from National Center for Biotechnology Information (NCBI), 2019

Exposure pathways to CPF include inhalation, oral and dermal exposures. Occupational workers present a higher risk of exposure because they are directly exposed to CPF, predominantly by inhalation or contact with the skin following application. Families are also considered a high-risk group. For the general population, the main pathways are dietary exposure to trace levels of

CPF in food and, to a lesser extent, residence in the vicinity of agricultural applications (Eaton et al., 2008; Reiss et al., 2015). Oral exposure mainly involves fruit and vegetables, water, milk and derived products. Pre- and postnatal exposure can also occur since CPF can easily cross the placenta and be transferred via breastfeeding (Abdel-Rahman et al., 2002; Mansour and Mossa, 2010; Saunders et al., 2012). In view of the above factors, it is difficult to calculate average daily exposure to CPF. However, several years ago, the US Department of Agriculture and the FDA (US Food and Drug Administration) were able to estimate 'typical' average daily exposure to CPF throughout diet in several age groups. Estimated average exposure was 0.005 μg/kg/day for adults, 0.014 μg/kg/day for toddlers, and 0.009 μg/kg/day for infants (Eaton et al. 2008). In 2014, the European Food Safety Authority established an acceptable daily intake of 0.001 mg/kg/day (*Human health risk assessment of the active substance chlorpyrifos, EFSA)*.

1.2.4. Absorption, distribution, biotransformation and excretion

CPF is normally well absorbed from the intestine, the skin and the lungs after inhalation. Due to its lipophilic nature, chlorpyrifos is absorbed through the skin, though less efficiently than via the respiratory or oral routes. With oral exposure, recovery as urine metabolites accounts for 70-93%, compared to 1% after dermal exposure (Griffin et al., 1999). However, not all the absorbed CPF is excreted in the urine. Although CPF is well absorbed from the intestine, the absorbed percentage may depend on the physical form and formulation properties. CPF is found in high concentrations in fat tissue, with a half-life of 62h, compared to the half-life of 18h reported in plasma (Suratman et al., 2015). CPF can be found attached to proteins such as albumin, which is the most abundant protein in plasma. The amount of albumin attached to CPF can be used as a biomarker for CPF exposure (Marsillach et al., 2013).

CPF is biotransformed by a cytochrome P450-mediated oxidative desulphurization reaction to the highly toxic form CPF-oxon (CPO), which irreversibly inhibits AChE. Alternatively, CPF can be detoxified by dearylation by specific cytochrome P450 and produce 3,5,6-trichloro-2-pyridinol (TCPy) and di-ethyl thiophosphate (DETP), which are eliminated in the urine. CPO can undergo spontaneous or enzymatic hydrolysis by paraoxonase 1 (PON1) and result in the formation of two metabolites: TCPy and di-ethyl phosphate (DEP), which are also eliminated in the urine (Figure 4) (Marsillach et al., 2016).

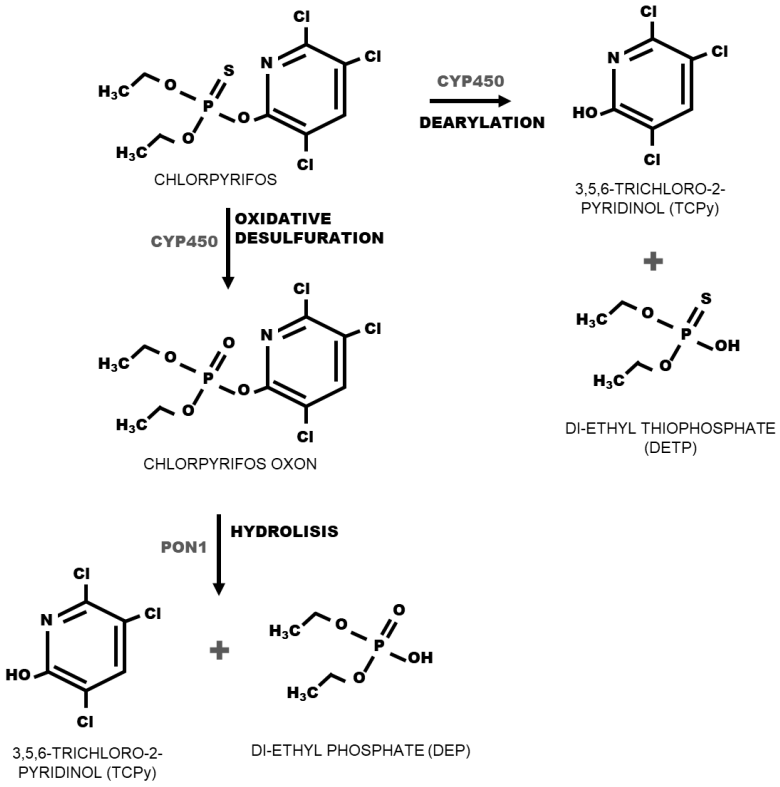


Figure 4. Schematic representation of CPF biotransformation.
Adapted from Marsillach et al. (2016)

Detoxification enzymes include PON1 and cytochrome P450. PON1 is a polymorphic A-esterase enzyme synthesized in the liver and involved in CPO detoxification. PON1 efficiency is modulated by a polymorphism (*Q192R)* and differences in PON1 phenotype modulate susceptibility to CPF toxicity (Furlong et al., 2005). Several studies have demonstrated the protective effect of PON1 against CPF and especially against CPO. For example, a study with a PON1 knockout model showed that the animals presented higher sensitivity to CPO (Shih et al., 1998). On the other hand, cytochrome P450 also present several isoforms that differ in their ability to metabolize CPF, with CYP2B6 being associated with low CPF concentrations and CYP3A4 being associated with higher CPF concentrations (Croom et al., 2010).

CPF metabolites are excreted, mainly through the urine. Chlorpyrifos metabolites such as TCPy (62%), DETP (40%) and DEP (4%) can be detected in the urine hours after CPF exposure, with the maximum peak at 12 h (Timchalk et al., 2007). TCPy, which is the main CPF metabolite identified in urine, has been used as a common biomarker. However, it can be misleading because, as well as representing exposure levels to the parent pesticide, it can be modified by direct exposure to the CPF metabolites, which are present in the environment (Morgan et al., 2011). Moreover, CPF metabolites can also be excreted by biliary and fecal elimination, while they have been detected in breast milk (Sanghi et al., 2003).

1.2.5. Toxicity

The primary target of CPF toxicity is the central and peripheral nervous system. Like most OPs, the toxic effect of CPF is mediated by the irreversible inhibition of the cholinesterase enzymes. As we discussed earlier, the oxon form, CPO, is largely responsible for the inhibition. Cholinesterase enzymes AChE and BChE were the first targets described. Inhibition of AChE above 50% generally leads to cholinergic symptoms including anatomic dysfunction, involuntary movements, respiratory depression, and death (Pope, 1999). Inhibition of AChE

is triggered by the formation of a covalent P-O bond in the catalytic active site of the enzyme (Gorecki et al., 2016). Inhibition of the enzymes responsible for hydrolyzing the neurotransmitter ACh leads to greater accumulation of ACh in the cholinergic synapsis, resulting in cholinergic overstimulation. This would ultimately lead to a downregulation of the muscarinic and nicotinic cholinergic receptors (Terry et al., 2007). Although inhibition of AChE is the main toxic effect, several other mechanisms have been reported at sub-toxic doses. For example, CPF can induce oxidative stress (Elsharkawy et al., 2013; Verma et al., 2007), cause histopathological alterations in the liver (Ezzi et al., 2016), alter gene expression in the hippocampus (Lee et al., 2016) and inhibit hepatic carboxylesterase and fatty acid amide hydrolase (Howell et al., 2018).

1.2.6. CPF and the cholinergic system

The cholinergic system is subject to the toxic effects of CPF since a direct effect is exerted on two of its cholinesterase enzymes (AChE and BChE). These enzymes are involved in signal transmission in the nervous system, where they are responsible for breaking down the neurotransmitter ACh and terminating the transmission. It has been suggested that, in humans, BChE is more sensitive to CPF than AChE (Eaton et al., 2008). As we discussed in the previous section, this inhibition can alter ACh levels and trigger a downregulation of AChRs. Altered levels of cholinergic system elements have therefore been reported after exposure to CPF. One of the most common parameter studied is the percentage of inhibition of ChEs, normally using the Ellman method (Ellman et al., 1961). Huff et al. (2001) found that a high dose of CPF (30 mg/kg) inhibits AChE activity in the brain and BChE activity in plasma at similar levels. Similarly, chronic exposure to CPF (18 mg/kg every other day) for 30 days significantly inhibited cerebral AChE and plasmatic ChE. This effect was still evident after a 14-day wash-out period in adult rats (Terry et al., 2007). Exposures during development triggered similar effects. For example, gestational exposure to CPF (10 mg/kg/day) inhibited ChE at 3 and 8 months in mice (Pallotta et al., 2017). Consistent findings are that ChE inhibition strongly depends on CPF dosage and

that inhibition percentages are normally highest after higher doses. The anticholinesterase effect of CPF was also corroborated using SN56 cells, a cholinergic neuroblastoma cell line. In addition to a decrease in AChE activity, they also reported that CPF induced cell death in this cholinergic model after acute and long-term exposure to the pesticide (del Pino et al., 2015). Karanth et al. (2006) reported higher ACh levels in the striatum 1 to 7 days after exposure to CPF in adult rats, depending on the dose administered. This is in line with AChE inhibition and highlights the fact that tissues with higher AChE activity take more time to recover their normal function (Karanth et al., 2006).

Other cholinergic elements reported to be altered by CPF exposure include ChAT and VAChT. Terry et al. (2007) found a decrease in VAChT levels in the hippocampus after a 2-week wash-out period following 30-day-exposure to CPF in adult rats. The same trend was observed in rats exposed to CPF from PND 1 to 21. Indeed, the higher dose group presented a decrease in VAChT activity at PND 30. Moreover, a decrease in ChAT activity was also observed (Richardson and Chambers, 2005). In another study, Slotkin et al. (2001) found lower levels of ChAT in several brain regions at PND 30 and PND 60 in rats exposed postnatally to CPF (1 or 5 mg/kg/day).

Both nAChR and mAChR cholinergic receptors are reported to be vulnerable to CPF exposure. Nostrandt et al. (1997) observed a downregulation in muscarinic receptor density in the striatum, pons and medulla in male adult rats after exposure to the highest dosage of CPF (100 mg/kg). Lower mAChR density was also observed in rats postnatally (PND 1 to 21) exposed to CPF (assessed at PND 12 and PND 22) (Richardson and Chambers, 2005). Lower levels were also reported at PND 4 and PND 8 in rats postnatally exposed to different doses of CPF, depending on the dose and region of the brain studied (Guo-Ross et al., 2007). Deficits in nicotinic receptors, particularly α7 nAChR in the forebrain and cerebellum, were observed after postnatal exposure (PND 11 to PND 14) to CPF

(5 mg/kg/day) in rats (Slotkin, 2004). All in all, these results highlight the cholinergic system as the main target for CPF effects.

1.2.7. CPF and behavioral alterations

Epidemiological studies have found a link between exposure to OPs and human chronic diseases, including Parkinson's disease and autism (Mostafalou and Abdollahi, 2018). Animal studies have also found neurobehavioral effects after CPF exposure in a wide range of parameters. Several studies have described a significant effect of the pesticide on anxiety-like behaviors. For example, adult rats exposed to CPF (1 mg/kg/day) for 5 days showed decreased levels of anxiety when assessed by the marble burying test (Savy et al., 2015). Similarly, male rats exposed to a single dose of CPF (250 mg/kg) presented an anxiolytic-like effect determined in the open field (López-Crespo et al., 2007). On the other hand, increased levels of anxiety were observed in rats exposed to an acute high dose of CPF (166 mg/kg or 250 mg/kg) during adulthood when assessed in the plus-maze (Sánchez-Amate et al., 2001). Exposure during development (PND 10 to PND 16) produced diminished anxiety behaviors in rats assessed at PND 25. Compared to controls, CPF-treated rats presented less preference to remain in the safe area, which suggests an anxiolytic effect of CPF at different doses (0.5, 0.75 and 1 mg/kg/day) (Carr et al., 2017). CPF exposure from PND 27 to PND 36 was reported to induce depressive-like behaviors, since a higher immobility time was observed in the treated group during the forced swimming test. Also, altered latencies were observed in the novelty-suppressing feeding test. These results are in line with epidemiologic evidence linking exposed populations with a higher incidence of depression (Chen et al., 2014).

Middlemore-Risher et al. (2010) described the detrimental effects of CPF on attention and inhibitory control in 2-month-old rats after exposure to the pesticide (18 mg/kg) every day for 14 days or every other day for 30 days. Results of the 5-choice serial reaction time task (5-CSRTT) showed that both

treatment protocols induced lower accuracy and a higher percentage of omissions, thus indicating altered attention. Moreover, alterations in inhibitory control were also detected since they presented higher premature responses (Middlemore-Risher et al., 2010). Another study in rats exposed to a single dose of CPF (250 mg/kg) and assessed in the 5-CSRTT observed higher perseverative responses, which suggests that CPF induces long-term compulsivity in these animals (Montes de Oca et al., 2013). The performance of mice exposed to CPF through diet was also affected in the 5-CSRTT. On this occasion, attention was affected in the treated group (Peris-Sampedro et al., 2016). CPF-treated rats also showed a more impulsive choice in the delay-discounting task (Cardona et al., 2011).

Exposure to CPF can also lead to alterations in spatial learning and memory. López-Granero et al. (2016) observed detrimental long-term effects in male rats several months after 6-month-dietary exposure to CPF. Results obtained in the MWM showed thigmotaxis behavior, which suggests that CPF leads to altered search patterns. In a similar study of male rats conducted several months after CPF exposure, the authors observed an increase in the time needed to find the platform, which indicates that CPF is able to trigger long-term alterations in spatial working memory (López-Granero et al., 2013). Alterations in the acquisition of MWM were observed by Terry et al. (2012), who found modest impairment in the task acquisition in the first part of the experiment in male rats after a washout period of 140 days. On the other hand, retention impairments, including more time to reach the former location of the platform and less time spent in the target quadrant were observed in adult rats exposed to different doses of CPF for four consecutive weeks, which suggests spatial memory retrieval impairments (Yan et al., 2012). Other studies with transgenic mice (Tg2576) showed that exposure to CPF (two weekly doses of 25 mg/kg for 4 weeks) impaired MWM retention, since the animals did not show any preference for the target quadrant in any of the retention sessions (Peris-Sampedro et al., 2014). Altogether, these studies suggest that spatial learning

and memory is susceptible to the detrimental effects of CPF depending on the dosage and the exposure protocol. Note that the toxic effects of CPF can also be influenced by other factors, such as sex. Several studies have confirmed that subjects can present different sensitivities to the toxic effects of the pesticide depending on sex. Gómez-Giménez et al. (2017), for instance, observed spatial learning and memory impairments in males but not in females after developmental exposure to different doses of CPF.

1.2.8. CPF and metabolic alterations

There is increasing evidence for the potential effect of CPF on metabolic disorders. Several studies have reported a significant increase in body weight after chronic exposure to CPF. For instance, 6-month-old rats exposed to CPF (5 mg/kg/day) over a 4-month-period presented higher body weight gain from month 2 onwards. They also presented higher perinephric fat pads, a marker of total body fat (Meggs and Brewer, 2007). The same effect was observed in adult male rats exposed to CPF (0.3 and 3 mg/kg/day) for 9 weeks (Fang et al., 2018). CPF-treated male rats presented a dose-dependent increase in body weight after gestational and postnatal exposure to different doses of CPF, but female rats did not (Lassiter and Brimijoin, 2008).

Chronic exposure to CPF (5 mg/kg/day) in mice revealed a potential effect on glucose homeostasis and insulin sensitivity, since higher concentrations of fasting glucose and insulin were observed. As well as greater body weight and fat pad weight compared to controls, CPF-treated mice presented impaired intestinal integrity. The authors of the study suggest that an increase in LPS, which can be underlying insulin resistance and obesity, may have been triggered (Liang et al., 2019). An acute single exposure to a high dose of CPF (50 mg/kg) was enough to trigger hyperglycemic and hyperlipidemic effects in adult rats (observed 2-24 h after exposure) including higher plasma glucose, LDL and triglyceride levels and lower HDL levels (Acker and Nogueira, 2012). Chronic

exposure triggered similar effects. Elsharkawy et al. (2013), for example, reported higher glucose and cholesterol levels after exposure to CPF (30 mg/kg) twice a week for 90 days. Developmental exposure (PND 1 to PND 4) increased the levels of cholesterol and triglycerides in a sex-dependent manner since only males were affected. Male mice also presented hyperinsulinemia (Slotkin et al., 2005). Reygner et al. (2016) reported dose-dependent metabolic effects after gestational and postnatal exposure to CPF.

1.2.9. Developmental exposure to CPF

The last few decades have seen increased interest in the effects of developmental exposure to CPF. Epidemiologic studies based on populations living in agricultural areas have observed a link between higher exposure levels during early childhood and adverse neurodevelopmental effects, with boys apparently being more sensitive to exposure than girls (Guo et al., 2019). Another study found that exposure to CPF during pregnancy may increase the risk of autism spectrum disorder. This effect may be further increased and associated with intellectual disability if the exposure takes place during the postnatal period (Von Ehrenstein et al., 2019). Moreover, three-year-old children prenatally exposed to high doses of CPF scored lower on psychomotor and development assessment tests and presented a higher incidence of attention problems and attention-deficit/hyperactivity disorder (Rauh et al., 2006). In the same line, another study reported a link between prenatal CPF levels and poorer performance on the Working Memory Index and Full-Scale IQ in 7-year-old children (Rauh et al., 2015).

Studies in animal models further corroborated these epidemiologic results, demonstrating that young animals are more sensitive to the toxic effects of CPF than older animals (Moser, 2000; Pope, 1999). Pope et al. (1991) compared the response to CPF of young (7-day-old) rats and adult rats and observed that the maximal tolerance dose was higher in adults (279 mg/kg) than in neonates (45

mg/kg), which suggests that neonates are more sensitive to acute CPF toxicity than adults. Moser and Padilla (1998) observed similar results, since young rats received a 5-7 times lower CPF dose than adult rats to obtain a similar effect, thus suggesting higher sensitivity in young subjects. Interestingly, these authors found that ChE activity tended to recover more rapidly in young subjects, probably due to higher protein synthesis during development (Moser and Padilla, 1998).

Differences in sensitivity between young and adult animals can be explained by differences in absorption, biotransformation and distribution. Moreover, children's detoxification systems need several years to fully develop. For instance, PON1 levels change with age. It has also been reported that PON1 activity in serum and the liver is very low at birth and increases with age (Costa et al., 2013), so it may be considered a potential factor behind the greater sensitivity of younger subjects to CPF. Another factor that accounts for this age-dependent vulnerability is the lower metabolic detoxication observed in young animals in comparison with adults (Costa et al., 2013). Moser (1998) observed differences in the levels of carboxylesterase and A-esterase in plasma and the liver depending on age. These enzymes, which are known to play a role in the detoxification of OPs, presented significantly lower activity in preweaning rats than in adult ones.

Exposure during development may also lead to changes in the normal maturation of several systems, and is especially relevant during neurodevelopment. A single oral dose of CPF (5 mg/kg) on PND 10 was enough to modify the levels of important proteins in mice hippocampus. More specifically, CPF decreased the levels of CaMKII and synaptophysin, two proteins that are involved in synaptogenesis, neuronal proliferation and myelination and play a key role in memory and learning. These differences may be behind the long-term cognitive impairments observed after 2 and 4 months

(Lee et al., 2015). Gestational and postnatal exposure to CPF in rats was also reported to alter the normal development of the serotoninergic system and cause effects in adulthood that can be linked to appetite control or affective disorders (Aldridge et al., 2004). Another study showed that developmental exposure to different doses of CPF can induce dose-dependent downregulation in genes that are involved in important processes such as synaptic transmission, plasticity and GABAergic, dopaminergic and glutamatergic signaling. These differences were clearly observed in 3-month-old mice and, to a lesser extent, in 8-month-old mice, which suggests long-lasting effects (Pallotta et al., 2017). Studies including developmental exposure to CPF showed differences in several aspects. For instance, postnatal exposure to CPF (1 or 3 mg/kg/day) induced higher locomotor activity on PND 25, as well as more active behavior in mice exposed to new environments during novelty seeking (Ricceri et al., 2003). In another study, with both gestational (GD 15 to GD 18) and postnatal (PND 11 to PND 14) exposure, CPF increased motor activity in the open field (OF) during adulthood and induced aggressive behaviors in males. Postnatal exposure to CPF also enhanced maternal response in females and decreased anxiety response in both males and females in the plus maze test (Ricceri et al., 2006). Similarly, behavioral alterations in lactating females were observed in mice postnatally exposed to 3 mg/kg/day of CPF. A reduction in anxious-like behaviors was observed in the Light/Dark paradigm (Venerosi et al., 2008). Social behaviors also seem to be susceptible to CPF effects, since female mice exposed to CPF (6mg/kg/day) during gestation (GD 14 to GD 17) presented longer social investigation times (De Felice et al., 2014).

It is worth noting that the cholinergic system plays a key role in neurodevelopment. As we previously suggested, the cholinergic system can be targeted by exposure to CPF, which suggests that developmental exposure to CPF may trigger long-lasting neurotoxic effects. For instance, a study in rats reported long-lasting effects of gestational exposure (GD 6 to GD 20) to the cholinergic neurons at two different doses of CPF. Results in the offspring

treated with the higher dose revealed ChE inhibition and lower mAChR levels, both of which lasted until PND 6. On the other hand, ChAT activity was unaffected until PND 9 when its activity presented a decrease at least until PND 30 (Richardson and Chambers, 2004, 2003). Another study reported that, during postnatal period (PND 1 to PND 21), developmental exposure to an increasing dose of CPF altered mAChR levels and ChAT activity in rats assessed on PND 30 (Richardson and Chambers, 2005).

Interestingly, numerous studies have reported various sex-dependent vulnerabilities to developmental CPF exposure. For example, gestational exposure (GD 12 to GD 15) to 5 mg/kg/day of CPF produced social preference impairments only in male mice but social conditioned place preference impairments in both males and females (Lan et al., 2019). Another study of gestational and postnatal exposure (GD 7 to PND 21) to different doses of CPF in rats observed sex-dependent behavioral deficiencies. That is, adult males exposed to higher doses of CPF (1 mg/kg/day) presented decreased learning and impaired retention during the MWM and the 8-arm radial maze task, whereas females performed in a similar way to controls. The authors found a link between these effects and increased levels of pro-inflammatory cytokines (IL-1b) and subunits of NMDA receptors in male hippocampus that may be behind these differences (Gómez-Giménez et al., 2017). Levin et al. (2001) exposed neonatal rats to CPF during two postnatal periods: PND 1 to PND 4 (1 mg/kg/day), and PND 11 to PND 14 (5 mg/kg/day). These are both periods with higher CNS events and critical phases of susceptibility to CPF in rodents. Exposure between PND 1 and PND 4 reduced the number of errors in females but increased the number in males during the first stages of training in the radial maze. Exposure between PND 11 and PND 14 slowed the response latency in the T-maze only in males, which suggests that these exposures resulted in alterations in cognitive performance that depended on the exposure period and sex (Levin et al., 2001). Motor activity and coordination can also be altered in a sex-dependent manner by developmental exposures (GD 7 to PND 21) to

different doses of CPF in rats. For instance, females exposed to CPF spent less time in the rotarod, which suggests that there are detrimental effects on motor coordination. This was not observed in their male counterparts. However, CPF exposure increased spontaneous motor activity in both males and females at 2-3 months of age. Biochemical tests determined a sexual-dimorphic distribution of NMDA receptors in the hippocampus after CPF exposure, which may influence the previously observed effects during adulthood (Gómez-Giménez et al., 2018).

1.3. Interaction between apoE and CPF

Genetic background can influence one's intrinsic response to certain environmental toxics, thus conferring different vulnerabilities on the subject. Several studies have confirmed the difference in sensitivities depending on the apoE genotype after exposure to toxicants such as lead (Engstrom et al., 2017; Prada et al., 2016) or mercury (Tratnik et al., 2017), with ε4 carriers being more susceptible to the toxic effects. In the last few years, our group has specifically studied the interaction between apoE isoforms and exposure to certain toxics. First, we studied decabromodiphenyl ether (BDE-209) exposures and showed that a single dose of 10 or 30 mg/kg on PND 10 was enough to trigger genotype-dependent impairments in learning and memory, a delay in eye opening, impaired cued fear learning and altered BDNF levels in the frontal cortex (Reverte et al., 2014a, 2014b, 2013).

We then focused on exposure to the widely used pesticide CPF both in adulthood and in the developmental period and observed effects over a wide range of behavioral and metabolic aspects depending on the apoE isoform. Our studies with apoE-TR male mice exposed to CPF (2 mg/kg/day) throughout their adult diet revealed genotype-dependent effects on spatial learning and memory, when assessed in the Barnes maze. More specifically, we observed a basal difference in performance between genotypes and a CPF effect during task acquisition only with apoE2 mice. Indeed, these mice presented a higher

velocity and a lower escape latency that were only detectable with this genotype. Also, retention was slightly impaired in apoE3 mice, since they performed fewer entries to the target hole, thus suggesting subtle memory impairments (Peris-Sampedro et al., 2015a).

A specific genotype-dependent effect of the pesticide was observed on metabolic assessment. First, apoE3 mice presented a greater increase in body weight after exposure to the pesticide than their counterparts (Peris-Sampedro et al., 2015b, 2015a), suggesting that apoE3 is more susceptible to the obesogenic effect of CPF. To gain further insight, metabolic biomarkers were studied in the plasma (Peris-Sampedro et al., 2015b). Results showed higher glucose levels, increased fasting plasma insulin and higher rates of insulin resistance in all groups after exposure to CPF. However, these effects were enhanced in apoE3-TR mice, which once more suggests the greater sensitivity of this genotype to CPF. Moreover, higher levels of leptin that correlated strongly with body weight were observed in E3. Overall, our results suggested that there were metabolic alterations in both genotypes after exposure to CPF but that these were more prominent in *APOE3* (Peris-Sampedro et al., 2015b). We therefore designed another study to analyze the leptin and insulin signaling pathways. Interestingly, CPF disrupted insulin and leptin homeostasis but triggered a decrease in the expression levels of PON genes. Once again, apoE3-TR mice were most affected by the pesticide's toxic effects. In fact, CPF-exposed apoE3 mice presented altered phosphorylation of elements involved in insulin and leptin homeostasis. Effects on PON1 and PON3 levels were also observed, with a decreased expression in apoE3-TR and C57BL/6 mice (Peris-Sampedro et al., 2018).

Female apoE-TR mice were also tested in the 5-CSRTT in order to study the effects of the pesticide and its interaction with the genetic background in terms of attention, motivation and impulsivity. The animals were first trained in the

apparatus in order to establish the baseline and then exposed to 3.75 mg/kg/day of CPF. During the baseline, differences between genotypes were observed, with apoE4 mice performing the worst. Interestingly, differences between genotypes were no longer observed when the animals were exposed to CPF, which suggests that CPF has a specific effect on apoE4-TR mice. During the wash-out period, the effects of CPF were detectable, with apoE3-TR mice showing attentional disruptions (Peris-Sampedro et al., 2016). All these studies reported an inhibition of plasma ChE, with activity values ranging from 17.76 to 32.12 % (Peris-Sampedro et al., 2015a, 2015b, 2016, 2018).

To assess whether these effects would also be observed after developmental exposure to CPF, we used both male and female apoE-TR mice exposed to either 0 or 1 mg/kg/day of CPF from PND 10 to PND 15. First we studied the short-term effects on development and the cholinergic system. For all groups, a general delay in eye opening was reported after CPF exposure as were impaired motor functions such as tail pull reflex and clinging and climbing ability. Study of the gene expression of cholinergic elements in the brain revealed a pesticide effect modulated by both *APOE* genotype and sex. For example: α7 nAChRs expression decreased in CPF-treated males; VAChT levels decreased in both CPF-treated apoE3-TR males and females; and PON2 levels decreased in CPF-treated apoE4 males but increased in CPF-treated apoE3 females. All in all, these results suggest that CPF exposure has a significant effect on the cholinergic system on PND 15 and PND 30 that is dependent on *APOE* genotype and sex (Basaure et al., 2018). The results of another study that included both postnatal exposure to CPF and re-exposure at 5 months of age suggested that CPF had detrimental effects on spatial learning and memory when the mice were assessed in a Barnes maze. Indeed, postnatal CPF impaired task acquisition and retention especially in apoE3-TR mice. Interestingly, adult CPF exposure enhanced performance only in apoE4-TR mice. A study of how the treatment affects the expression of cholinergic elements showed postnatal effects on ChAT in apoE4-TR mice. Adult CPF exposure affected expression of α4 nAChR, AChE-

S and α7 nAChR in apoE4 females. These results suggest that the differences are modulated by CPF exposure (both postnatal and adult), genotype and sex (Basaure et al., 2019).

In general, these data show that the *APOE* genotype is involved in response to environmental toxics, and specifically to the pesticide CPF. It is therefore important to take into account the potential influence of genetic background and sex in toxicological studies.

1.4. Microbiota and short-chain fatty acids

Recent evidence recognizes the critical role played by microbiota in multiple aspects related to health and disease. Gut microbiota are the large number of microorganisms (estimated at approximately 10^{14}) that colonize the intestine in a symbiotic relationship with the host. These microorganisms are involved in numerous functions and can influence general health. Some microorganisms can produce metabolites such as short-chain fatty acids (SCFAs), which are important mediators of microbiota-gut-brain communication.

1.4.1. Microbiota

Microorganisms colonize the surfaces of the human body that are exposed to the environment but the highest percentage of them are found in the intestinal tract. Gut microbiota include not only bacteria but also archaea, viruses and unicellular eukaryotes. The genes they encode constitute the microbiome (Clemente et al., 2012; Sekirov et al., 2010). The composition of gut microbiota can vary during one's lifetime but becomes relatively stable in adulthood. The diversity of microbiota in newborns is initially very low and it depends strongly on the mode of delivery whether they resemble the microbes of the mother's vagina or those of the mother's skin (Nicholson et al., 2012; Tamburini et al.,

2016). During development, diversity increases, shifting from mainly aerotolerant microorganisms to anaerobes characteristic of the adult gut (Palmer et al., 2007). Although there is interpersonal variability in community composition, the adult gut is largely dominated by *Bacteroidetes* and *Firmicutes,* followed by *Proteobacteria, Verrucomicrobia, Actinobacteria* and *Fusobacteria* (Eckburg et al., 2005).

Microbiota establish a symbiotic relationship with the host, being involved in functions such as digestion, maintenance of the immune system and energy homeostasis (Yadav et al., 2018). More specifically, microbiota may help to control body weight and energy homeostasis by regulating the energy harvested from the diet (Bäckhed et al., 2007; Turnbaugh et al., 2006). Microbiota may also interact with both the innate and adaptative immune systems and promote the development of the normal immune functions and the maturation of immune cells (Chow et al., 2010). Differences in microbiota composition exist between healthy subjects, which may be influenced by environmental and genetic factors and the interaction between them (Rothschild et al., 2018; Turpin et al., 2016). Studies corroborating the genetic contribution have assessed a wide range of genes, including *APOE*, which has been shown to be behind differences in microbiota composition in both animals and humans (Tran et al., 2019).

The composition of gut microbiota can be disrupted by several factors that lead to an imbalance in the microbial community known as dysbiosis (Hawrelak and Myers, 2004). Petersen and Round (2014) divided dysbiosis into three categories: a) loss of beneficial microorganisms, b) increase in potentially harmful microorganisms and c) loss of diversity. Factors that can induce dysbiosis include use of antibiotics (Fröhlich et al., 2016), dietary changes such as the introduction of a high-fat diet (Miller et al., 2019), and stress (Golubeva et al., 2015). Gut microbiota dysbiosis has been linked to a wide range of diseases, including asthma (Abrahamsson et al., 2014), colorectal cancer (Ahn

et al., 2013), Crohn's disease (Manichanh et al., 2006), autism (Kang et al., 2013), obesity (Turnbaugh et al., 2009), type 2 diabetes (Wang et al., 2012), irritable bowel syndrome (Carroll et al., 2012), cardiovascular affections (Katsimichas et al., 2019) and neurodegenerative diseases (Marizzoni et al., 2017). All the above studies corroborate the important influence of microbiota on health and disease.

Since microbiota can be directly exposed to pesticides throughout the ingestion of treated food, they have been proposed as recipients of pesticide toxicity (Yuan et al., 2019). Previous studies have reported alterations in the composition of microbiota after exposure to pesticides and, more specifically, to the widely used CPF both in animal models (Condette et al., 2015; Fang et al., 2018; Liang et al., 2019; Zhao et al., 2016) and the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) (Joly et al., 2013; Réquilé et al., 2018; Reygner et al., 2016a). Generally speaking, these studies agree on the potential capacity of CPF to alter the composition of gut microbiota and induce dysbiosis.

1.4.2. Short-chain fatty acids (SCFAs)

SCFAs generally refer to fatty acids composed by 2-6 carbon atoms. They are mainly produced by gut microbiota after the fermentation of non-digestible carbohydrates in the gut. Acetate, propionate and butyrate are the most abundant SCFAs in the human body though others have been described in lower proportions. SCFA levels depend on factors such as diet and microbiota composition (Dalile et al., 2019). Studies have shown that SCFAs (especially butyrate) can be metabolized and used as energy substrate for colonocytes in the intestine (Donohoe et al., 2011; Maslowski, 2019; Morrison and Preston, 2016). However, some SCFAs may leave the gut, enter the portal circulation, and eventually reach the liver, where they can be metabolized. A small percentage of SCFA can bypass portal circulation to reach systemic circulation and ultimately peripheral tissues such as the pancreas, the liver, adipocytes and

skeletal muscle (Boets et al., 2017). In fact, SCFA receptors have been observed in various tissues, which suggests they have a role in the crosstalk between the gut and peripheral tissues (Canfora et al., 2015). Some SCFAs can reach the brain by crossing the blood brain barrier though in minimal proportions of around 2-3% (Frost et al., 2014). SCFAs are not only produced by the microbiota in the gut but can also be produced by endogenous synthesis during fatty acid oxidation or glucose metabolism (Bourassa et al., 2016; Pouteau et al., 2003). The diet is also a source of SCFAs for the organism. For example, butyric acid and propionic acid are present in dairy products such as whole cow's milk, butter and cheese (Al-Lahham et al., 2010; Stilling et al., 2016).

SCFA are responsible for certain local effects in the gut, such as maintenance of the intestinal barrier and regulation of intestinal inflammation. However, they also play an important role in gut-brain-axis communication, since they can bind to G-protein-coupled receptors. Importantly, SCFAs can also inhibit histone deacetylases (HDACs), which remove the acetyl groups of the histones and lead to condensed chromatin. The inhibition of HDACs by certain SCFAs can therefore modulate transcription and gene expression (Dalile et al., 2019). SCFAs are also reported to influence inflammation and immune response and to interact with vagal signaling (Tan et al., 2014). Other studies have demonstrated a role for SCFAs in regulating the host metabolism, since they are involved in, for example, glucose homeostasis, lipid metabolism and appetite regulation (Morrison and Preston, 2016). The involvement of SCFAs in neurological disorders has also been reported, e.g. a neuroprotective effect of butyrate against vascular dementia (Liu et al., 2015) and cerebral injury (Sun et al., 2016) was reported in mice. However, elevated levels of certain SCFAs such as propionic acid have been observed in children with non-genetically related Autism Spectrum Disorder (MacFabe, 2015). In summary, SCFAs may have important beneficial and detrimental effects on host physiology.

To sum up, SCFAs may be an important target for certain disorders mainly related to metabolism. They may also be modulated throughout the diet, for example by increasing dietary fiber or using prebiotics, with SCFAs acting as mediators between diet, microbiota and the health or illness of the host.

2. HYPOTHESIS AND OBJECTIVES

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POSTNATAL CHLORPYRIFOS EXPOSURE INFLUENCES THE GUT MICROBIOTA AND THE EXPRESSION OF BIOLOGICAL AND NEUROBEHAVIORAL CHARACTERISTICS OF THE APOE GENOTYPE IN AN AGE-DEPENDENT MANNER
Laia Guardia Escoté

2. HYPOTHESIS AND OBJECTIVES

Genetic-driven differences in the response to toxic exposure have been the main focus for our laboratory in the last few years. More specifically, we are interested in the three most common human *APOE* genetic variants, which confer not only different susceptibilities to cognitive impairments, cardiovascular risk and AD but also different vulnerabilities to environmental toxics such as pesticides. Organophosphate pesticides – particularly CPF – are used extensively worldwide. While exposed workers and their families constitute a high risk group, the general population is exposed to low doses of the pesticide, mainly in the diet (Reiss et al., 2015). Regulations have been introduced aimed at reducing the use of CPF, especially in residential areas but it is still used extensively. CPF residues have been found, for example, in fruit and vegetables, water, and milk and its derivatives, so it reaches virtually the whole population (Saunders et al., 2012). Epidemiological and experimental studies have found a link between chronic exposure to non-toxic low doses of CPF and metabolic and neurobehavioral disorders. Indeed, it has been hypothesized that exposure to pesticides can be behind the growing incidence of neurodegenerative diseases (Mostafalou and Abdollahi, 2018).

In a humanized apoE-TR mouse model, we have previously shown that *APOE* genetic background may influence intrinsic response to CPF exposure during adulthood, in terms of spatial learning and memory, metabolic alterations and attention, motivation and impulsivity (Peris-Sampedro et al., 2015a, 2015b, 2016, 2018). Certain sub-populations are reported to present higher vulnerability to CPF exposure, for example when this exposure occurs during development (Moser, 2000; Pope, 1999). Although several studies have focused on the higher sensitivity to toxic effects at early ages, information on how this is influenced by *APOE* genotype and sex is scarce. Our research has therefore focused on the interaction between developmental CPF exposure, *APOE*

genotype and sex (Basaure et al., 2018, 2019) in behavioral and biochemical processes.

This thesis follows the work conducted in our laboratory using apoE-TR mice carrying human *APOE3* and *APOE4* as well as wild type C57BL/6 mice. The selected exposure period (PND 10–PND 15) coincides with important brain maturation processes such as neurogenesis, synaptogenesis, differentiation and myelination (Semple et al., 2013) and corresponds to the last trimester of gestation or birth in humans (Watson et al., 2006). Though significantly lower than in other studies, the CPF dose used in the present study (1 mg/kg/day) is still different from that estimated in humans. As we mentioned earlier, it is difficult to determine the real average daily dose in humans, though it has been estimated at 0.005 μg/kg/day in adults and 0.009 μg/kg/day in infants (Eaton et al., 2008). Although the dosage used is several times higher, we should bear in mind that extrapolation between species would reduce the initial difference. According to the FDA, to calculate the human equivalent dosage we should divide the mouse dosage by a factor of 12.3 (Nair and Jacob, 2016). In general, this thesis follows the work previously established in our laboratory. It attempts to provide further insight into the specific traits of the *APOE* genotype and determine how, together with sex, this genotype influences response to postnatal CPF exposure at different stages of life.

2.1. Hypothesis

The *APOE* genotype and sex confer different vulnerabilities to the detrimental effects of postnatal exposure to CPF, leading to different neurobehavioral and biochemical phenotypes expressed at different moments and situations during lifespan.

2.2. Objectives

2.2.1. Main objective

The main objective of this thesis is to assess how genetic background influences the short-, mid- and long-term effects of developmental exposure to the pesticide CPF on different biological and behavioral endpoints.

2.2.2. Specific objectives

1. To assess the specific changes in C57BL/6, apoE3- and apoE4-TR mice induced by the short-term effects of developmental exposure to CPF on the composition of gut microbiota and how this interaction influences SCFA levels in the brain.

- To study the differences between genotypes.
- To study the interactions between genotype and treatment.
- To study the possible relationship between gut microbiota and SCFA in the brain.

2. To determine the detrimental effects of CPF exposure during development and the influence of *APOE* genotype and sex on recognition memory in young adult mice.

- To study the response to the cholinergic drugs scopolamine and rivastigmine depending on sex, genotype and treatment.
- To study the response to the GABAergic agonist alprazolam depending on sex, genotype and treatment.

3. To study the involvement of the *APOE4* genotype and sex in the long-term response to postnatal exposure to CPF in spatial learning and memory and the

long-term effects on the expression of several key elements of the cholinergic neurotransmitter system.

3. RESULTS

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3. RESULTS

This thesis includes three original research articles, either already published or in the process of being published. Table 2 shows the specific objectives of the thesis and their correspondence with these articles.

Table 2. Specific objectives of the thesis and corresponding publications

Specific objectives	Publications
1	Guardia-Escote L, Basaure P, Biosca-Brull J, Cabré M, Blanco J, Pérez-Fernández C, Sánchez-Santed F, Domingo JL, Colomina MT. ***APOE genotype and postnatal chlorpyrifos exposure modulate gut microbiota and cerebral short-chain fatty acids in preweaning mice.*** Food and Chemical Toxicology, 2019 (submitted)
2	Guardia-Escote L, Basaure P, Peris-Sampedro F, Biosca-Brull J, Cabré M, Sánchez-Santed F, Domingo JL, Colomina MT. ***APOE genetic background and sex confer different vulnerabilities to postnatal chlorpyrifos exposure and modulate the response to cholinergic drugs.*** Behavioral Brain Research, 2019. 376: 112195
3	Guardia-Escote L, Basaure P, Blanco J, Cabré M, Pérez-Fernández C, Sánchez-Santed F, Domingo JL, Colomina MT. ***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.*** Food and Chemical Toxicology, 2018. 122: 1-10.

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3.1. Publication I

***APOE* genotype and postnatal chlorpyrifos exposure modulate gut microbiota and cerebral short-chain fatty acids in preweaning mice.**

Laia Guardia-Escote, Pia Basaure, Judit Biosca-Brull, Maria Cabré, Jordi Blanco, Cristian Pérez-Fernández, Fernando Sánchez-Santed, Maria Teresa Colomina.

Food and Chemical Toxicology, 2019 (submitted)

Study I overview.	
What do we already know?	The *APOE* genotype can modulate the composition of adult gut microbiota. CPF can induce dysbiosis and alter SCFA levels in the gut. To date, little research has been conducted on the composition of SCFAs in the brain.
What does this study add?	This is the first study to assess the differences in microbiota between *APOE* genotypes at early ages. The effects of CPF exposure observed strengthen existing evidence of its ability to induce dysbiosis even at young ages. They also provide information about SCFAs in the brain and their correlation with gut microbiota.
Highlights	ApoE4-TR mice had different microbiota composition at young ages and were the animals most effected by CPF exposure. *A. muciniphila* is influenced by genotype and CPF exposure. Higher levels of SCFAs in the brain were observed in apoE3-TR mice and mice exposed to CPF

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APOE genotype and postnatal chlorpyrifos exposure modulate gut microbiota and cerebral short-chain fatty acids in preweaning mice.

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Summary

The gut microbiota is composed of a large number of microorganisms residing in the intestine in a symbiotic relationship with the host. Its composition can be altered by genetic and environmental factors. The host's genetic background, including the different isoforms of the apolipoprotein E (*APOE*) gene, can also exert an influence over microbiota composition. Exposure to pesticides in general, and more specifically to the widely-used chlorpyrifos (CPF), can lead to dysbiosis. These changes can in turn alter the levels of metabolites produced by the microbiota, such as short-chain fatty acids (SCFAs), involved in gut-brain communication. This study was aimed at assessing the specific contribution of the *APOE* genetic background and early exposure to CPF to the composition of gut microbiota and SCFA levels in the brain. For it, apoE3- and apoE4-TR mice along with wild C57BL/6 mice postnatally exposed to CPF, were used. Microbiota composition was assessed at PND 15 in the gut, while SCFA levels were determined in the brain. We observed differences between genotypes at

different taxonomic levels, with *APOE4* presenting alterations in most cases compared to the other groups. *APOE4* was also most susceptible to the toxic effects of CPF, which induced dysbiosis in general. Interestingly, *A. muciniphila* belonging to the phylum *Verrucomicrobia* showed greater abundance in the *APOE4* genotype, but was reduced by the effect of the pesticide. Furthermore, it emerged as a potential modulatory factor on the basis of previously observed metabolic and cognitive differences. *APOE* and postnatal exposure to CPF also influenced cerebral levels of SCFA, with the *APOE3* genotype and CPF exposure being the factors inducing the highest concentrations. Overall, these results provide further knowledge about the gut microbiota and cerebral SCFA composition at early ages and how they can be modulated by *APOE* and postnatal exposure to CPF.

Keywords:
Chlorpyrifos, APOE, Gut microbiota, SCFA, short-chain fatty acids, *Akkermansia muciniphila*

1. Introduction

In recent years there has been increasing interest in the functions of the gut microbiota, i.e. the large number of microorganisms residing in the intestine in a symbiotic relationship with their host (Gomes et al., 2018; Young and Schmidt, 2009). These microorganisms are involved in different functions such as digestion, metabolism (Joyce and Gahan, 2014) and immune function (Kamada et al., 2013), and they can influence the health status in general (Clemente et al., 2012; Sekirov et al., 2010). The gut microbiota undergoes a number of changes from birth (Bäckhed et al., 2015) and achieves a relatively stable composition by adulthood (Palmer et al., 2007). Nevertheless, it can be disrupted by several factors that may lead to dysregulation or dysbiosis (Hawrelak and Myers, 2004), with early postnatal ages being the most sensitive period to external factors. Microbiota dysbiosis has been linked to several diseases, such as autism (Needham et al., 2018), obesity and diabetes (Lee et al., 2019), cardiovascular

affections (Katsimichas et al., 2019), allergy and asthma (Fujimura and Lynch, 2015), as well as neurodegenerative diseases (Marizzoni et al., 2017).

Microbiota composition can be affected by changes in diet (Makki et al., 2018; Sonnenburg and Bäckhed, 2016; Turnbaugh et al., 2008), drugs (Lange et al., 2016) and exposure to environmental toxics such as pesticides (Yuan et al., 2019). Exposure to organophosphate pesticides, in particular the widely-used chlorpyrifos (CPF), results in alterations to core microbial communities. Previous studies have reported gut dysbiosis after exposure to CPF in male rats (Condette et al., 2015; Fang et al., 2018) and male mice (Liang et al., 2019; Zhao et al., 2016) and with the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) (Joly et al., 2013; Réquilé et al., 2018; Reygner et al., 2016). A link between alterations in microbiota composition and higher gut permeability has also been observed after exposure to different doses of the pesticide (Liang et al., 2019; Zhao et al., 2016). The general population is exposed to CPF mainly through diet (Reiss et al., 2015), being the gut microbiota directly exposed to CPF. CPF elicits its toxicity by the irreversible inhibition of the acetylcholinesterase (AChE) enzyme, and there is evidence that developing organisms are more sensitive to this effect than adults (Burke et al., 2017; Moser, 1998). This is of particular relevance, since several studies have reported the existence of a crucial window during development when microbiota alterations can cause important long-lasting effects (Borre et al., 2014; Cox et al., 2014; Torow and Hornef, 2017).

Gut microbiota composition is also shaped by individual factors such as the genetic profile of the host (Bonder et al., 2016; Turpin et al., 2016), which can interact with environmental factors. A large study including monozygotic and dizygotic twins revealed that the relative abundance of certain microbiota elements is influenced by the genetic background of the subject (Goodrich et al., 2014). Tran et al. (2019) studied the influence of the different isoforms of

apolipoprotein E (apoE) – a protein involved in lipid transport and homeostasis – on the composition of microbiota and its associated metabolites in both humans and transgenic mice. The results revealed the influence of the APOE genotype on the relative abundance of several bacteria taxa, which may in turn explain the differences observed in the associated metabolite levels (Tran et al., 2019). In humans, three isoforms – apoE2, apoE3 and apoE4 – which are functionally different, are mainly found, with apoE4 being a well-established risk factor for the development of dementia and Alzheimer's disease (Hersi et al., 2017). Recent studies using the targeted replacement (TR) mouse model expressing the different isoforms of apoE (apoE-TR) showed different vulnerabilities to CPF exposure depending on *APOE* genetic background, including neurobehavioral (Basaure et al., 2019; Guardia-Escote et al., 2018) and metabolic disorders (Peris-Sampedro et al., 2015a, 2015b, 2018).

Gut microbiota send signals to the CNS via changes in immune reactivity, gut and serum metabolites and vagal nerve sensory innervation (Vuong et al., 2017). The short-chain fatty acids (SCFAs), which are produced by some microorganisms by the fermentation of non-digestible carbohydrates in the gut (Morrison and Preston, 2016) are key signaling factors of the gut-brain communication (Dalile et al., 2019). Different functions have been attributed to SCFAs, for instance that they serve as an energy substrate for the gut cells, contributing to colonic epithelial integrity (Guilloteau et al., 2010; Morrison and Preston, 2016). Moreover, they can be transported via the portal vein, act as energy substrate or signaling molecules and influence the host metabolism in various tissues (De Vadder et al., 2014). Finally, a low percentage of SCFAs can cross the blood-brain barrier and enter the brain (Frost et al., 2014). A few authors have reported that levels of SCFA in the brain are low compared to those in the gut (Dalile et al., 2019). However, a correlation has been observed between the amount of some SCFAs in the gut and the brain, including butyrate, which may be involved in the modulation of several cerebral functions (Liu et al., 2015; Sun et al., 2016). In addition of being a source of energy for several

cells, butyrate exerts its effects by acting as an inhibitor of histone deacetylases (HDACs) contributing in this way to the increase in gene expression in the host (Tan et al., 2014). This latter function can be of special relevance in developing organisms.

The aim of this investigation is to assess the effects on the composition of the gut microbiota of the most common apoE isoforms and those of postnatal exposure to the pesticide CPF. How they modulate the levels of SCFA in the brain at 15 days of age has also been investigated. To the best of our knowledge, this is the first study in which the gut microbiota and SCFA composition in the brain - at such a young age - in mice postnatally exposed to CPF has been assessed.

2. Material and methods

2.1. Animals and care

Male apoE-TR mice and C57BL/6 mice were used. Mice homozygous for the ε3 and ε4 allele were purchased from Taconic (Taconic Europe, Lille Skensved, Denmark). The apoE-TR model has its murine apoE gene replaced by the different alleles of human apoE (Sullivan et al., 1997). The C57BL/6 mice were obtained from Charles River (Charles River, Barcelona, Spain). Mice of the same genotype were mated during a one-week period, after which the females' body weight was monitored. Pregnant females were kept in individual cages and the day of delivery was considered postnatal day (PND) 0. The animal room was maintained under standard conditions (temperature 22 ± 2 °C and relative humidity 50 ± 10%) with a 12h light/dark automatic light cycle. All the mice were allowed free access to water and 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. Chemicals and treatment

CPF [0,0-diethyl O-(3,5,6-trichloropyridin-2-yl) phosphorothioate], 99.5% purity (Sigma-Aldrich Co. LLC., Madrid, Spain) was orally administered to the CPF-treated groups during PND 10-15. The compound was adjusted to administer 1 mg/kg in 1 μL/g of body weight. The control groups were given corn oil as the vehicle during the same period. A total of 36 male mice were divided into six experimental groups (n=6 animals/group).

2.3. Sacrifice and sampling

Biological samples were collected at PND 15, four hours after the last dose was administered. Animals were deeply anesthetized with isoflurane before being euthanized by decapitation. Gut and brain samples were collected and immediately stored at -80ºC.

2.4. Bacterial DNA extraction

Bacterial DNA was purified from the entire gut content obtained from 15-day-old mice using the PureLink™ Microbiome DNA Purification Kit (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. The DNA concentration in each sample was assessed by fluorometric quantification.

2.5. 16S metagenomics

The 16S metagenomics study was carried out in an external laboratory (Biomedica Molecular Medicine, Madrid, Spain). The specific region of the bacterial 16S rDNA gene V3+V4 was amplified by PCR with the conditions

adapted to the DNA input in each sample. After marking, barcoding and quantifying, the resulting DNA was pooled and sequenced. Sequencing was performed using Illumina® MiSeq technology in order to obtain an approximate mean of 100,000 reads per sample. After the quality filter and barcode separation, the processing of the sequence reads was performed by Illumina 16S Metagenomics, version 1.0.1.0. Taxonomic assignation was performed in accordance with the Greengenes database. Each 16S rRNA sequence was assigned at different taxonomic levels, such as phylum, genus and species.

2.6. Analysis of short-chain fatty acids

SCFA determination was carried out in an external laboratory (Centre for Omic Sciences (COS), Reus, Spain). Whole brain samples were homogenized and lipids were extracted from a total volume of 100 mg of the homogenized tissue. Chromatographic separation was performed with a gradient elution using a Kinetex polar C18, 2.6 µm 2.1 x 100 mm analytical column (Phenomenex, Torrance, CA, USA). Acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, 3-methylvaleric acid, 4-methylvaleric acid and hexanoic acid were quantified in all samples. Metabolite concentration was normalized using tissue weight and expressed as pmol/mg or pmol/g tissue.

2.7. Statistical analysis

Data were analyzed using SPSS 25.0 software (IBM Corp, Chicago, USA). Principal component analysis (PCA) was performed in order to observe intrinsic clusters in the gut microbiota data. A two-way analysis of variance (ANOVA) was used to study the relative abundance of the different microbiota components and the concentration of SCFAs. A one-way ANOVA and *post-hoc* Tukey's tests of variance were used to analyze differences between groups. Pearson correlations were used to investigate associations between microbiota composition and SCFAs levels. Variance homogeneity was assessed by a Levene test. The results

are represented as mean values ± S.E.M. Statistical significance was set at $p<0.05$.

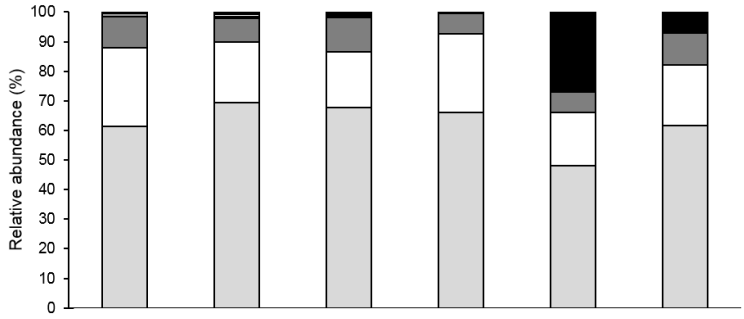
3. Results

3.1. Gut microbiome

The relative abundance of each phylum, genus and species was calculated by dividing each read count by the total read count for each sample. There were no differences between groups regarding diversity, assessed using the Shannon diversity index (Kim et al., 2017) (data not shown).

3.1.1. The phylum Verrucomicrobia was highly present in apoE4 mice, while postnatal exposure to CPF tended to equal the levels between genotypes

The gut bacteria assigned at phylum level are shown in Figure 1, with only the most abundant phyla represented (i.e. subjects with a >1% relative abundance). It can be seen that the predominant phylum is *Bacteroidetes* (48.05-69.39%), followed by *Firmicutes* (18.01-26.69%) and *Proteobacteria* (7.03-11.50%). Interestingly, *Verrucomicrobia* was highly present (26.34%) only in apoE4 mice. Indeed, a two-way ANOVA (genotype x treatment) indicated significant effects of the genotype on this phylum [$F(2,30)=9.490$, $p=0.001$] and a tendency towards significant effects of the treatment [$F(1,30)=4.091$, $p=0.052$] and the interaction between genotype and treatment [$F(2,30)=3.295$, $p=0.051$]. In order to further study these differences, we performed a one-way ANOVA (group) (Fig. 2). *Post-hoc* analysis revealed that the control-apoE4 group presented significantly higher levels of *Verrucomicrobia*, suggesting a basal difference between apoE4 and the other genotypes, which is attenuated by postnatal exposure to CPF (Fig. 2A).



	Control C57BL/6	CPF-Treated C57BL/6	Control apoE3	CPF-Treated apoE3	Control apoE4	CPF-Treated apoE4
Bacteroidetes	61.38±6.07	69.39±3.08	67.85±4.54	65.94±7.47	48.05±5.93	61.63±5.98
Firmicutes	26.55±6.60	20.60±4.26	18.75±3.97	26.69±8.62	18.01±2.92	20.38±3.63
Proteobacteria	10.65±2.56	7.96±3.36	11.50±2.24	7.03±3.46	7.03±1.17	10.84±1.25
Verrucomicrobia	0.02±0.003^{a}	0.01±0.004^{a}	1.49±1.48^{a}	0.02±0.005^{a}	26.34±9.12^{b}	6.65±4.93^{a}
Cyanobacteria	0.99±0.06	0.39±0.19	0.23±0.05	0.15±0.02	0.16±0.05	0.18±0.03
Tenericutes	0.20±0.10	0.98±0.97	0.06±0.02	0.08±0.05	0.05±0.02	0.08±0.05
Deferribacteres	0.01±0.00	0.46±0.29	0.002±0.00	0.001±0.00	0.11±0.07	0.12±0.08
Other	0.21±0.09	0.21±0.05	0.10±0.01	0.09±0.01	0.24±0.07	0.12±0.01

Figure 1| Relative abundance of the most abundant phyla (i.e. subjects with a >1% relative abundance). Different letters represent significant differences between groups at $p<0.05$.

Further study of the less abundant phyla showed significant differences. For example, the phylum *Nitrospirae* showed a significant influence of the genotype [$F(2,30)=6.709$, $p=0.004$] and the treatment [$F(1,30)=7.268$, $p=0.011$], and a tendency towards interaction between genotype and treatment [$F(2,30)=3.253$, $p=0.053$] (Fig. 2B). The phylum *Thermi* displayed significant effects of the genotype [$F(2,30)=4.280$, $p=0.023$] (Fig. 2C), while the phyla *Thermotogae* and *Chrysiogenetes* showed effects of the treatment ([$F(1,30)=7.401$, $p=0.011$] and [$F(1,30)=8.155$, $p=0.008$] respectively) (Fig. 2D and 2E). Finally, the phylum *Planctomycetes* presented an effect of the genotype [$F(2,30)=9.103$, $p=0.001$], the treatment [$F(1,30)=6.929$, $p=0.013$] and the interaction between genotype and treatment [$F(2,30)=5.783$, $p=0.008$] (Fig. 2F). Further *post-hoc* analysis

revealed that *Nitrospirae* and *Planctomycetes* showed the same pattern of differences between groups as that previously described for *Verrucomicrobia*, with the control apoE4 being the group showing the highest levels of the microorganism compared to the other groups.

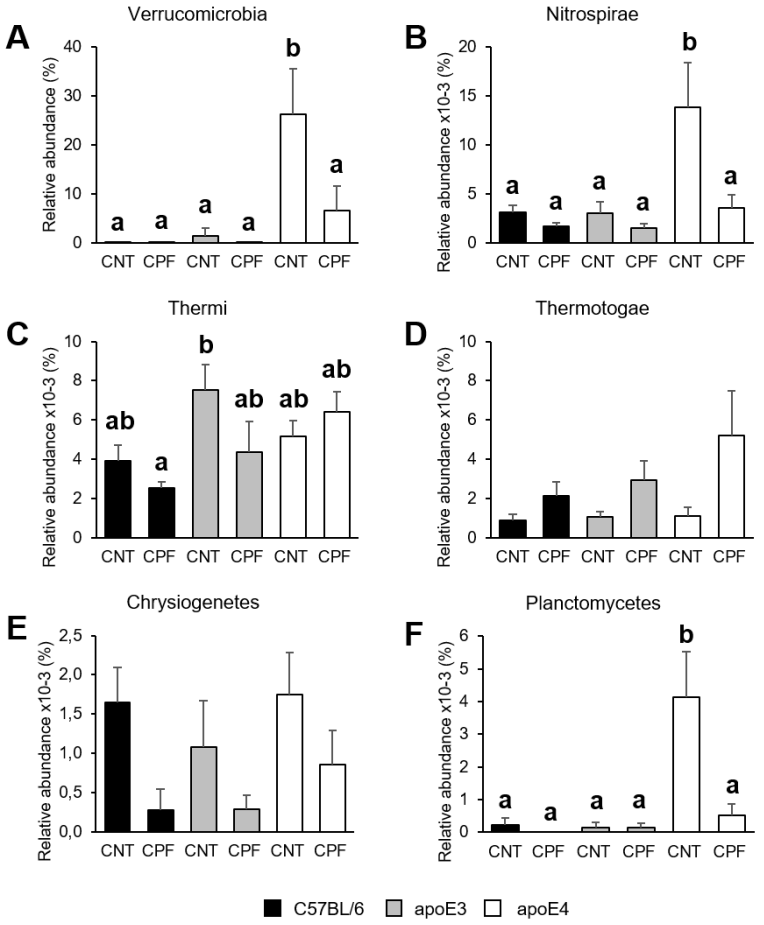


Figure 2| Relative abundance of phyla presenting significant differences between groups. (A) *Verrucomicrobia*, (B) *Nitrospirae*, (C) *Thermi*, (D) *Thermotogae*, (E) *Chrysiogenetes* and (F) *Planctomycetes*. Different letters represent significant differences between groups at $p<0.05$.

3.1.2. The APOE4 genotype differs from the other genotypes in the Akkermansia, Luteolibacter and Rubritalea genera, with the APOE4 genotype being the only one affected by CPF exposure.

We performed a principal component analysis (PCA) on the most abundant gut bacteria (i.e. subjects presenting >1% relative abundance) assigned at genus level (Fig. 3). PCA was used to explore the different clustering patterns of the genera studied, with the variance explained by the principal components (PC). PC 1 explained 16.54% of the variance, while PC 2 explained 13.78% and PC 3 explained 8.88%. We found some genera that were clustered together. Therefore, to study them in a greater detail we performed a two-way ANOVA (genotype x sex) (Supplementary Table 1). Significant effects of the genotype [$F(2,30)=9.748$, $p=0.001$], the treatment [$F(1,30)=4.240$, $p=0.048$] and interaction between genotype and treatment [$F(2,30)=3.425$, $p=0.046$] on the genus *Akkermansia* were observed (Fig. 4A). The effects of genotype were also noticed in *Escherichia* [$F(2,30)=4.235$, $p=0.024$] (Fig. 4B), *Helicobacter* [$F(2,30)=3.611$, $p=0.039$] (Fig. 4C), *Parapedobacter* [$F(2,30)=3.745$, $p=0.035$] (Fig. 4D), *Enterococcus* [$F(2,30)=3.452$, $p=0.045$] (Fig. 4E), *Serratia* [$F(2,30)=4.512$, $p=0.019$] (Fig. 4F), *Luteolibacter* [$F(2,30)=9.439$, $p=0.001$] (Fig. 4G) and *Rubritalea* [$F(2,30)=4.008$, $p=0.001$] (Fig. 4H). Furthermore, *Helicobacter* and *Streptococcus* (Fig. 4I) also presented significant interaction between genotype and treatment ([$F(2,30)=5.121$, $p=0.012$] and [$F(2,30)=5.289$, $p=0.011$] respectively). In order to further study these differences, we performed a one-way ANOVA (group). *Post-hoc* analysis revealed that the control-apoE4 group presented an enrichment of *Akkermansia, Luteolibacter* and *Rubritalea* compared to the other groups, whereas the CPF-treated apoE4 mice presented an enrichment of *Helicobacter*. These results suggest that *APOE4* carriers differ from the other genotypes, being the *APOE4* genotype the most susceptible to the effects of the pesticide on gut microbiota composition.

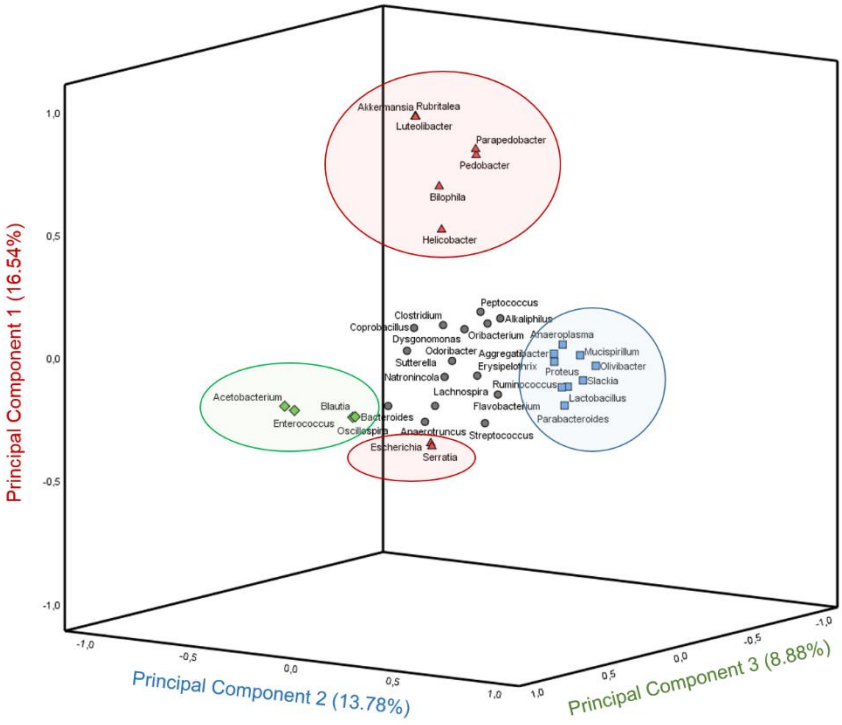


Figure 3| Principal component analysis (PCA) with the relative abundance of the most abundant genera (i.e. subjects with a >1% relative abundance). PC1 included *Akkermansia*, *Escherichia*, *Helicobacter*, *Pedobacter*, *Parapedobacter*, *Serratia*, *Luteolibacter*, *Rubritalea* and *Bilophila*. PC2 included *Parabacteroides*, *Lactobacillus*, *Aggregatibacter*, *Ruminococcus*, *Olivibacter*, *Anaeroplasma*, *Slackia*, *Mucispirillum* and *Proteus*. PC3 included *Blautia*, *Bacteroides*, *Oscispirilla*, *Enterococcus* and *Acetobacterium*.

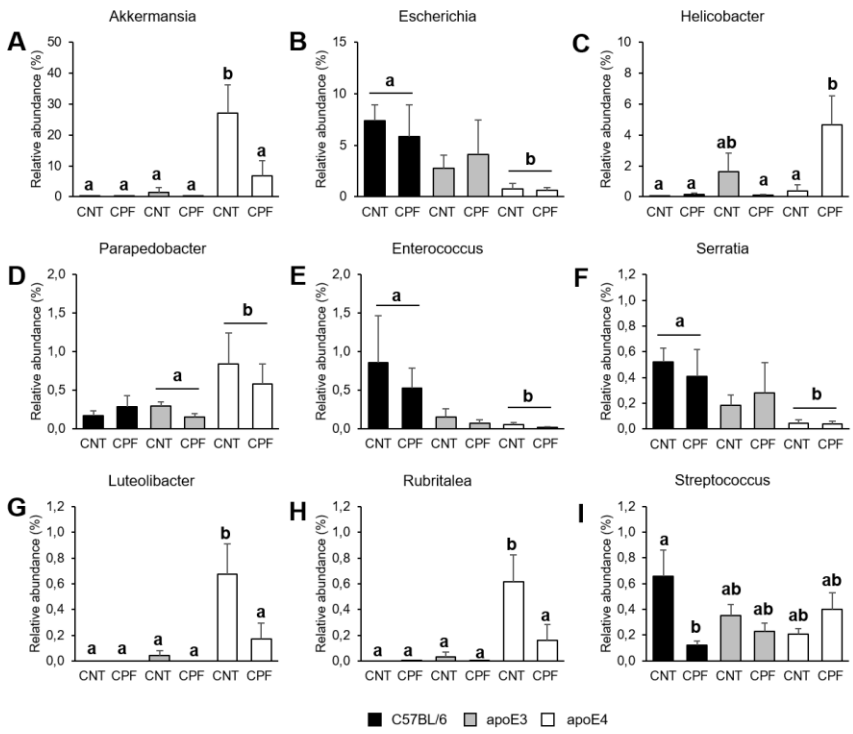


Figure 4| Relative abundance of the most abundant genera (i.e. subjects with a >1% relative abundance) showing significant differences between groups. (A) *Akkermansia*, (B) *Escherichia*, (C) *Helicobacter*, (D) *Parapedobacter*, (E) *Enterococcus,* (F) *Serratia,* (G) *Luteolibacter*, (H) *Rubritalea* and (I) *Streptococcus.* Different letters represent significant differences at $p<0.05$.

3.1.3. The APOE4 genotype differs from the other genotypes in the species Akkermansia muciniphila.

In order to gain further insight into the effects already observed, we studied the species in those genera showing significant differences. Those whose relative abundance was significantly affected by the *APOE* genotype and CPF exposure are shown in Supplementary Table 2. The results point to the importance of *Akkermansia Muciniphila.* A two-way ANOVA (genotype x treatment) showed a significant effect of the genotype [$F(2,30)=9.748$, $p=0.001$], the treatment

[F(1,30)=4.240, p=0.048] and the interaction between genotype and treatment [F(2,30)=3.425, p=0.046]. Post-hoc analysis revealed that the apoE4 control group was significantly different from the other groups, accounting for more than 24-fold the amount found in the C57BL/6 control group. ApoE4 was again the group showing the highest levels, while postnatal CPF exposure considerably diminished the presence of this species. Interestingly, a similar effect was also observed at a considerably lower percentage in different species belonging to the same *Verrucomicrobia* phylum (i.e. *Luteolibacter algae, Prosthecobacter fluviatilis* and *Coraliomargarita akajimensis*).

3.2. The APOE3 genotype and CPF treatment increased the levels of certain SCFAs.

We studied the concentrations of different SCFAs in the brain (Fig. 5). Differences between genotypes were found in three of the most abundant SCFAs: acetic acid [F(2,30)=11.069, p<0.001] (Fig. 5A), propionic acid [F(2,30)=3.399, p=0.047] (Fig. 5B) and butyric acid [F(2,30)=33.692, p<0.001] (Fig. 5C). The latter also presented an interaction between genotype and treatment [F(2,30)=4.044, p=0.028]. In all cases, the apoE3-TR mice showed higher concentrations compared to the other groups. Differences between treatments (Fig. 6) were observed in isovaleric acid [F(1,30)=1.204, p=0.044] (Fig. 6A) and 4-methylvaleric acid [F(1,30)=4.432, p=0.044] (Fig. 6B). In both cases, animals in the CPF-treated group presented higher concentrations than those in the control group. The remaining SCFAs studied (isobutyric acid, 3-methylvaleric acid, valeric acid and hexanoic acid) did not show significant differences between groups.

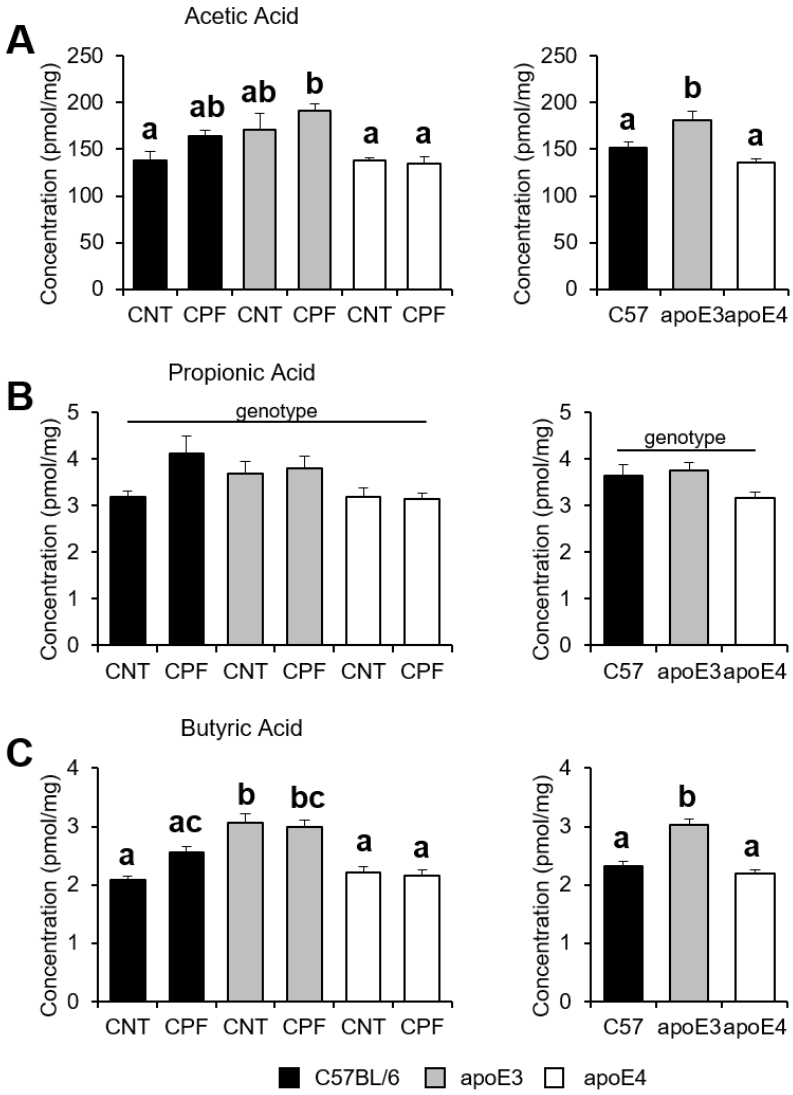


Figure 5| Brain concentrations of SCFAs presenting significant differences between genotypes. (A) Acetic acid, (B) Propionic acid and (C) Butyric acid. Different letters represent significant differences at $p<0.05$.

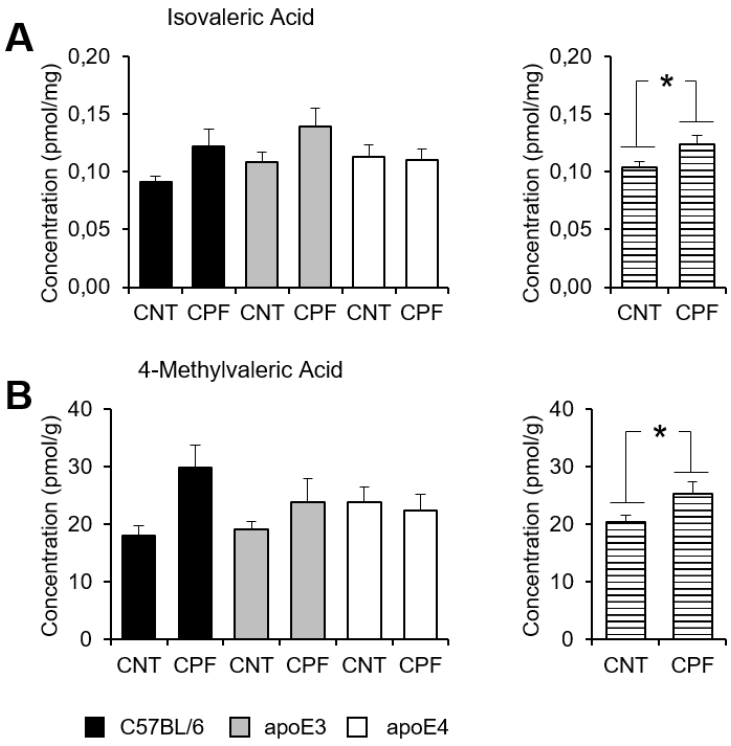


Figure 6| Brain concentrations of SCFAs presenting significant effects of the postnatal treatment. (A) Isovaleric acid and (B) 4-Methylvaleric acid. Different letters represent significant differences at $p<0.05$.

3.3. Certain SCFAs presented mild correlations with the relative abundance of several intestinal species.

In order to identify any correlations between the relative abundance of the microbiota species of interest and SCFA concentrations in the brain, we performed a Pearson's correlation (Table 1). Mild correlations were noticed between isobutyric acid and the relative abundance of *Coraliomargarita akajimensis, Selenomonas infelix* and *Candidatus Scalindua brodae.* Furthermore, a relative abundance of *Methylobacillus glycogenes* positively correlated with

the concentration of valeric acid, 3-methylvaleric acid and hexanoic acid, suggesting that SCFA production in the gut might influence the brain levels.

Table 1. Correlation between gut microbiota species and SCFA composition

Specie	SCFA	Correlation	p-value
Coraliomargarita akajimensis	IBA	r =0.532	p=0.001
Selenomonas infelix	IBA	r =0.554	p<0.001
Candidatus Scalindua brodae	IBA	r =0.544	p<0.001
Methylobacillus glycogenes	VA	r =0.523	p=0.001
Methylobacillus glycogenes	3-MVA	r =0.810	p<0.001
Methylobacillus glycogenes	HA	r =0.560	p<0.001

Abbreviations: IBA, isobutyric acid; VA, valeric acid; 3-MVA, 3-methylvaleric acid; HA, hexanoic acid.

4. - Discussion

The aim of the present study was to assess the effects of the *APOE* genotype and postnatal exposure to the pesticide CPF on gut microbiota composition and SCFA concentrations in the brain. The relative abundance of the microbiota at PND 15, four hours after exposure to CPF, was studied at different taxonomic levels: phylum, genus and species. The results revealed not only changes induced by the pesticide but also important changes depending on the host's *APOE* genetic background – and the interaction between the two – implying that the microbiota can be modulated by both the host's genetics and environmental factors. SCFA concentrations in the brain also proved to be susceptible to the effects of the genotype and the treatment, presenting a mild correlation with the changes observed in microbiota composition. Infant microbiota is influenced by factors such as the delivery process, the maternal environment and milk composition (Borre et al., 2014). Differences between lactation and adulthood periods have been reported (Bäckhed et al., 2015; Palmer et al., 2015), but little is known about milk composition at early stages and its influence on brain development.

In the present study, microbiota composition differed between genotypes at different taxonomic levels, with the *APOE4* genotype being the one diverging from the others in the vast majority of cases. Since no differences in diversity as assessed using the Shannon index were observed, it can be stated that apoE4 differed from the other genotypes in the proportion of some microorganisms. A recent study including both human and apoE3- and apoE4-TR mice at two different ages during adulthood also found that the *APOE* genotype influenced the relative abundance of microbiota (Tran et al., 2019). Specifically, they observed differences in the most abundant phyla: apoE4-TR mice presented a higher amount of *Deferribacteres* and a lower relative abundance of *Candidatus Saccharibacteria* and *Proteobacteria* compared to apoE3-TR mice (Tran et al., 2019). In the current study, no differences in the same phyla were found, but we did observe a higher relative abundance of *Verrucomicrobia*, *Nitrospirae* and *Planctomycetes* in apoE4-TR mice in comparison with the rest of genotypes. However, we found genotype differences in genera belonging to the phylum *Proteobacteria*: *Helicobacter*, *Escherichia*, *Enterobacter* and *Serratia*, among others. Both studies used the same animal model, but determinations in the previous study (Tran et al., 2019) used fecal samples and adult mice, whereas the determinations in our study were performed on total gut content and lactating mice. Microbiota composition can change during the postnatal period (Pantoja-Feliciano et al., 2013), which makes it difficult to compare results from different developmental stages. Tran et al. (2019) also found differences between genotypes in human samples, since E2/E3 presented higher amounts of *Firmicutes* than E3/E4 and E4/E4, which were found neither in adult mice (Tran et al., 2019) nor in our lactating mice. However, it must be taken into account that the data in human studies may be compromised by diet variations, infections or antibiotic use, as well as environmental factors. In the present case, given that the determinations were conducted during the lactation period, we cannot rule out that any differences in the nutritional composition of maternal milk or differences in the nutrient conversion between genotypes may be contributing to the observed differences. In fact, breast milk plays an important role in establishing a healthy gut microbiota (Walker and Iyengar, 2015), and

differences in its composition could enhance the proliferation of different microorganisms. We have recently found different maturation patterns for apoE3- and apoE4-TR mice (Basaure et al., 2018). Since the presence of *Akkermansia muciniphila* increases with age (Collado et al., 2007; Derrien et al., 2008), an earlier maturation of apoE4 could also be underlying its higher relative abundance compared to the other genotypes.

In recent years, the impact of CPF on the gut microbiota has been thoroughly explored with all studies agreeing on the potential effect of CPF to induce dysbiosis. In the present investigation we assessed the effects of postnatal exposure to CPF, in contrast to most studies, which have mainly focused only on adult exposure. Nevertheless, Condette et al. (2015) studied in male rats the effects of a gestational and developmental exposure to CPF until 60 days of age. It was noticed that CPF exposure during this period induced dysbiosis, and lower levels of microorganisms related to healthy phenotypes (i.e. *Lactobacillus spp*) and higher levels of microorganisms related to unhealthy phenotypes (i.e. *Enterococcus* spp, *Clostridium* spp and *Staphylococcus* spp) at PND 21 and PND 60 were found in different parts of the intestine (Condette et al., 2015). Furthermore, they also found detrimental effects on the normal development of the intestine (Condette et al., 2015) and compromised integrity of the epithelial barrier function in rats (Condette et al., 2014). Exposure during adulthood has also revealed detrimental effects in gut permeability and alterations in the relative abundance of key microorganisms, with a decrease in the relative abundance in the phylum *Firmicutes,* and an increase in *Bacteroidetes* in the CPF-exposed mice (Zhao et al., 2016). Despite the fact that we did not observe any differences in those phyla, we found some changes in the same direction after CPF exposure. More specifically, exposure to CPF decreased the relative abundance of *Streptococcus* in C57BL/6 mice but increased *Rhodothermus* in apoE4-TR mice. It is worth noting that CPF can have a direct effect on the relative abundance of some microorganisms. Overall, these results corroborate the

disturbing effects of CPF on microbiota composition and identify new microorganisms affected by the genotype and CPF at very early ages.

The three most abundant phyla (*Bacteroidetes, Firmicutes* and *Proteobacteria*) presented no differences between groups, but differences were found in Verrucomicrobia and in some of the less abundant phyla. Interestingly, when we further studied the genus and species belonging to the *Verrucomicrobia* phylum, we observed the same pattern. The species *Akkermansia muciniphila, Luteolibacter algae, Phrosthecobacter fluvialis*, and *Coraliomargarita akajimensis* all presented the same differences between groups, i.e. the control apoE4 presented a significantly higher abundance than the other groups. This implies a basal difference between genotypes, but also a significant effect of the treatment only in the *APOE4* genotype, as postnatal exposure to CPF decreased the relative abundance of this group of microorganisms. In fact CPF exposure would decrease the existing variability between genotypes, making *APOE4* more comparable to the other genotypes. Of special relevance is the *A. muciniphila* species, a mucin-degrading bacterium (Derrien et al., 2004), which is present from early life in infants (Collado et al., 2007), and it is positively correlated with a healthier metabolic status (Dao et al., 2016) and negatively correlated with obesity and diabetes (Everard et al., 2013). Several studies suggest *A. muciniphila* as a new treatment for obesity and diabetes (Cani and de Vos, 2017; Zhang et al., 2019). Indeed, it has been demonstrated that administration of viable A. muciniphila would ameliorate the metabolic profile, improve the gut barrier function and reduce inflammation, and reverse diet-induced metabolic disorders in mice (Everard et al., 2013). Differences between the metabolic profiles of *APOE* genotypes have been observed before (Huebbe et al., 2015). The *APOE3* genotype has been associated with greater vulnerability to the development of obesity, while *APOE4* has normally been associated with a leaner phenotype (Huebbe et al., 2015). We hypothesize that this different vulnerability to obesity and diabetes of apoE3- and apoE4-TR mice may in part

be influenced by the presence of *A. muciniphila* since early life, conditioning the long-term metabolic health of the subject.

In this sense, in a previous study, apoE3-TR male mice presented more signs of insulin resistance, obesity and metabolic diseases after dietary exposure to CPF during adulthood compared to their peers: apoE4-TR and C57BL/6 mice (Peris-Sampedro et al., 2015a, 2015b). It is known that CPF can impact the metabolism and increase the incidence of several metabolic conditions (Acker and Nogueira, 2012; Elsharkawy et al., 2013). Studies including a dietary challenge with a high-fat diet have suggested that CPF may alter microbiota composition and be associated with an obese phenotype in a diet-specific manner (Fang et al., 2018). In fact Liang et al. (2019) hypothesize that these effects may be the final outcome of disruptions in the gut microbiota composition that alter gut permeability and consequently increase the release of LPS to the body, resulting in inflammation. *A. muciniphila* normally resides in the mucus layer of the intestinal epithelium (Ouwerkerk et al., 2016). Thus, we can speculate that alterations in this species could compromise its integrity. It has been reported that low-grade inflammation after CPF exposure enhanced body weight increase in mice, impaired glucose homeostasis and induced insulin resistance (Liang et al., 2019). Indeed, a higher presence of *A. muciniphila* has been observed in children with autism (De Angelis et al., 2013) and in studies with the BTBR inbred mouse model, which presents the full spectrum of symptoms associated with autism spectrum disorder (Needham et al., 2018). Higher levels of *A. muciniphila* have also been detected in Parkinson disease patients (Bedarf et al., 2017), suggesting the potential implication of *A. muciniphila* in altered neurobiological processes.

In the recent years important attention has been paid to the SCFA produced by microorganisms in the gut and its signaling effects. Previous studies suggest an important role of SCFAs in the cerebral function. For instance, SCFAs can carry out an important function in communication between gut and microglia.

Moreover, acetate acts as an important energy source for astrocytes (Deelchand et al., 2009) while butyrate can play a neuroprotective role (Sun et al., 2016). However, physiological levels of SCFAs in the brain are largely unknown, especially during developmental period. The current results reveal that SCFA composition is modulated by the APOE genotype and also by postnatal exposure to CPF in the brain of lactating mice. In the present study, the levels of acetic acid, butyric acid and propionic acid, which are considered to be the three most common SCFAs (Cummings et al., 1987), were influenced by the genotype. Specifically, apoE3 presented higher concentrations than the other genotypes. We found that levels of butyrate in brains of our mice are about five to ten times higher than those previously found in mature mice (Liu et al., 2015; Sun et al., 2016). Since butyrate serve several functions such as being an energy substrate for cells (Donohoe et al., 2011; Maslowski, 2019); signaling via interaction with G-protein-coupled receptors and the inhibition of histone deacetylases stimulating gene expression (Tan et al., 2014), the current results provide new insights to be explored as a source of early variations related to the APOE genotype. We hypothesize that these differences may already be influencing the cerebral function, conferring higher neuroprotection to apoE3 in contrast to apoE4. This would be in accordance with the higher susceptibility of apoE4 to develop cognitive impairments and AD later in life. An important question is, which are the sources of SCFAs in brain? It is known that only a small part of the SCFAs produced would pass through the blood-brain barrier and enter the brain (Frost et al., 2014). Another important source of SCFAs is the diet being dairy products an important source for butyrate (Stilling et al., 2016). It is also worth considering that SCFAs in the form of acetate, butyrate and propionate can be derived from endogenous sources such as fatty acid oxidation and glucose metabolism in a lower level (Bourassa et al., 2016; Pouteau et al., 2003; Wolever et al., 1997). In the present case, it must be taken into account that the composition of mother's milk, as the only source of diet in lactating mice, can be an important factor contributing to the differences found. Albeit speculative, if differences between maternal milk composition exist, early interventions could be planned.

We observed an effect of CPF on the cerebral concentrations of isovaleric acid and 4-methylvaleric acid, increasing the concentration in all genotypes except for apoE4, which remained unaltered. As mentioned above, CPF can alter gut permeability, and therefore it can be hypothesized that it may favor the release of less abundant SCFAs into the blood, which may then reach the brain at higher concentrations. A link between higher levels of isovaleric acid in stool and depression has been reported in human patients (Szczesniak et al., 2016), correlated with higher levels of certain microorganisms previously linked to this condition. This study suggests that isovaleric acid may be inducing this effect through the inhibition of Na^+, K^+-ATPase (Szczesniak et al., 2016), and thus it can be conjectured that increased levels could cause important damage to the developing brain. Zhao et al. (2016) have reported changes in urine metabolic profiles after exposure to CPF, which are strongly correlated with changes in SCFA-producing bacteria. These metabolites, which included SCFAs such as isobutyrate and valerate, showed changes after CPF exposure (Zhao et al., 2016). It is worth noting that we observed a general effect of the treatment in several metabolites in the brain, but apoE4-TR mice remained unaltered. This would suggest that APOE3 carriers are more sensitive to the effects of CPF, while APOE4 carriers would not be affected by the pesticide in this aspect. This finding has important implications because of the Ɛ3 allele is the most conserved in the human population worldwide, reaching a frequency of 73.3% (SD=12.1). In contrast, the Ɛ4 allele is only present in 14.5% (SD=8.5) (Eisenberg et al., 2010), indicating that a large number of people could be susceptible to these toxic-induced changes in their SCFA levels. Insofar as SCFA can influence the regulation of feeding behavior, these differences are along similar lines to the metabolic differences between apoE3 and apoE4, which have been discussed above. Even though the direct relationship between SCFAs in the gut and microbiota composition is incontestable, information on cerebral SCFAs is scarce. It has been previously reported that supplementation with the butyrate-producer specie *Clostridium butyricum* would have a direct effect by increasing butyrate levels in the brain and by ameliorating cognitive impairments in a

mouse model of vascular dementia (Liu et al., 2015). However, in the present case we did not find a strong correlation between the two factors.

The results of this study were obtained 4 h after exposure to low doses of CPF from PND 10 to PND 15. This would raise the question of whether the results obtained represent a permanent effect or only an acute response that could disappear over time. Changes in the gut microbiota due to environmental toxics during this critical development period have shown to create permanent impairments that can condition the whole life of the subject (Torow and Hornef, 2017). However, further investigation is required to assess whether this also happens with CPF, and to study the evolution over time of the differences between genotypes at such an early age. Last but not least, as most of these studies with mouse models, our results are based only on males. However, considering that we recently observed sexual-dimorphic differences and vulnerabilities to toxic exposures (Basaure et al., 2018; Guardia-Escote et al., 2018), we believe that we might be missing important information by leaving the females out of these studies. Consequently, we propose a long-term follow-up study including both sexes, with a strict control of all possible parameters influencing the gut microbiota.

In summary, the current results provide information about the composition of gut microbiota in early life and its modulation by the *APOE* genetic background and postnatal exposure to CPF. We also included an assessment of SCFA levels in the brain and studied the potential relationship between *APOE* and CPF. Basal differences between genotypes were observed, and CPF showed its capacity to induce gut dysbiosis. *A. muciniphila* emerged as a potential modulatory element providing an explanation for previously-observed metabolic and cognitive differences between groups, especially related to *APOE4* genotype. Genetic and environmental effects on SCFA composition were also found, with potential implications for cognitive functioning. Finally, these findings raise a number of

intriguing questions regarding the nature and extent of the involvement of the gut microbiota and its metabolites in different aspects underlying health and disease. At the same time, they show the scope of postnatal exposure to low doses of pesticides such as CPF and corroborate the influence of the *APOE* genotype.

Declaration of Interests

The authors declare no competing interests.

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References

Acker, C.I., Nogueira, C.W., 2012. Chlorpyrifos acute exposure induces hyperglycemia and hyperlipidemia in rats. Chemosphere 89, 602–608. https://doi.org/10.1016/j.chemosphere.2012.05.059

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., Li, Y., Xia, Y., Xie, H., Zhong, H., Khan, M.T., Zhang, J., Li, J., Xiao, L., Al-Aama, J., Zhang, D., Lee, Y.S., Kotowska, D., Colding, C., Tremaroli, V., Yin, Y., Bergman, S., Xu, X., Madsen, L., Kristiansen, K.,

Dahlgren, J., Wang, J., 2015. Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. Cell Host Microbe 17, 690–703. https://doi.org/10.1016/j.chom.2015.04.004

Basaure, P., Guardia-Escote, L., Cabré, M., Peris-Sampedro, F., Sánchez-Santed, F., Domingo, J.L., Colomina, M.T., 2019. Learning, memory and the expression of cholinergic components in mice are modulated by the pesticide chlorpyrifos depending upon age at exposure and apolipoprotein E (APOE) genotype. Arch. Toxicol. 93, 693–707. https://doi.org/10.1007/s00204-019-02387-9

Basaure, P., Guardia-Escote, L., Cabré, M., Peris-Sampedro, F., Sánchez-Santed, F., Domingo, J.L., Colomina, M.T., 2018. Postnatal chlorpyrifos exposure and apolipoprotein E (APOE) genotype differentially affect cholinergic expression and developmental parameters in transgenic mice. Food Chem. Toxicol. 118, 42–52. https://doi.org/10.1016/j.fct.2018.04.065

Bedarf, J.R., Hildebrand, F., Coelho, L.P., Sunagawa, S., Bahram, M., Goeser, F., Bork, P., Wüllner, U., 2017. Functional implications of microbial and viral gut metagenome changes in early stage L-DOPA-naïve Parkinson's disease patients. Genome Med. 9, 1–13. https://doi.org/10.1186/s13073-017-0428-y

Bonder, M.J., Kurilshikov, A., Tigchelaar, E.F., Mujagic, Z., Imhann, F., Vila, A.V., Deelen, P., Vatanen, T., Schirmer, M., Smeekens, S.P., Zhernakova, D. V., Jankipersadsing, S.A., Jaeger, M., Oosting, M., Cenit, M.C., Masclee, A.A.M., Swertz, M.A., Li, Y., Kumar, V., Joosten, L., Harmsen, H., Weersma, R.K., Franke, L., Hofker, M.H., Xavier, R.J., Jonkers, D., Netea, M.G., Wijmenga, C., Fu, J., Zhernakova, A., 2016. The effect of host genetics on the gut microbiome. Nat. Genet. 48, 1407–1412. https://doi.org/10.1038/ng.3663

Borre, Y.E., O'Keeffe, G.W., Clarke, G., Stanton, C., Dinan, T.G., Cryan, J.F., 2014. Microbiota and neurodevelopmental windows: implications for brain disorders. Trends Mol. Med. 20, 509–518. https://doi.org/10.1016/j.molmed.2014.05.002

Bourassa, M.W., Alim, I., Bultman, S.J., Ratan, R.R., 2016. Butyrate, neuroepigenetics and the gut microbiome: Can a high fiber diet improve brain health? Neurosci. Lett. 625, 56–63. https://doi.org/10.1016/j.neulet.2016.02.009

Burke, R.D., Todd, S.W., Lumsden, E., Mullins, R.J., Mamczarz, J., Fawcett, W.P., Gullapalli, R.P., Randall, W.R., Pereira, E.F.R., Albuquerque, E.X., 2017. Developmental neurotoxicity of the organophosphorus insecticide chlorpyrifos: from clinical findings to preclinical

models and potential mechanisms. J. Neurochem. 142, 162–177. https://doi.org/10.1111/jnc.14077

Cani, P.D., de Vos, W.M., 2017. Next-generation beneficial microbes: The case of Akkermansia muciniphila. Front. Microbiol. 8, 1–8. https://doi.org/10.3389/fmicb.2017.01765

Clemente, J.C., Ursell, L.K., Parfrey, L.W., Knight, R., 2012. The impact of the gut microbiota on human health: An integrative view. Cell 148, 1258–1270. https://doi.org/10.1016/j.cell.2012.01.035

Collado, M.C., Derrien, M., Isolauri, E., De Vos, W.M., Salminen, S., 2007. Intestinal integrity and Akkermansia muciniphila, a mucin-degrading member of the intestinal microbiota present in infants, adults, and the elderly. Appl. Environ. Microbiol. 73, 7767–7770. https://doi.org/10.1128/AEM.01477-07

Condette, C.J., Bach, V., Mayeur, C., Gay-Quéheillard, J., Khorsi-Cauet, H., 2015. Chlorpyrifos Exposure during Perinatal Period Affects Intestinal Microbiota Associated with Delay of Maturation of Digestive Tract in Rats. J. Pediatr. Gastroenterol. Nutr. 61, 30–40. https://doi.org/10.1097/MPG.0000000000000734

Condette, C.J., Khorsi-Cauet, H., Morlière, P., Zabijak, L., Reygner, J., Bach, V., Gay-Quéheillard, J., 2014. Increased gut permeability and bacterial translocation after chronic chlorpyrifos exposure in rats. PLoS One 9, 1–10. https://doi.org/10.1371/journal.pone.0102217

Cox, L.M., Yamanishi, S., Sohn, J., Alekseyenko, A. V., Leung, J.M., Cho, I., Kim, S.G., Li, H., Gao, Z., Mahana, D., Zárate Rodriguez, J.G., Rogers, A.B., Robine, N., Loke, P., Blaser, M.J., 2014. Altering the Intestinal Microbiota during a Critical Developmental Window Has Lasting Metabolic Consequences. Cell 158, 705–721. https://doi.org/10.1016/j.cell.2014.05.052

Cummings, J.H., Pomare, E.W., Branch, H.W.J., Naylor, C.P.E., MacFarlane, G.T., 1987. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut 28, 1221–1227. https://doi.org/10.1136/gut.28.10.1221

Dalile, B., Van Oudenhove, L., Vervliet, B., Verbeke, K., 2019. The role of short-chain fatty acids in microbiota–gut–brain communication. Nat. Rev. Gastroenterol. Hepatol. 16, 461–478. https://doi.org/10.1038/s41575-019-0157-3

Dao, M.C., Everard, A., Aron-Wisnewsky, J., Sokolovska, N., Prifti, E., Verger, E.O., Kayser, B.D., Levenez, F., Chilloux, J., Hoyles, L., Dumas, M.-E., Rizkalla, S.W., Doré, J., Cani, P.D., Clément, K., 2016. Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology. Gut 65, 426–436. https://doi.org/10.1136/gutjnl-2014-308778

De Angelis, M., Piccolo, M., Vannini, L., Siragusa, S., De Giacomo, A., Serrazzanetti, D.I., Cristofori, F., Guerzoni, M.E., Gobbetti, M., Francavilla, R., 2013. Fecal Microbiota and Metabolome of Children with Autism and Pervasive Developmental Disorder Not Otherwise Specified. PLoS One 8, e76993. https://doi.org/10.1371/journal.pone.0076993

De Vadder, F., Kovatcheva-Datchary, P., Goncalves, D., Vinera, J., Zitoun, C., Duchampt, A., Bäckhed, F., Mithieux, G., 2014. Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. Cell 156, 84–96. https://doi.org/10.1016/j.cell.2013.12.016

Deelchand, D.K., Shestov, A.A., Koski, D.M., Uğurbil, K., Henry, P.G., 2009. Acetate transport and utilization in the rat brain. J. Neurochem. 109, 46–54. https://doi.org/10.1111/j.1471-4159.2009.05895.x

Derrien, M., Collado, M.C., Ben-Amor, K., Salminen, S., De Vos, W.M., 2008. The mucin degrader Akkermansia muciniphila is an abundant resident of the human intestinal tract. Appl. Environ. Microbiol. 74, 1646–1648. https://doi.org/10.1128/AEM.01226-07

Derrien, M., Vaughan, E.E., Plugge, C.M., de Vos, W.M., 2004. Akkermansia municiphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. Int. J. Syst. Evol. Microbiol. 54, 1469–1476. https://doi.org/10.1099/ijs.0.02873-0

Donohoe, D.R., Garge, N., Zhang, X., Sun, W., O'Connell, T.M., Bunger, M.K., Bultman, S.J., 2011. The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. Cell Metab. 13, 517–526. https://doi.org/10.1016/j.cmet.2011.02.018

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. https://doi.org/10.1002/ajpa.21298

Elsharkawy, E.E., Yahia, D., El-Nisr, N.A., 2013. Sub-chronic exposure to chlorpyrifos induces hematological, metabolic disorders and oxidative stress in rat: Attenuation by

glutathione. Environ. Toxicol. Pharmacol. 35, 218–227. https://doi.org/10.1016/j.etap.2012.12.009

Everard, A., Belzer, C., Geurts, L., Ouwerkerk, J.P., Druart, C., Bindels, L.B., Guiot, Y., Derrien, M., Muccioli, G.G., Delzenne, N.M., de Vos, W.M., Cani, P.D., 2013. Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. Proc. Natl. Acad. Sci. 110, 9066–9071. https://doi.org/10.1073/pnas.1219451110

Fang, B., Li, J.W., Zhang, M., Ren, F.Z., Pang, G.F., 2018. Chronic chlorpyrifos exposure elicits diet-specific effects on metabolism and the gut microbiome in rats. Food Chem. Toxicol. 111, 144–152. https://doi.org/10.1016/j.fct.2017.11.001

Frost, G., Sleeth, M.L., Sahuri-Arisoylu, M., Lizarbe, B., Cerdan, S., Brody, L., Anastasovska, J., Ghourab, S., Hankir, M., Zhang, S., Carling, D., Swann, J.R., Gibson, G., Viardot, A., Morrison, D., Louise Thomas, E., Bell, J.D., 2014. The short-chain fatty acid acetate reduces appetite via a central homeostatic mechanism. Nat. Commun. 5, 3611. https://doi.org/10.1038/ncomms4611

Fujimura, K.E., Lynch, S. V., 2015. Microbiota in Allergy and Asthma and the Emerging Relationship with the Gut Microbiome. Cell Host Microbe 17, 592–602. https://doi.org/10.1016/j.chom.2015.04.007

Gomes, A.C., Hoffmann, C., Mota, J.F., 2018. The human gut microbiota: Metabolism and perspective in obesity. Gut Microbes 9, 308–325. https://doi.org/10.1080/19490976.2018.1465157

Goodrich, J.K., Waters, J.L., Poole, A.C., Sutter, J.L., Koren, O., Blekhman, R., Beaumont, M., Van Treuren, W., Knight, R., Bell, J.T., Spector, T.D., Clark, A.G., Ley, R.E., 2014. Human Genetics Shape the Gut Microbiome. Cell 159, 789–799. https://doi.org/10.1016/j.cell.2014.09.053

Guardia-Escote, L., Basaure, P., Blanco, J., Cabré, M., Pérez-Fernández, C., Sánchez-Santed, F., Domingo, J.L., Colomina, M.T., 2018. 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. Food Chem. Toxicol. 122, 1–10. https://doi.org/10.1016/j.fct.2018.09.069

Guilloteau, P., Martin, L., Eeckhaut, V., Ducatelle, R., Zabielski, R., Van Immerseel, F., 2010. From the gut to the peripheral tissues: the multiple effects of butyrate. Nutr. Res. Rev. 23, 366–384. https://doi.org/10.1017/s0954422410000247

Hawrelak, J.A., Myers, S.P., 2004. The causes of intestinal dysbiosis: a review. Altern. Med. Rev. 9, 180–97.

Hersi, M., Irvine, B., Gupta, P., Gomes, J., Birkett, N., Krewski, D., 2017. Risk factors associated with the onset and progression of Alzheimer's disease: A systematic review of the evidence. Neurotoxicology 61, 143–187. https://doi.org/10.1016/j.neuro.2017.03.006

Huebbe, P., Dose, J., Schloesser, A., Campbell, G., Glüer, C.-C., Gupta, Y., Ibrahim, S., Minihane, A.-M., Baines, J.F., Nebel, A., Rimbach, G., 2015. Apolipoprotein E (APOE) genotype regulates body weight and fatty acid utilization-Studies in gene-targeted replacement mice. Mol. Nutr. Food Res. 59, 334–343. https://doi.org/10.1002/mnfr.201400636

Joly, C., Gay-Quéheillard, J., Léké, A., Chardon, K., Delanaud, S., Bach, V., Khorsi-Cauet, H., 2013. Impact of chronic exposure to low doses of chlorpyrifos on the intestinal microbiota in the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) and in the rat. Environ. Sci. Pollut. Res. 20, 2726–2734. https://doi.org/10.1007/s11356-012-1283-4

Joyce, S.A., Gahan, C.G.M., 2014. The gut microbiota and the metabolic health of the host. Curr. Opin. Gastroenterol. 30, 120–127. https://doi.org/10.1097/MOG.0000000000000039

Kamada, N., Seo, S.U., Chen, G.Y., Núñez, G., 2013. Role of the gut microbiota in immunity and inflammatory disease. Nat. Rev. Immunol. 13, 321–335. https://doi.org/10.1038/nri3430

Katsimichas, T., Antonopoulos, A.S., Katsimichas, A., Ohtani, T., Sakata, Y., Tousoulis, D., 2019. The intestinal microbiota and cardiovascular disease. Cardiovasc. Res. 62, 85. https://doi.org/10.1093/cvr/cvz135

Kim, B.-R., Shin, J., Guevarra, R.B., Lee, J.H., Kim, D.W., Seol, K.-H., Lee, J.-H., Kim, H.B., Isaacson, R.E., 2017. Deciphering Diversity Indices for a Better Understanding of Microbial Communities. J. Microbiol. Biotechnol. 27, 2089–2093. https://doi.org/10.4014/jmb.1709.09027

Lange, K., Buerger, M., Stallmach, A., Bruns, T., 2016. Effects of Antibiotics on Gut Microbiota. Dig. Dis. 34, 260–268. https://doi.org/10.1159/000443360

Lee, C.J., Sears, C.L., Maruthur, N., 2019. Gut microbiome and its role in obesity and insulin resistance. Ann. N. Y. Acad. Sci. 1–16. https://doi.org/10.1111/nyas.14107

Liang, Y., Zhan, J., Liu, D., Luo, M., Han, J., Liu, X., Liu, C., Cheng, Z., Zhou, Z., Wang, P., 2019. Organophosphorus pesticide chlorpyrifos intake promotes obesity and insulin resistance through impacting gut and gut microbiota. Microbiome 7, 19. https://doi.org/10.1186/s40168-019-0635-4

Liu, J., Sun, J., Wang, F., Yu, X., Ling, Z., Li, H., Zhang, H., Jin, J., Chen, W., Pang, M., Yu, J., He, Y., Xu, J., 2015. Neuroprotective Effects of Clostridium butyricum against Vascular Dementia in Mice via Metabolic Butyrate. Biomed Res. Int. 2015. https://doi.org/10.1155/2015/412946

Makki, K., Deehan, E.C., Walter, J., Bäckhed, F., 2018. The Impact of Dietary Fiber on Gut Microbiota in Host Health and Disease. Cell Host Microbe 23, 705–715. https://doi.org/10.1016/j.chom.2018.05.012

Marizzoni, M., Provasi, S., Cattaneo, A., Frisoni, G.B., 2017. Microbiota and neurodegenerative diseases. Curr. Opin. Neurol. 30, 630–638. https://doi.org/10.1097/WCO.0000000000000496

Maslowski, K.M., 2019. Metabolism at the centre of the host–microbe relationship. Clin. Exp. Immunol. cei.13329. https://doi.org/10.1111/cei.13329

Morrison, D.J., Preston, T., 2016. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes 7, 189–200. https://doi.org/10.1080/19490976.2015.1134082

Moser, V., 1998. Age- and Gender-Related Differences in Sensitivity to Chlorpyrifos in the Rat Reflect Developmental Profiles of Esterase Activities. Toxicol. Sci. 46, 211–222. https://doi.org/10.1006/toxs.1998.2526

Needham, B.D., Tang, W., Wu, W.L., 2018. Searching for the gut microbial contributing factors to social behavior in rodent models of autism spectrum disorder. Dev. Neurobiol. 78, 474–499. https://doi.org/10.1002/dneu.22581

Ouwerkerk, J.P., van der Ark, K.C.H., Davids, M., Claassens, N.J., Finestra, T.R., de Vos, W.M., Belzer, C., 2016. Adaptation of Akkermansia muciniphila to the Oxic-Anoxic Interface of

the Mucus Layer. Appl. Environ. Microbiol. 82, 6983–6993. https://doi.org/10.1128/aem.01641-16

Palmer, C., Bik, E.M., DiGiulio, D.B., Relman, D.A., Brown, P.O., 2007. Development of the human infant intestinal microbiota. PLoS Biol. 5, 1556–1573. https://doi.org/10.1371/journal.pbio.0050177

Palmer, J., Albrecht, J., Park, Y., Dutcher, S., Rattinger, G., Simoni-Wastila, L., Walker, L., Zuckerman, I., 2015. Use of Drugs with Anticholinergic Properties among Nursing Home Residents with Demencia: A national analysis of Medicare Beneficiaries 2007-2008. Drugs Aging 32, 79–86. https://doi.org/10.1016/j.jocn.2013.06.015.

Pantoja-Feliciano, I.G., Clemente, J.C., Costello, E.K., Perez, M.E., Blaser, M.J., Knight, R., Dominguez-Bello, M.G., 2013. Biphasic assembly of the murine intestinal microbiota during early development. ISME J. 7, 1112–1115. https://doi.org/10.1038/ismej.2013.15

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. https://doi.org/10.1016/j.physbeh.2015.03.006

Peris-Sampedro, F., Blanco, J., Cabré, M., Basaure, P., Guardia-Escote, L., Domingo, J.L., Sánchez, D.J., Colomina, M.T., 2018. New mechanistic insights on the metabolic-disruptor role of chlorpyrifos in apoE mice: a focus on insulin- and leptin-signalling pathways. Arch. Toxicol. 92, 1717–1728. https://doi.org/10.1007/s00204-018-2174-3

Peris-Sampedro, F., Cabré, M., Basaure, P., Reverte, I., Domingo, J.L., Colomina, M.T., 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. https://doi.org/10.1016/j.envres.2015.06.036

Pouteau, E., Nguyen, P., Ballevre, O., Krempf, M., 2003. Production rates and metabolism of short-chain fatty acids in the colon and whole body using stable isotopes. Proc. Nutr. Soc. 62, 87–93. https://doi.org/10.1079/PNS2003208

Reiss, R., Chang, E.T., Richardson, R.J., Goodman, M., 2015. A review of epidemiologic studies of low-level exposures to organophosphorus insecticides in non-occupational populations. Crit. Rev. Toxicol. 45, 531–641. https://doi.org/10.3109/10408444.2015.1043976

Réquilé, M., Gonzàlez Alvarez, D.O., Delanaud, S., Rhazi, L., Bach, V., Depeint, F., Khorsi-Cauet, H., 2018. Use of a combination of in vitro models to investigate the impact of chlorpyrifos and inulin on the intestinal microbiota and the permeability of the intestinal mucosa. Environ. Sci. Pollut. Res. 25, 22529–22540. https://doi.org/10.1007/s11356-018-2332-4

Reygner, J., Condette, C.J., Bruneau, A., Delanaud, S., Rhazi, L., Depeint, F., Abdennebi-Najar, L., Bach, V., Mayeur, C., Khorsi-Cauet, H., 2016. Changes in composition and function of human intestinal microbiota exposed to chlorpyrifos in oil as assessed by the SHIME® model. Int. J. Environ. Res. Public Health 13. https://doi.org/10.3390/ijerph13111088

Sekirov, I., Russell, S.L., Antunes, L.C.M., Finlay, B.B., 2010. Gut Microbiota in Health and Disease. Physiol. Rev. 90, 859–904. https://doi.org/10.1152/physrev.00045.2009

Sonnenburg, J.L., Bäckhed, F., 2016. Diet–microbiota interactions as moderators of human metabolism. Nature 535, 56–64. https://doi.org/10.1038/nature18846

Stilling, R.M., van de Wouw, M., Clarke, G., Stanton, C., Dinan, T.G., Cryan, J.F., 2016. The neuropharmacology of butyrate: The bread and butter of the microbiota-gut-brain axis? Neurochem. Int. 99, 110–132. https://doi.org/10.1016/j.neuint.2016.06.011

Sullivan, P.M., Mezdour, H., Aratanti, Y., Knouff, C., Najib, J., Reddick, R.L., Quarfordt, S.H., Maeda, N., 1997. Targeted Replacement of the Mouse Apolipoprotein E Gene with the Common Human APOE3 Allele Enhances Diet-induced Hypercholesterolemia and Atherosclerosis. J. Biol. Chem. 272, 17972–17980. https://doi.org/10.1074/jbc.272.29.17972

Sun, J., Ling, Z., Wang, F., Chen, W., Li, H., Jin, J., Zhang, H., Pang, M., Yu, J., Liu, J., 2016. Clostridium butyricum pretreatment attenuates cerebral ischemia/reperfusion injury in mice via anti-oxidation and anti-apoptosis. Neurosci. Lett. 613, 30–35. https://doi.org/10.1016/j.neulet.2015.12.047

Szczesniak, O., Hestad, K.A., Hanssen, J.F., Rudi, K., 2016. Isovaleric acid in stool correlates with human depression. Nutr. Neurosci. 19, 279–283. https://doi.org/10.1179/1476830515Y.0000000007

Tan, J., McKenzie, C., Potamitis, M., Thorburn, A.N., Mackay, C.R., Macia, L., 2014. The Role of Short-Chain Fatty Acids in Health and Disease, 1st ed, Advances in Immunology. Elsevier Inc. https://doi.org/10.1016/B978-0-12-800100-4.00003-9

Torow, N., Hornef, M.W., 2017. The Neonatal Window of Opportunity: Setting the Stage for Life-Long Host-Microbial Interaction and Immune Homeostasis. J. Immunol. 198, 557–563. https://doi.org/10.4049/jimmunol.1601253

Tran, T.T.T., Corsini, S., Kellingray, L., Hegarty, C., Le Gall, G., Narbad, A., Müller, M., Tejera, N., O'Toole, P.W., Minihane, A.-M., Vauzour, D., 2019. APOE genotype influences the gut microbiome structure and function in humans and mice: relevance for Alzheimer's disease pathophysiology. FASEB J. 33, 8221–8231. https://doi.org/10.1096/fj.201900071R

Turnbaugh, P.J., Bäckhed, F., Fulton, L., Gordon, J.I., 2008. Marked alterations in the distal gut microbiome linked to diet- induced obesity. Cell Host Microbe 3, 213–223. https://doi.org/10.1016/j.chom.2008.02.015

Turpin, W., Espin-Garcia, O., Xu, W., Silverberg, M.S., Kevans, D., Smith, M.I., Guttman, D.S., Griffiths, A., Panaccione, R., Otley, A., Xu, L., Shestopaloff, K., Moreno-Hagelsieb, G., Paterson, A.D., Croitoru, K., 2016. Association of host genome with intestinal microbial composition in a large healthy cohort. Nat. Genet. 48, 1413–1417. https://doi.org/10.1038/ng.3693

Vuong, H.E., Yano, J.M., Fung, T.C., Hsiao, E.Y., 2017. The Microbiome and Host Behavior. Annu. Rev. Neurosci. 40, 21–49. https://doi.org/10.1146/annurev-neuro-072116-031347

Walker, W.A., Iyengar, R.S., 2015. Breast milk, microbiota, and intestinal immune homeostasis. Pediatr. Res. 77, 220–228. https://doi.org/10.1038/pr.2014.160

Wolever, T.M.S., Josse, R.G., Leiter, L.A., Chiasson, J.L., 1997. Time of day and glucose tolerance status affect serum short-chain fatty acid concentrations in humans. Metabolism. 46, 805–811. https://doi.org/10.1016/S0026-0495(97)90127-X

Young, V.B., Schmidt, T.M., 2009. Overview of the Gastrointestinal Microbiota. Adv Exp Med Biol. 635, 29–40. https://doi.org/10.1007/978-0-387-09550-9_3

Yuan, X., Pan, Z., Jin, C., Ni, Y., Fu, Z., Jin, Y., 2019. Gut microbiota: an underestimated and unintended recipient for pesticide-induced toxicity. Chemosphere 227, 425–434. https://doi.org/10.1016/j.chemosphere.2019.04.088

Zhang, T., Li, Q., Cheng, L., Buch, H., Zhang, F., 2019. Akkermansia muciniphila is a promising probiotic. Microb. Biotechnol. https://doi.org/10.1111/1751-7915.13410

Zhao, Y., Zhang, Y., Wang, G., Han, R., Xie, X., 2016. Effects of chlorpyrifos on the gut microbiome and urine metabolome in mouse (Mus musculus). Chemosphere 153, 287–293. https://doi.org/10.1016/j.chemosphere.2016.03.055

Supplementary tables:

Table S1. Significant differences between groups at genus level.

Genus	Genotype	Treatment	Interaction	Group differences
Akkermansia	p=0.001	p=0.048	p=0.046	E4-CNT vs. all
Escherichia	p=0.024	n.s.	n.s.	n.s.
Helicobacter	p=0.039	n.s.	p=0.012	E4-CPF vs. all (except E3-CNT)
Parapedobacter	p=0.035	n.s.	n.s.	n.s.
Streptococcus	n.s.	n.s.	p=0.011	C57-CNT vs. C57-CPF
Enterococcus	p=0.045	n.s.	n.s.	n.s.
Serratia	p=0.019	n.s.	n.s.	n.s.
Luteolibacter	p=0.001	p=0.051	p=0.051	E4-CNT vs. all
Rubritalea	p=0.001	n.s.	n.s.	E4-CNT vs. all
Rhodothermus	n.s.	n.s.	p=0.036	n.s.
Prevotella	p=0.024	n.s.	n.s.	n.s.
Prosthecobacter	p=0.000	n.s.	n.s.	E4-CNT vs. all
Enterobacter	p=0.034	n.s.	n.s.	n.s.
Butyricimonas	p=0.032	n.s.	n.s.	n.s.
Gallibacterium	p=0.023	n.s.	n.s.	n.s.
Sharpea	p=0.021	n.s.	n.s.	n.s.
Lysobacter	p=0.001	n.s.	n.s.	E4-CNT vs. all (except E4-CPF)
Thermo-desulfovibrio	p=0.003	p=0.011	p=0.050	E4-CNT vs. all
Thermus	n.s.	p=0.025	p=0.039	30 vs 31, 50 vs 51
Psychrobacter	p=0.011	n.s.	n.s.	E4-CPF vs. C57-CPF
Mycoplasma	n.s.	n.s.	p=0.032	n.s.
Polaribacter	p=0.024	n.s.	n.s.	E4-CNT vs. E3-CPF and C57-CPF
C. Glomeribacter	n.s.	n.s.	p=0.036	n.s.
Rhodospirillum	n.s.	p=0.002	n.s.	n.s.

Campylobacter	p=0.020	n.s.	p=0.004	E4-CPF vs. all (except E3-CNT)
Thermicanus	n.s.	n.s.	p=0.027	n.s.
Caldicellulo-siruptor	p=0.001	n.s.	n.s.	E4-CNT vs. all (except E4-CPF)
C. Methyl-acidiphilum	p=0.002	n.s.	n.s.	E4-CNT vs. all (except E4-CPF)
Steno-trophomonas	p=0.043	n.s.	0.026	n.s.
Thiobacillus	p=0.027	n.s.	n.s.	n.s.
Vibrio	p=0.009	n.s.	n.s.	n.s.
Chelonobacter	p=0.041	n.s.	n.s.	n.s.
Vagococcus	p=0.006	n.s.	n.s.	n.s.
Coraliomargarita	p=0.002	n.s.	n.s.	E4-CNT vs. all (except E4-CPF)
Desulfuri-spirillum	n.s.	p=0.011	n.s.	n.s.
Thiocapsa	p=0.035	p=0.042	n.s.	n.s.
Thiorhodococcus	p=0.016	n.s.	n.s.	n.s.
Cystobacter	p=0.003	n.s.	n.s.	E4-CNT vs. all (except E4-CPF)
Avibacterium	n.s.	n.s.	p=0.021	n.s.
Selenomonas	p=0.012	n.s.	n.s.	n.s.
Leptospira	p=0.042	n.s.	n.s.	n.s.
C. Scalindua	p=0.000	p=0.013	p=0.003	E4-CNT vs. all
Carboxydocella	p=0.010	n.s.	n.s.	E3-CNT vs. C57-CNT
Thiohalorhabdus	n.s.	n.s.	p=0.050	n.s.
Chondromyces	0.023	n.s.	n.s.	E4-CNT vs. E3-CNT, E3-CPF and C57-CNT
Fervido-bacterium	n.s.	p=0.049	p=0.022	E4-CNT vs. E4-CPF
Trabulsiella	p=0.038	n.s.	n.s.	n.s.
Roseospira	p=0.049	n.s.	n.s.	n.s.

Glycomyces	n.s.	n.s.	0.051	n.s.
Thiomonas	p=0.027	n.s.	n.s.	n.s.
Melissococcus	p=0.017	n.s.	n.s.	n.s.
Methylobacillus	p=0.009	n.s.	n.s.	E3-CPF vs. E4-CPF and C57-CPF
Kitasatospora	n.s.	p=0.021	n.s.	n.s.
Glaciecola	n.s.	n.s.	p=0.020	n.s.
Klebsiella	n.s.	p=0.045	n.s.	n.s.
Thermoanaerobacterium	n.s.	p=0.049	n.s.	n.s.
Leptolyngbya	p=0.050	n.s.	p=0.050	C57-CPF vs. all
Petrotoga	n.s.	p=0.018	n.s.	n.s.
Thioalkalivibrio	n.s.	n.s.	p=0.035	C50-CNT vs. all (except E4-CPF)
Oscillatoria	p=0.014	n.s.	n.s.	n.s.

Table S2. Significant differences between groups at species level.

Specie	Genotype	Treatment	Interaction	Group differences
Verrucomicrobia				
Akkermansia muciniphila	p=0.000	p=0.047	p=0.044	E4-CNT vs. all
Luteolibacter algae	p=0.001	p=0.049	p=0.049	E4-CNT vs. all
Prosthecobacter fluviatilis	p=0.001	n.s.	n.s.	E4-CNT vs. all (except E4-CPF)
Coraliomargarita akajimensis	p=0.003	n.s.	n.s.	E4-CNT vs. all (except E4-CPF)
Proteobacteria				
Helicobacter ganmani	p=0.022	n.s.	p=0.010	E4-CPF vs. all (except E3-CNT)

Helicobacter mesocricetorum	*p*=0.041	n.s.	*p*=0.017	E4-CPF vs. all (except E3-CNT)
Helicobacter suncus	*p*=0.033	n.s.	*p*=0.002	E4-CPF vs. all (except E3-CNT)
Helicobacter mastomyrinus	n.s.	n.s.	p=0.023	n.s.
Helicobacter rodentium	*p*=0.040	n.s.	*p*=0.041	E4-CPF vs. all (except E4-CNT and E3-CNT)
Escherichia albertii	*p*=0.044	n.s.	n.s.	n.s.
Escherichia coli	*p*=0.000	*p*=0.010	*p*=0.002	C57-CNT vs. all
Serratia entomophila	*p*=0.019	n.s.	n.s.	n.s.
Enterobacter aceae	*p*=0.039	n.s.	n.s.	n.s.
Enterobacter nickellidurans	*p*=0.003	n.s.	n.s.	n.s.
Gallibacterium melopsittaci	*p*=0.019	n.s.	n.s.	n.s.
Campylobacter faecalis	*p*=0.002	n.s.	*p*=0.001	41 vs all
Vibrio litoralis	*p*=0.048	n.s.	n.s.	n.s.
Chelonobacter oris	*p*=0.038	n.s.	n.s.	n.s.
Thiorhodococcus pfennigii	*p*=0.031	n.s.	n.s.	n.s.
Thiohalorhabdus denitrificans	n.s.	n.s.	*p*=0.035	n.s.
Chondromyces pediculatus	*p*=0.028	n.s.	n.s.	E4-CNT vs. all (except E4-CPF and C57-CPF)
Trabulsiella odontotermitis	*p*=0.038	n.s.	n.s.	n.s.

Thiomonas thermosulfata	p=0.020	n.s.	n.s.	n.s.
Methylobacillus glycogenes	p=0.033	n.s.	n.s.	n.s.
Glaciecola nitratireducens	n.s.	n.s.	p=0.028	n.s.
Klebsiella granulomatis	n.s.	p=0.038	n.s.	n.s.
Thioalkalivibrio jannaschii	n.s.	n.s.	p=0.033	C57-CNT vs. all (except E4-CPF)
Bacteroidetes				
Parapedobacter koreensis	p=0.045	n.s.	n.s.	n.s.
Butyricimonas virosa	p=0.042	n.s.	n.s.	n.s.
Firmicutes				
Streptococcus milleri	n.s.	n.s.	p=0.006	C57-CNT vs. C57-CPF
Streptococcus lactarius	n.s.	n.s.	p=0.015	n.s.
Enterococcus faecalis	p=0.037	n.s.	n.s.	n.s.
Enterococcus lactis	p=0.032	n.s.	n.s.	n.s.
Sharpea azabuensis	p=0.027	n.s.	n.s.	n.s.
Vagococcus teuberi	p=0.012	n.s.	n.s.	n.s.
Selenomonas infelix	p=0.030	n.s.	n.s.	n.s.
Carboxydocella ferrireducens	p=0.011	n.s.	n.s.	E4-CNT vs. C57-CNT
Thermoanaerobacterium islandicum	n.s.	p=0.044	n.s.	n.s.

Chrysiogenetes				
Desulfurispirillum alkaliphilum	n.s.	p=0.018	n.s.	n.s.
Planctomycetes				
Candidatus Scalindua brodae	p=0.000	p=0.012	p=0.003	E4-CNT vs. all
Cyanobacteria				
Oscillatoria corallinae	p=0.021	n.s.	n.s.	n.s.
Thermotogae				
Fervidobacterium islandicum	n.s.	p=0.049	p=0.019	E4-CPF vs. all (except C57-CNT and C57-CPF)
Thermi				
Thermus rehai	n.s.	n.s.	p=0.002	C57-CNT vs. C57-CPF
Thermus scotoductus	n.s.	p=0.015	n.s.	n.s.

UNIVERSITAT ROVIRA I VIRGILI
POSTNATAL CHLORPYRIFOS EXPOSURE INFLUENCES THE GUT MICROBIOTA AND THE EXPRESSION OF BIOLOGICAL AND NEUROBEHAVIORAL CHARACTERISTICS OF THE APOE GENOTYPE IN AN AGE-DEPENDENT MANNER
Laia Guardia Escoté

3.2. Publication II

APOE genetic background and sex confer different vulnerabilities to postnatal chlorpyrifos exposure and modulate the response to cholinergic drugs

Laia Guardia-Escote, Pia Basaure, Fiona Peris-Sampedro, Judit Biosca-Brull, Maria Cabré, Fernando Sánchez-Santed, José L. Domingo, Maria Teresa Colomina.

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Study II overview.	
What do we already know?	*APOE* influenced learning and memory, which can be disrupted by exposure to the pesticide CPF. The cholinergic system plays an important role in learning and memory. Differences depending on the *APOE* genotype have been reported.
What does this study add?	Differences in recognition memory between *APOE* genotypes confer different vulnerabilities to the toxic effects of CPF in a sex-dependent manner.
Highlights	*APOE3* mice did not acquire the task. CPF-induced detrimental effects during task retention, especially on apoE4-TR mice. ApoE4-TR mice presented lower sensitivities to scopolamine and responded differently to rivastigmine depending on sex.

*No edited version of the online article

UNIVERSITAT ROVIRA I VIRGILI
POSTNATAL CHLORPYRIFOS EXPOSURE INFLUENCES THE GUT MICROBIOTA AND THE EXPRESSION OF BIOLOGICAL AND NEUROBEHAVIORAL CHARACTERISTICS OF THE APOE GENOTYPE IN AN AGE-DEPENDENT MANNER
Laia Guardia Escoté

APOE genetic background and sex confer different vulnerabilities to postnatal chlorpyrifos exposure and modulate the response to cholinergic drugs

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Abstract.

Chlorpyrifos (CPF) is an extensively used organophosphate pesticide. Exposure to CPF has been related to neurobehavioral disorders, particularly during neurodevelopment. Apolipoprotein E (apoE) is a lipid and cholesterol carrier and a susceptibility factor for cognitive impairment which can influence the response to toxic exposures. The study was aimed at assessing the effects of postnatal exposure to CPF on object recognition memory and its modulation by sex and apoE genotype. Human apoE3 and apoE4 targeted replacement mice and C57BL/6 mice were postnatally exposed to 0 or 1 mg/kg/day of CPF. Recognition memory was evaluated in an Object Recognition Test (ORT). In order to study the contribution of cholinergic and GABAergic neurotransmitter systems to recognition memory, a pharmacological challenge was included. Sex,

genotype and postnatal exposure to CPF were key factors throughout the testing period. Specifically, CPF increased exploratory behavior and impaired discrimination performance. We observed that administering scopolamine, a cholinergic antagonist, was detrimental to recognition memory. However, discrimination in C57BL/6 and apoE4 males improved with the administration of the cholinergic agonist rivastigmine, but the same drug worsened retention in apoE4 females. Finally, the GABAergic agonist alprazolam altered performance in a sex- and genotype-dependent manner. Overall, these results suggest complex interactions between sex, *APOE* genotype and postnatal CPF exposure and indicate a different functioning of both the cholinergic and GABAergic neurotransmitter system between groups.

Keywords:

Chlorpyrifos, Pesticide, APOE, Cholinergic system, Recognition memory, Brain development

1. Introduction

The impact of environmental toxic exposure during brain development has enormous consequences for the general population. As far as subclinical behavioral or cognitive deficits caused by toxic exposure are elusive in diagnoses, most of the neurotoxic agents affecting the brain lack specific environmental regulations. This is the case of the worldwide used pesticide chlorpyrifos (CPF), which targets the cholinergic neurotransmitter system. Despite the fact that its residential use was banned in 2001, CPF is still one of the most widespread compounds. The general population is permanently exposed to nontoxic low levels of the pesticide through the diet (Reiss et al., 2015), being known that young subjects are more sensitive than adults to CPF toxicity (Moser and Padilla, 1998; Pope et al., 1991). The main detrimental

effects of CPF are caused by the irreversible inhibition of cholinesterase enzymes (ChE) such as acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) (Casida and Quistad, 2004). To date, a number of studies have reported changes in the cholinergic system after exposure to CPF. For instance, Slotkin et al. (2004) found a decreased binding in both α7 and α4β2 nicotinic receptors (nAChRs) in different brain regions of rats including the forebrain and cerebellum, following postnatal (postnatal days (PND) 11-14) exposure to the pesticide. In the same line, Richardson and Chambers (2005) observed a considerable dose-dependent decrease in muscarinic receptors (mAChRs) binding in the brain after postnatal (PND 1-21) exposure to CPF. The observed effects, which cover a wide spectrum, seem to be dependent on the sex and the period of exposure. For example, postnatal exposure to CPF has been related to increased aggressive behavior in adult males and an enhanced maternal response in females, while anxiety-like behaviors have been reduced in both sexes (Ricceri et al., 2006). Likewise, postnatal exposure to CPF is known to induce changes in locomotor activity (Dam et al., 2000; Ricceri et al., 2003), as well as thyroid alterations (De Angelis et al., 2009).

To date, a number of studies have focused on the gene of apolipoprotein E (apoE) as another potential factor underlying cholinergic alterations. The apoE protein is implicated in lipid and cholesterol transport in the brain (Getz and Reardon, 2009). Three different isoforms have been described in humans: apoE2, apoE3 and apoE4. E4 is the isoform related to diminished functioning of the cholinergic system (Allen et al., 1997), being the major genetic risk factor for the development of Alzheimer's Disease (AD) (Bales, 2010; Roses, 1996). Previous results from our group, using the targeted replacement (TR) mouse model that expresses the different human isoforms of apoE (apoE-TR), showed a wide variety of functional differences between genotypes as well as different vulnerabilities to the detrimental effects of CPF exposure. There are differences between genotypes, for example, in terms of spatial learning and memory (Reverte et al., 2012), attention, impulsivity and compulsivity (Peris-Sampedro

et al., 2016; Reverte et al., 2016), and these influence the effects of CPF during adulthood (Peris-Sampedro et al., 2015) or throughout the developmental period (Basaure et al., 2019). Likewise, differences in the gene expression of cholinergic elements were observed between genotypes after postnatal exposure to the pesticide, together with developmental alterations (Basaure et al., 2018).

Behavioral testing has played a crucial role in evaluating the effects of toxic exposures and in unraveling their interactions with environmental factors. The Object Recognition Test (ORT) is a behavioral task used to study recognition memory, which is based on the innate preference of rodents for novelty (i.e., to explore a new object over a familiar one). This innate novelty preference is ultimately used to test recognition memory because mice preferring a novel object would need to successfully recognize a familiar one (Ennaceur and Delacour, 1988). In mice, the brain structures likely to be implicated in object recognition memory are the hippocampus and rhinal cortices. More specifically, the perirhinal cortex is a crucial structure in the acquisition, consolidation and retrieval of information (Broadbent et al., 2009; Brown et al., 2012; Melichercik et al., 2012). The use of ORT has increased in recent years because of advantages over other well-established tasks: it does not require water or food deprivation, reinforcing stimuli or the learning of associations, being suitable for pharmacological screening (Dere et al., 2007; Grayson et al., 2015). The integrity of the cholinergic system appeared to be determinant for correct recognition memory. The use of the cholinergic antagonist scopolamine has become a widely established pharmacological model for cognitive impairments on object recognition (Dodart et al., 1997; Klinkenberg and Blokland, 2010). On the other hand, the use of nAChR agonists and AChE inhibitors (McLean et al., 2016; Pichat et al., 2007) has been associated with an improvement in the performance of the task. The GABAergic system has also been reported to play a role in recognition memory as the administration of γ-Aminobutyric acid (GABA) induced long-term improvements in recognition memory in rats (Thanapreedawat et al.,

2013). Amnesic effects and subsequent impairments have also been described in recognition memory after administration of an allosteric modulator of GABAA receptor such as alprazolam (Bertaina-Anglade et al., 2006; Soni and Parle, 2017).

The purpose of this investigation was to assess the effects of postnatal exposure to CPF on recognition memory and to investigate how individual intrinsic variables including sex and APOE genotype influence its toxicity. We also aimed to shed light on the neuropharmacological basis of these effects. To explore the role of both cholinergic and GABAergic systems on recognition memory and their interactions with the *APOE* genotype and CPF, a pharmacological challenge with agonist and antagonist drugs was used.

2. Materials and Methods

2.1. Animals and care

In the present study, we used male and female apoE3- and apoE4-TR mice from Taconic (Taconic Europe, Lille Skensved, Denmark) and C57BL/6 mice from Charles River (Charles River, Barcelona, Spain). The apoE-TR mouse model was originally created by Sullivan et al. (1997). These mice have a C57BL/6NTac background and their murine *apoE* gene was replaced by the human allele *APOE*. Females and males from the same genotype were subjected to mating sessions up to pregnancy. The day at birth was recorded as PND 0 and litters were left undisturbed until treatment. After weaning, animals were housed under standard conditions with 2-5 individuals per cage of the same genotype, sex and treatment group. Behavioral testing was conducted when mice were two months old. The animal room was maintained at a temperature of 22 ± 2 °C, a relative humidity of 50 ± 10%, and a 12 h light/dark automatic light cycle (light on 8:00-20:00). All animals were allowed free access to food and water and given 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. Chemicals and treatment

CPF [0,0-diethyl O-(3,5,6-trichloropyridin-2-yl) phosphorothioate], purity 99.5%, was provided by Sigma-Aldrich Co. LLC. (Madrid, Spain). The compound was dissolved in corn oil and adjusted to administer 1 mg/kg in 1 µL/g of body weight by oral gavage, using a micropipette. The treatment was conducted from PND10 to PND15. Control groups were administered corn oil throughout the same period of time. Animals were periodically monitored and kept under standard conditions for two months prior to starting the behavioral assessment.

To study the influence of GABAergic and cholinergic systems on the behavioral task performance, the following drugs were used: Rivastigmine L-Tartrate (CAS Number: 129101-54-8, TCI Europe N.V., Zwijndrecht, Belgium), scopolamine hydrobromide (CAS Number: 6533-68-2, Sigma-Aldrich, Madrid, Spain) and alprazolam (Trankimazin 0.75mg/ml, CAS Number: 28981-97-7, Pfizer, S.A., Alcobendas, Spain).

2.3. Object Recognition Test

Recognition memory was tested in an ORT, a one-trial learning paradigm based on the natural tendency of mice to prefer a new object over a familiar one. The experimental protocol was designed based on the original setup first described by Ennaceur and Delacour (1988). The apparatus consisted of a square open field box (60 cm x 60 cm x 50 cm). During the pre-training period, mice were

habituated to the testing area for 30 min. During habituation, the total distance traveled and the time spent in the center were measured to test general motor activity and anxiety-like behavior. A typical ORT session consisted of two parts: the acquisition and the retention phase. During acquisition, two identical objects were placed in the open field. Each individual was allowed to freely explore the objects for 15 min. The mouse was considered to be exploring an object when: (a) the head of the mouse was directed towards the object at a distance of 2 cm or less from it, or (b) when the animal was sniffing it. During acquisition, we studied the time spent exploring the two identical objects. The retention part was carried out 4 h after the acquisition session. During the retention session, one of the previous two objects was replaced with a new one, and each animal was allowed to explore the new setting for 5 min. A discrimination index (time spent exploring the new object/time spent exploring the familiar object) was used to test retention memory. All objects were white, being of similar sizes, but with perfectly distinguishable shapes. All objects and the open field box were carefully cleaned with 70% ethanol after each trial to reduce olfactory cues. All objects used were previously tested in another set of animals in order to discard any object preference bias (data not shown). Experiments were automatically recorded and analyzed using a video-tracking software (EthoVision®, Noldus Information Technologies, the Netherlands).

2.3.1. Inclusion criteria

A total of 146 animals were used in this study. As far as retention could be biased by exploration activity, we set an inclusion criteria (at least 2 seconds exploring one object and at least 10 seconds of total time exploring the objects) to gain access to the retention phase. Therefore, the number of animals in each group during the different parts was the following: habituation n=11-13, acquisition n=11-13 and retention n=8-13 (Table 1).

Table 1 | Number of animals in each experimental group

Group		HAB	ACQ	Retention			
				Sal	***Sc***	***Riv***	***Apz***
Males	CNT C57	12	12	10	10	11	9
	CPF C57	12	11	10	10	11	11
	CNT E3	11	12	10	8	10	11
	CPF E3	12	12	9	9	10	11
	CNT E4	12	12	11	9	11	11
	CPF E4	13	13	12	8	9	11
Females	CNT C57	12	12	11	9	10	10
	CPF C57	13	13	13	12	13	13
	CNT E3	12	12	12	10	12	12
	CPF E3	12	12	11	9	10	12
	CNT E4	12	12	12	9	12	12
	CPF E4	12	12	11	10	11	12

Abbreviations: CNT, Control; CPF, CPF-treated; C57, C57BL/6; HAB, habituation; ACQ, acquisition; Sal, saline; Sc, scopolamine; Riv, rivastigmine; Apz, alprazolam.

2.3.2. Basal condition and pharmacological challenge

Mice were habituated to the injection daily over a period of three days before the experiment using 0.9% saline. Animals in the basal conditions received intraperitoneal infusions of 0.9% saline 30 min. before the acquisition session started. During the pharmacological challenge, animals received the drugs in different sessions: the cholinergic agonist rivastigmine (0.25 mg/kg), the cholinergic antagonist scopolamine (1.6 mg/kg) and the GABAergic agonist alprazolam (0.12 mg/kg). Saline and the drugs were administered in a Latin square design 30 min. (scopolamine), 40 min. (rivastigmine) and 2 h (alprazolam) before starting the acquisition session. The dose selection was based on previous studies (Reverte et al. 2016) and the time of administration was chosen according to each compound's average time to reach peak plasma concentration. Provided that we administered the drug before the acquisition part, we expected it to affect the encoding of the object information during acquisition. All drugs were intraperitoneally injected except rivastigmine, which was administered subcutaneously. Individual mice were subjected to each

treatment on different days, separated by at least 48 h. To this end, a large set of objects was used in order to avoid repetition and bias.

2.4. Study of the estrous cycle

The stage of the reproductive cycle was assessed in females right after the retention part of each session in order to test possible influences on behavioral performance. The stage of the cycle was assessed on the basis of the type of cells observed in the vaginal smears as described elsewhere (Caliglioni, 2009). In short, nucleated epithelial cells indicate the proestrus stage, while cornified squamous epithelial cells indicate the estrous stage. A mix of cell types is characteristic of the metestrus, while leukocytes are characteristic of the diestrus stage. The four stages were divided into two groups: proestrus/estrus and metestrus/diestrus, as described in previous studies (Contreras et al., 2000; van Goethem et al., 2012).

2.5. Statistical analysis

Data were analyzed with the SPSS 25.0 software (IBM Corp, Chicago, USA). A three-way analysis of variance (ANOVA) for repeated measures was used to study the general activity in the open field, while a three-way ANOVA for each drug condition was used in both the acquisition and retention parts. A *t*-test for independent samples was used to asess the specific contribution of the postnatal treatment and the effects of the different drugs in each genotype and sex. A one-sample *t*-test allowed to compare the recognition memory with a neutral chance level during retention. 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. General activity during habituation

General activity during habituation was analyzed by a three-way ANOVA (sex x genotype x treatment) for repeated measures. The 5-minute fractions of time were the within-subject factor and the distance traveled as well as the time spent in the center of the open field were studied as the dependent variables. A decrease over time in the total distance traveled [$F(5,129) = 69.268$, $p<0.001$] indicated habituation to the space (Fig. 1A and 1B). An interaction between time and genotype [$F(10,260) = 3.107$, $p=0.001$] indicated that the habituation pattern depended on the *APOE* genotype. A three-way ANOVA (sex x genotype x treatment) showed the general effects of sex [$F(1,133) = 11.569$, $p=0.001$] and genotype [$F(2,133) = 3.208$, $p=0.044$], as well as an interaction between genotype and treatment [$F(2,133) = 5.461$, $p=0.005$] on total distance traveled. While females traveled greater distances than males (Fig. 1C), the postnatal CPF treatment only affected apoE4 mice, which traveled a greater total distance than the respective controls (Fig. 1D). On the other hand, the time spent in the center of the open field, as a measure of anxiety-like behavior, was similar between groups (data not shown).

3.2. Acquisition

Exploration performance during the acquisition session was analyzed by a three-way ANOVA (sex x genotype x treatment) for basal (saline) and pharmacological conditions. Treatment effects were further analyzed within each sex and genotype group by a t-test for independent samples. A t-test for independent samples was also used to compare the mice's exploratory behavior between each drug and the basal conditions

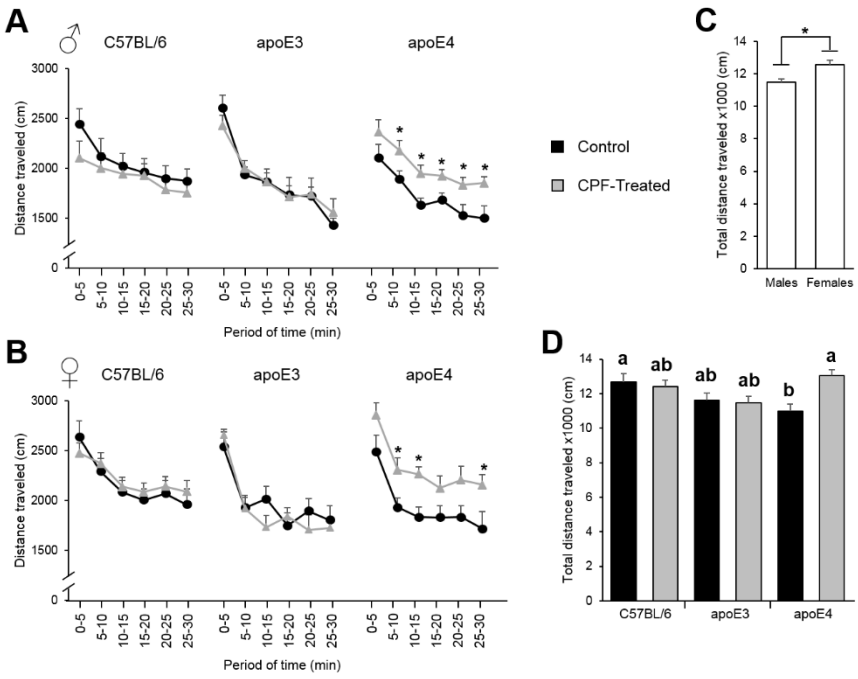


Figure 1| Distance traveled divided into fractions of 5 min. in an open field during the 30 min. of habituation to the working space of the ORT in: males (A) and females (B). Total distance traveled by sex (C) and by genotype and treatment (D). An asterisk indicates that performance is significantly different at $p<0.05$ from the respective control group. Groups with different letters (a,b) are significantly different ($p<0.05$).

3.2.1. Basal condition

The cumulative time exploring the two identical objects in basal conditions was influenced by genotype [$F(2,132) = 4.072$, $p=0.019$] and postnatal CPF treatment [$F(1,132) = 10.619$, $p=0.001$] (Fig. 2A and 2B). We found a triple interaction between sex, genotype and treatment [$F(2,531) = 6.484$, $p=0.002$]. Therefore, we split the groups up by genotype and sex to study the postnatal treatment effects. The results showed that the time exploring the objects in CPF-treated apoE4 females was significantly higher than their respective control group [$t = 3.716$; d.f.23; $p=0.001$] (Fig. 3B).

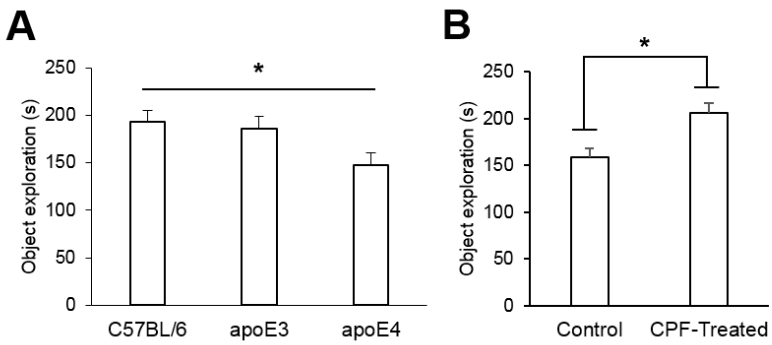


Figure 2| Cumulative time exploring two identical objects during the acquisition phase of the ORT. General effect of genotype (A) and treatment (B) in basal conditions after saline administration. An asterisk indicates that performance is significantly different at $p<0.05$.

3.2.2. Pharmacological challenge

Exploratory activity during acquisition upon different pharmacological drugs, showed that the cholinergic antagonist scopolamine affected exploratory behavior, as the general effects of genotype and postnatal treatment observed in the basal conditions were no longer detected. Specifically C57BL/6 exposed mice and apoE3 either exposed or control significantly decreased their activity (Fig. 3C and 3D) compared to the basal conditions (Fig. 3A and 3B) while control C57BL/6 and apoE4 mice were not affected by scopolamine. Rivastigmine treatment (Fig. 3E and 3F) and alprazolam treatment (Fig. 3G and 3H) did not change basal conditions. A significant effect of the genotype [$F(2,133) = 3.588$, $p=0.030$] was still observed under the influence of rivastigmine. Similarly, postnatal treatment effects observed in basal conditions were also evident under the influence of rivastigmine [$F(1,133) = 12.178$, $p=0.001$] and of alprazolam [$F(1,133) = 9.437$, $p=0.003$]. In fact, apoE4 females postnatally exposed to CPF mantained high exploration levels on both rivastigmine [$t = 5.014$; d.f.24; $p<0.001$] and alprazolam [$t = 2.365$; d.f.24; $p=0.026$] conditions (Fig. 3F and 3H, respectively).

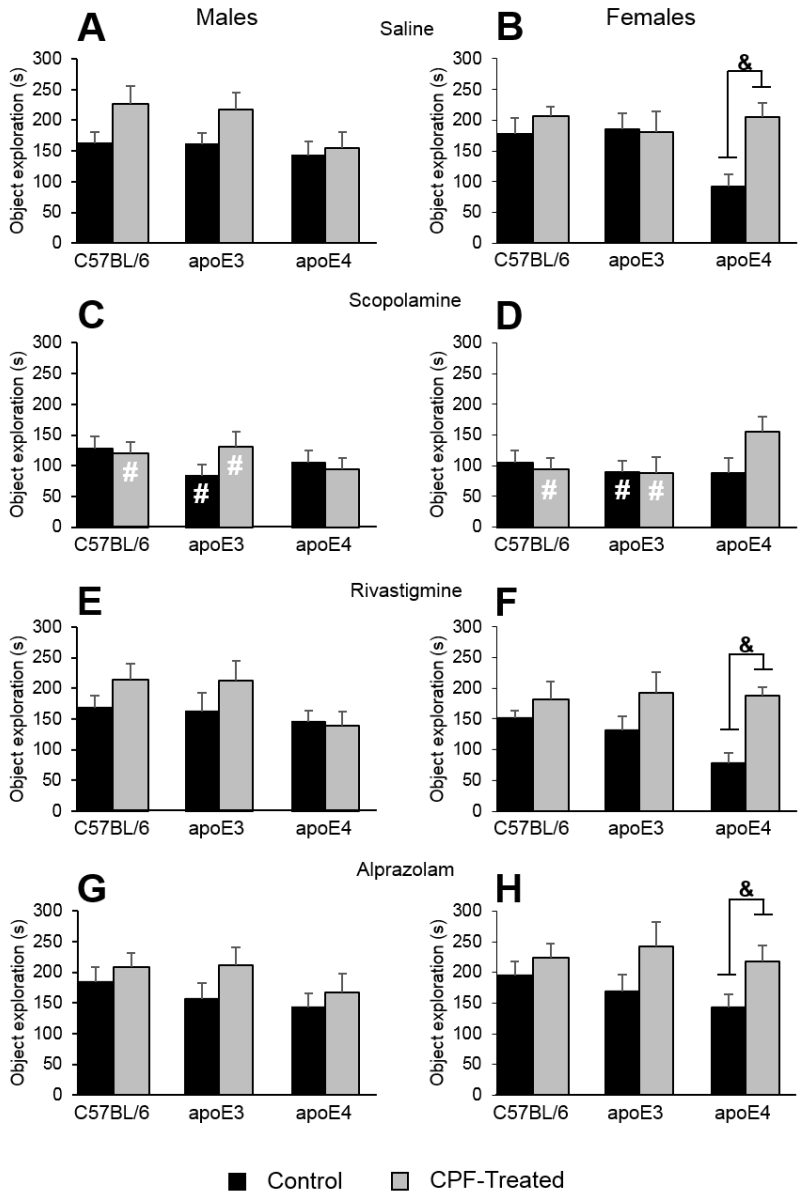


Figure 3| Time exploring two identical objects during the acquisition phase of the ORT after the administration of saline (A,B), scopolamine (C,D), rivastigmine (E,F) and alprazolam (G,H). The symbol (&) indicates that performance is significantly different to the respective control at $p<0.05$. The symbol (#) indicates significant differences with respect to the saline group ($p<0.05$).

3.3. Retention

The discrimination ratio between familiar and novel objects during the retention session was analyzed by a three-way ANOVA (sex x genotype x treatment). We also used a one-sample *t*-test to analyze the discrimination ratio in comparison to the chance level (a theoretical value that assumes that mice spend the same time exploring familiar and novel objects). Finally, to establish the specific contribution of each drug, we compared the results of the different drugs in basal conditions using a *t*-test for independent samples.

3.3.1. Basal condition

The discrimination ratio in basal conditions was influenced by sex [$F(1,120) = 7.752$, $p=0.006$] and postnatal treatment [$F(1,120) = 6.328$, $p=0.013$]. Female mice performed better than males (Fig. 4A), with postnatal CPF exposure impairing retention (Fig. 4B). When the discrimination ratio was compared to chance level, control apoE4 males [$t = 2.860$; d.f.10; $p=0.017$], control apoE4 females [$t = 4.958$; d.f.11; $p<0.001$], and control [$t = 2.948$; d.f.10; $p=0.015$] and CPF-treated [$t = 2.493$; d.f.10; $p=0.032$] C57BL/6 females presented a significant preference for the novel object (Fig. 5A and 5B). It is worth noting that neither apoE3 males and females nor C57BL/6 males displayed any retention of the test in basal conditions.

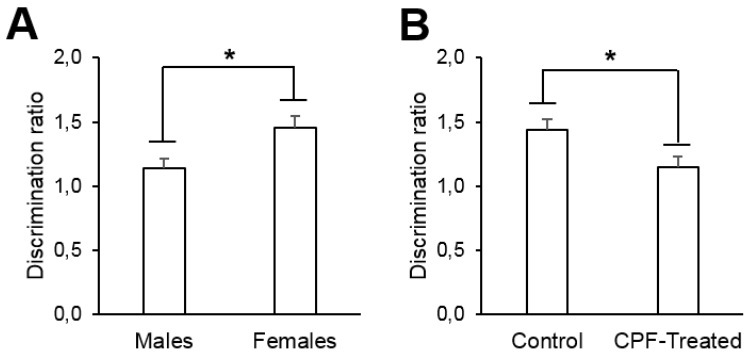


Figure 4| Discrimination ratio (time exploring the novel object/time exploring the familiar object) during the retention session. General effects of sex (A) and postnatal treatment (B) on basal conditions after saline administration. An asterisk indicates that performance is significantly different at $p<0.05$.

3.3.2. Pharmacological challenge

Pharmacological testing showed an overall effect of the genotype [F(2,462) = 8.874, $p<0.001$], postnatal treatment [F(1,462) = 4.695, $p=0.031$] and an interaction between postnatal treatment and sex [F(1,462) = 9.199, $p=0.003$]. To better understand these effects, each drug condition was analyzed for each genotype, sex and treatment (Fig. 5).

None of the groups under the influence of scopolamine showed recognition memory. Although control apoE4 males explored the novel object more than the familiar one, the differences with chance level or basal conditions were not significant. It must be highlighted that compared to basal conditions, female mice showed impairments in those groups that had good retention in the basal condition: C57BL/6 control [t = 2.741; d.f.18; $p=0.013$] and CPF-treated [t = 2.150; d.f.19; $p=0.045$], and apoE4 control mice [t = 2.598; d.f.18; $p=0.018$] (Fig. 5 C and 5D).

A tendency towards an interaction between sex and treatment [$F(1,118) = 3.684$, $p=0.057$] was noted after administration of rivastigmine. This drug improved retention in control C57BL/6 male mice, which displayed a preference for the novel object compared to the chance level [$t = 3.377$; d.f.11; $p=0.006$]. This also occurred in both control [$t = 3.068$; d.f.10; $p=0.012$] and CPF-treated [$t = 2.310$; d.f.9; $p=0.046$] apoE4 males (Fig. 5E). Among the female groups, only the control C57BL/6 [$t = 3.596$; d.f.10; $p=0.006$] showed a preference for the novel object. In addition, control apoE4 females showed a decrease in the discrimination ratio after rivastigmine administration [$t = 2.536$; d.f.19; $p=0.020$] compared to basal conditions (Fig. 5F).

Lastly, the administration of alprazolam led to an effect of genotype [$F(2,123) = 5.657$, $p=0.004$] and an interaction between sex and treatment [$F(1,123) = 3.993$, $p=0.048$]. Control apoE4 was the only group with a significant preference for the novel object in both males [$t = 3.682$; d.f.10; $p=0.004$] and females [$t = 2.397$; d.f.10; $p=0.037$] compared with the chance level (Fig. 5G and 5H). No effects of alprazolam were observed in comparison to basal conditions in males (Fig. 5G). In females, discrimination in control apoE4 mice decreased with respect to basal conditions after alprazolam administration [$t = 2.075$; d.f.21; $p=0.015$] (Fig. 5H).

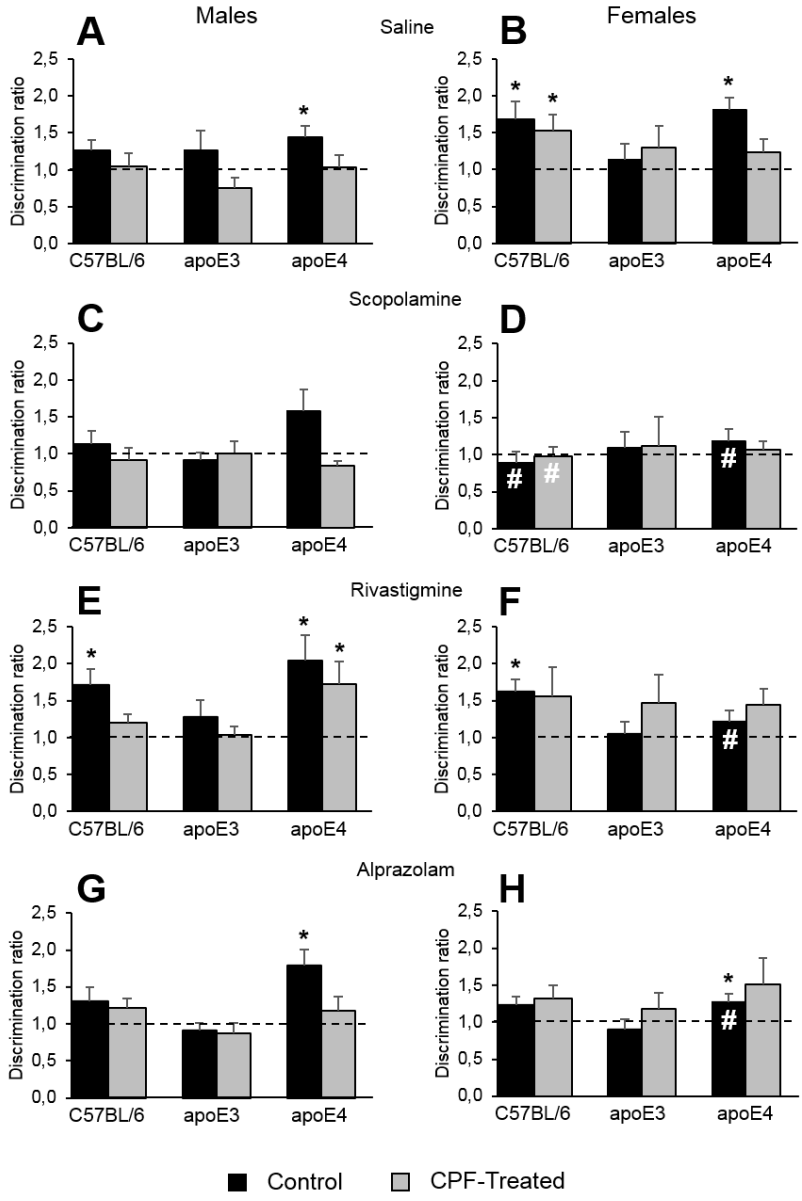
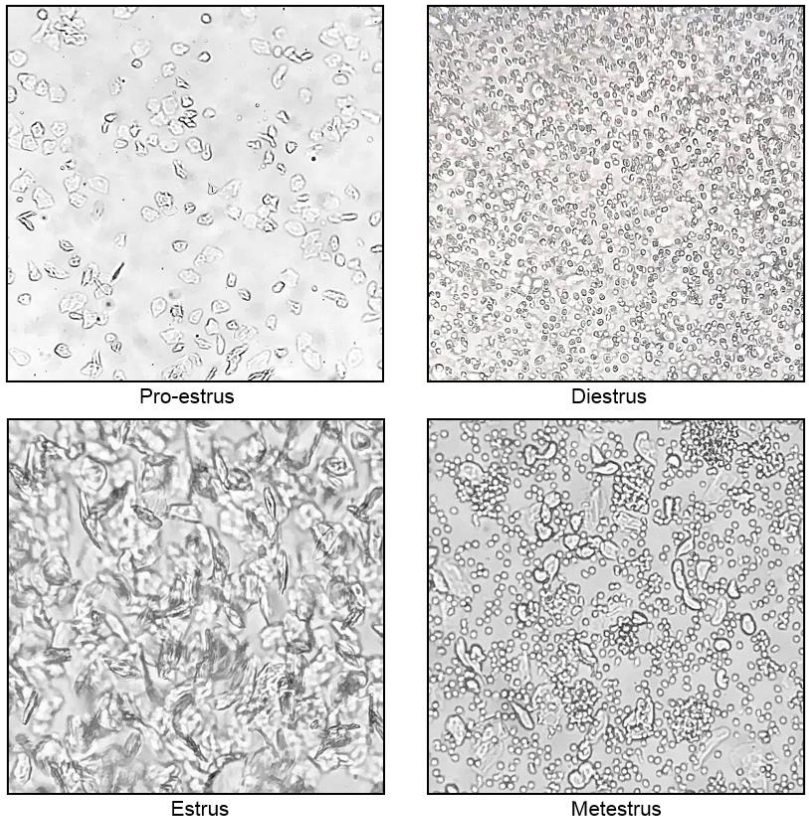


Figure 5| Discrimination ratio during the retention session after the administration of saline (A,B), scopolamine (C,D), rivastigmine (E,F) and alprazolam (G,H). The symbol (#) indicates significant differences with respect to the saline group (p<0.05) and the asterisk (*) indicates significant differences with the chance level (equal time exploring novel and familiar object) at p<0.05.

Taken together the results obtained during the retention sesions show long lasting effects produced by the postnatal treatment with CPF on apoE4 mice, a lack of retention in apoE3 mice, a lack of effects produced by scopolamine and alprazolam in males and the divergent effects produced by rivastigmine depending on *APOE* genotype and sex.

3.4. Influences of the estrous cycle

The influence that the stage of the reproductive cycle has on exploration during the various parts of the task was analyzed by a four-way ANOVA (estrous cycle stage x genotype x treatment x drug). Since an interaction between the drug and the cycle was found [$F(3,243) = 5.102$, $p=0.002$], we studied each drug separately. The stage of the cycle had an effect only in the saline group [$F(1,60) = 10.291$, $p=0.002$]. Female mice in the diestrus/metestrus phase of the cycle explored significantly less than the females in the pro-estrus/estrus (Fig. 6). This effect was no longer observed after the drugs were administered, suggesting that they can mask the differences related to the physiology of the reproductive cycle. Furthermore, this effect was not detected during the retention part of the test, suggesting that the phase of the cycle did not significantly affect recognition memory recall.



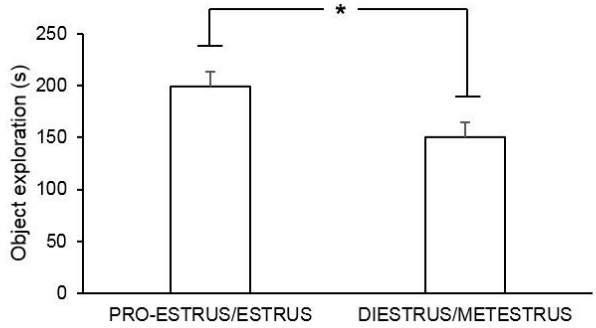


Figure 6| Time exploring two equal objects during the acquisition session. Involvement of the estrous cycle in the exploration activity. An asterisk indicates that performance is significantly different at $p<0.05$.

4. Discussion

The current investigation was aimed at studying in mice the effects of postnatal exposure to the pesticide CPF on recognition memory. An ORT was used to assess recognition memory, based on the premise that preference for a novel object means that the representation of the familiar object still exists in the memory of the animals. The potential implication of both the GABAergic and the cholinergic systems was assessed according to sex, *APOE* genotype and postnatal treatment using pharmacological challenges.

During acclimatization to the ORT working space, mice explored the new environment. All groups decreased the distance traveled over time, indicating habituation. Differences between sex and genotype were observed in terms of total distance traveled, with females having the highest locomotor activity. Previous studies with apoE3- and apoE4-TR mice (Bour et al., 2008; Grootendorst et al., 2005) at different ages (4-5 months and 15 months of age, respectively), and with young C57BL/6 mice (2-3 months of age) (Van Swearingen et al., 2013) also found that females were more active than males during habituation to a novel environment. In the same line, other studies reported an increase in activity after exposure to CPF (Ricceri et al., 2006, 2003) while others did not find effects on locomotor activity (López-Granero et al., 2016; Savy et al., 2015). Likewise, CPF-treated apoE4 mice traveled greater distances in the open field than their controls, reaching levels of activity similar to those of C57BL/6 mice. Taking into account that behavioral deficits associated to apoE4 have been related to an impaired cholinergic system (Allen et al., 1997), and that CPF targets the AChE enzyme and produces long-term effects on AChE-S and AChE-R mRNA at low doses (López-Granero et al., 2013, 2014), exposure to CPF during development may help to counterbalance a possible basal deficiency in cholinergic neurotransmission inherent to this genotype. Recent studies have already reported an enhancement of the performance of apoE4 mice after exposure to CPF, and thus, a resemblance with

other genotypes including *APOE3* or wild-type C57BL/6 mice (Basaure et al., 2019; Guardia-Escote et al., 2018; Peris-Sampedro et al., 2016).

During the acquisition session, the exploratory behavior of groups exposed to postnatal CPF was greater than that of controls in basal conditions. In general, this effect was reversed by the cholinergic antagonist scopolamine. However, apoE4 mice and C57BL/6 control mice proved to be less sensitive to this drug.

Retention of the task was affected by sex, with females showing better discrimination index scores. Although there is no consensus in this regard, most data report that females perform better, especially in long-lasting retrieval memory (Ghi et al., 1999; Sutcliffe et al., 2007). Enhanced object recognition memory in females has also been reported in humans (Levy et al., 2005). Other studies suggest no differences between males and females (Benice et al., 2006), or go even further, demonstrating that males perform better than females (Frick and Gresack, 2003).

Differences in genotype were observed in the saline group during retention. Firstly, apoE3 mice failed to recognize the novel object during this part of the experiment, and none of the pharmacological conditions led to any improvement. For this reason, it is not possible to draw any conclusion on this genotype. On the other hand, both male and female control apoE4, and C57BL/6 female mice showed a significant preference for the novel object. The fact that apoE4-TR mice presented better retention than apoE3-TR, or even C57BL/6 mice, is in contrast to most effects described in the scientific literature. Non-apoE4 carriers have been observed to perform better than apoE4 carriers in most human (Berteau-Pavy et al., 2007) and animal studies (Chouinard-Watkins et al., 2017; Salomon-Zimri et al., 2013). For instance, studies using ORT found a clear advantage of apoE3-TR over apoE4-TR mice at 4 (Salomon-Zimri et al.,

2013) and 12 months of age (Chouinard-Watkins et al., 2017). It is important to consider that apart from the differences in age between studies, both used different protocols, including shorter inter-trial times, and different parameters for assessing retention. Consequently, they are difficult to compare. Nevertheless, this is not the first study to report that apoE4 perform better than their apoE3 counterparts. Siegel et al. (2012) reported that during Morris water maze (MWM) acquisition and passive avoidance training, apoE4 females performed better than apoE2- and apoE3-TR mice at different ages. This better performance of apoE4 correlated with higher levels of anxiety-like behavior in the elevated zero maze and the elevated plus maze (Siegel et al., 2012). Likewise, in a previous study we also found that apoE4 needed fewer sessions than apoE3 mice to meet the learning criteria in the five-choice serial reaction time task (5-CSRTT) (Reverte et al., 2016).

CPF exposure impaired retention in apoE4 mice, which lost their preference for the novel object during this part of the test. Slotkin et al. (2015) also observed a detrimental effect of postnatal (PND 1-4) CPF exposure on recognition memory. They reported this effect only in females, which performed better in basal conditions. Other studies testing different doses of CPF during the gestational period or throughout aduthood in males showed no differences between treatments in this task (Lan et al., 2017; Savy et al., 2015; Terry et al., 2007). Altogether, these results suggest that the detrimental effects on recognition memory are dependent on the exposure period to the pesticide and highly influenced by sex.

The cholinergic system and its neurotransmitter ACh play a key role in the central and peripheral nervous system. ACh synthesis from choline and acetyl coenzyme A takes place in the cytoplasm of cholinergic neurons, being ACh then stored in synaptic vesicles. Once the vesicles with the neurotransmitter are released into the synaptic cleft, ACh binds to mAChRs and nAChRs, located at the

pre- or postsynaptic neurons. ACh is then inactivated by cholinesterase enzymes such as AChE and BChE, which share high homology, but not equal efficiency to break the ACh, which terminates the cholinergic neurotransmission (Abreu-Villaça et al., 2011; Hrabovska and Krejci, 2014; Soreq and Seidman, 2001). In a previous study assessing the gene expression of the various elements involved in cholinergic neurotransmission in the brain of nine-month-old mice, we observed differences between C57BL/6 and apoE4-TR mice (Guardia-Escote et al., 2018). More specifically, apoE4 males expressed lower levels of M1 and M2 mAChRs in the frontal cortex and females expressed higher levels of BChE in the hippocampus compared with C57BL/6 mice (Guardia-Escote et al., 2018). These constitutive differences in the cholinergic system may underlie the effects observed under the influence of the cholinergic drugs scopolamine and rivastigmine.

Administration of scopolamine impaired retention in the groups displaying good retention in the basal condition. As a result, none of the groups showed a significant preference for novelty. However, the control apoE4 male group still spent more time exploring the novel object. In previous studies, we found that apoE4 females were less sensitive to scopolamine during attention assessment in the 5-CSRTT (Reverte et al., 2016). Scopolamine is a cholinergic antagonist targeting the muscarinic receptors. It has been reported that apoE4 males express lower levels of muscarinic receptor M1 and M2 in the hippocampus and frontal cortex than their C57BL/6 counterparts (Guardia-Escote et al., 2018). These differences have not been observed in females, which suggests constitutive cholinergic variances between sexes. Likewise, the effect was no longer observed in CPF-treated apoE4 females, which suggests a compensating effect induced by exposure to CPF. Further investigations are required to unravel the underlying mechanisms of this conceivable compensation.

The cholinergic agonist rivastigmine improved performance in control C57BL/6 mice and in both control and CPF-exposed apoE4 males. Interestingly, rivastigmine did not produce the same effect in apoE4 females which, in the case of the control group, showed decreased retention after rivastigmine administration. These results suggest a sex-dimorphic effect of rivastigmine on recognition memory. The discrepancy between males and females has important implications because rivastigmine is one of the drugs currently used in the treatment of AD (Anand et al., 2014). Previous studies with rats have reported that inhibition levels of ChEs in different brain areas in females are higher than in males after rivastigmine administration (Wang et al., 2000). In general terms, it seems that these differences can be attributed once more to constitutive differences in the cholinergic system between males and females. In recent studies we found that expression levels of BChE were lower in apoE4-TR mice than in C57BL/6 mice, but only in females (Guardia-Escote et al., 2018). Therefore, apoE4 females may be less sensitive to the effects of rivastigmine because they express lower levels of BChE, one of its targets. Animals with decreased levels of BChE would present higher levels of ACh, just as they do after rivastigmine administration (Cerbai et al., 2007). Hence, it can be hypothesized that these females undergo an overstimulation of the cholinergic system, which leads to the observed impairments in recognition memory. The results of the present study raises the possibility that the effective dose in females should be reviewed and maybe adapted to the intrinsic requirements defined by sex and genotype. Other studies have reported memory improvements in males after dietary exposures to rivastigmine in the MWM test (Basaure et al., 2017). However, the effects of rivastigmine on recognition memory have received little attention. Still, several studies using donepezil, another drug used in the treatment of AD, have reported positive effects on task performance (Kendall et al., 2011). These two drugs share a similar mechanism of action, as donepezil also inhibits AChE, but it does not inhibit BChE (Lane et al., 2004).

Alprazolam had a detrimental effect on both control and CPF-treated C57BL/6 females. After its administration, these animals lost the preference for novelty observed in basal conditions. Interestingly, the retention of control apoE4 females was worse than that of the saline group. This detrimental effect is in line with the amnesic propeties of alprazolam and low doses of the drug can impair object recognition in mice (Bertaina-Anglade et al., 2006). It has been reported that the GABAergic system can be influenced by apoE4 isoform in a sex-dependent manner (Li et al., 2016), giving a plausible explanation for the different responses of the groups. Further investigations are required to establish the exact mechanisms underlying these observations.

One of the major limitations of the data analysis is that the different ORT protocols make it difficult to compare studies. Nonetheless, Akkerman et al. (2012) pointed out that some factors are crucial to the correct performance of the task (for example, the habituation to treatment procedures or the use of meeting criteria regarding minimum exploration times). Other important factors may be the inter-trial time, the kind of objects used or the experimental design. Furthermore, the phase of the estrous cycle may also be a variability factor. We found that females in pro-estrus/estrus showed an advantage during the acquisition part of the task, but this advantage was imperceptible during the retention part and after the drug had been administered. The better performance during pro-estrus/estrus matches previous findings on differences in rats (van Goethem et al., 2012) and mice (Cordeira et al., 2018) during acquisition. However, unlike these researchers, we did not find any differences during retention. Other researchers have found that the estrous cycle did not lead to differences in any of the test parts (Sutcliffe et al., 2007). Overall, our results do not support the exclusion of females arguing variability caused by the estrous cycle.

In summary, the findings of the current research provide insights into the detrimental effects of postnatal exposure to CPF on recognition memory in apoE4 mice. The use of scopolamine revealed different sensitivities of apoE4 males, while the use of rivastigmine revealed differences between apoE4 males and females. Altogether, these results show that the cholinergic system is clearly involved in recognition memory and provide valuable information about its modulation. They also raise questions about the current use of rivastigmine for AD in females. Alprazolam also influenced recognition memory in a genotype- and sex-dependent manner with apoE4 females being the most impaired, thus raising questions about the use of GABA agonists in apoE4 females. In conclusion, sex differences observed in apoE4 mice in response to cholinergic and GABAergic drug challenges give rise to an important debate about therapeutic approaches, which can be personalized according to genotype and sex.

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References

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

Akkerman, S., Blokland, A., Reneerkens, O., van Goethem, N.P., Bollen, E., Gijselaers, H.J.M., Lieben, C.K.J., Steinbusch, H.W.M., Prickaerts, J., 2012. Object recognition testing: Methodological considerations on exploration and discrimination measures. Behav. Brain Res. 232, 335–347. https://doi.org/10.1016/j.bbr.2012.03.022

Allen, S.J., MacGowan, S.H., Tyler, S., Wilcock, G.K., Robertson, A.G.S., Holden, P.H., Smith, S.K.F., Dawbarn, D., 1997. Reduced cholinergic function in normal and Alzheimer's disease brain is associated with apolipoprotein E4 genotype. Neurosci. Lett. 239, 33–36. https://doi.org/10.1016/S0304-3940(97)00872-0

Anand, R., Gill, K.D., Mahdi, A.A., 2014. Therapeutics of Alzheimer's disease: Past, present and future. Neuropharmacology 76, 27–50. https://doi.org/10.1016/j.neuropharm.2013.07.004

Bales, K.R., 2010. Brain lipid metabolism, apolipoprotein E and the pathophysiology of Alzheimer's disease. Neuropharmacology 59, 295–302. https://doi.org/10.1016/j.neuropharm.2010.01.005

Basaure, P., Guardia-Escote, L., Cabré, M., Peris-Sampedro, F., Sánchez-Santed, F., Domingo, J.L., Colomina, M.T., 2019. Learning, memory and the expression of cholinergic components in mice are modulated by the pesticide chlorpyrifos depending upon age at exposure and apolipoprotein E (APOE) genotype. Arch. Toxicol. 93, 693–707. https://doi.org/10.1007/s00204-019-02387-9

Basaure, P., Guardia-Escote, L., Cabré, M., Peris-Sampedro, F., Sánchez-Santed, F., Domingo, J.L., Colomina, M.T., 2018. Postnatal chlorpyrifos exposure and apolipoprotein E (APOE) genotype differentially affect cholinergic expression and developmental parameters in transgenic mice. Food Chem. Toxicol. 118, 42–52. https://doi.org/10.1016/j.fct.2018.04.065

Basaure, P., Peris-Sampedro, F., Cabré, 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. https://doi.org/10.1016/j.bbr.2016.10.014

Benice, T.S., Rizk, A., Kohama, S., Pfankuch, T., Raber, J., 2006. Sex-differences in age-related cognitive decline in C57BL/6J mice associated with increased brain microtubule-associated protein 2 and synaptophysin immunoreactivity. Neuroscience 137, 413–423. https://doi.org/10.1016/j.neuroscience.2005.08.029

Bertaina-Anglade, V., Enjuanes, E., Morillon, D., Drieu la Rochelle, C., 2006. The object recognition task in rats and mice: A simple and rapid model in safety pharmacology to detect amnesic properties of a new chemical entity. J. Pharmacol. Toxicol. Methods 54, 99–105. https://doi.org/10.1016/j.vascn.2006.04.001

Berteau-Pavy, F., Park, B., Raber, J., 2007. Effects of sex and APOE ε4 on object recognition and spatial navigation in the elderly. Neuroscience 147, 6–17. https://doi.org/10.1016/j.neuroscience.2007.03.005

Bour, A., Grootendorst, J., Vogel, E., Kelche, C., Dodart, J.-C., Bales, K., Moreau, P.-H., Sullivan, P.M., Mathis, C., 2008. Middle-aged human apoE4 targeted-replacement mice show retention deficits on a wide range of spatial memory tasks. Behav. Brain Res. 193, 174–182. https://doi.org/10.1016/j.bbr.2008.05.008

Broadbent, N.J., Gaskin, S., Squire, L.R., Clark, R.E., 2009. Object recognition memory and the rodent hippocampus. Learn. Mem. 17, 5–11. https://doi.org/10.1101/lm.1650110

Brown, M.W., Barker, G.R.I., Aggleton, J.P., Warburton, E.C., 2012. What pharmacological interventions indicate concerning the role of the perirhinal cortex in recognition memory. Neuropsychologia 50, 3122–3140. https://doi.org/10.1016/j.neuropsychologia.2012.07.034

Caligioni, C., 2009. Assessing Reproductive Status/Stages in Mice. Curr Protoc Neurosci. Appendix–4I. https://doi.org/10.1002/0471142301.nsa04is48.

Casida, J.E., Quistad, G.B., 2004. Organophosphate toxicology: Safety aspects of nonacetylcholinesterase secondary targets. Chem. Res. Toxicol. 17, 983–998. https://doi.org/10.1021/tx0499259

Cerbai, F., Giovannini, M.G., Melani, C., Enz, A., Pepeu, G., 2007. N1phenethyl-norcymserine, a selective butyrylcholinesterase inhibitor, increases acetylcholine release in rat cerebral cortex: A comparison with donepezil and rivastigmine. Eur. J. Pharmacol. 572, 142–150. https://doi.org/10.1016/j.ejphar.2007.06.053

Chouinard-Watkins, R., Vandal, M., Léveillé, P., Pinçon, A., Calon, F., Plourde, M., 2017. Docosahexaenoic acid prevents cognitive deficits in human apolipoprotein E epsilon 4-targeted replacement mice. Neurobiol. Aging 57, 28–35. https://doi.org/10.1016/j.neurobiolaging.2017.05.003

Contreras, C.M., Molina, M., Saavedra, M., Martínez-Mota, L., 2000. Lateral septal neuronal firing rate increases during proestrus-estrus in the rat. Physiol. Behav. 68, 279–284. https://doi.org/10.1016/S0031-9384(99)00169-9

Cordeira, J., Kolluru, S.S., Rosenblatt, H., Kry, J., Strecker, R.E., McCarley, R.W., 2018. Learning and memory are impaired in the object recognition task during

metestrus/diestrus and after sleep deprivation. Behav. Brain Res. 339, 124–129. https://doi.org/10.1016/j.bbr.2017.11.033

Dam, K., Seidler, F.J., Slotkin, T.A., 2000. Chlorpyrifos exposure during a critical neonatal period elicits gender-selective deficits in the development of coordination skills and locomotor activity. Dev. Brain Res. 121, 179–187. https://doi.org/10.1016/S0165-3806(00)00044-4

De Angelis, S., Tassinari, R., Maranghi, F., Eusepi, A., Di Virgilio, A., Chiarotti, F., Ricceri, L., Venerosi Pesciolini, A., Gilardi, E., Moracci, G., Calamandrei, G., Olivieri, A., Mantovani, A., 2009. Developmental exposure to chlorpyrifos induces alterations in thyroid and thyroid hormone levels without other toxicity signs in Cd-1 mice. Toxicol. Sci. 108, 311–319. https://doi.org/10.1093/toxsci/kfp017

Dere, E., Huston, J.P., De Souza Silva, M.A., 2007. The pharmacology, neuroanatomy and neurogenetics of one-trial object recognition in rodents. Neurosci. Biobehav. Rev. 31, 673–704. https://doi.org/10.1016/j.neubiorev.2007.01.005

Dodart, J.C., Mathis, C., Ungerer, A., 1997. Scopolamine-induced deficits in a two-trial object recognition task in mice. Neuroreport 8, 1173–1178. https://doi.org/10.1097/00001756-199703240-00023

Ennaceur, A., Delacour, J., 1988. A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. Behav. Brain Res. 31, 47–59. https://doi.org/10.1016/0166-4328(88)90157-X

Frick, K.M., Gresack, J.E., 2003. Sex differences in the behavioral response to spatial and object novelty in adult C57BL/6 mice. Behav. Neurosci. 117, 1283–1291. https://doi.org/10.1037/0735-7044.117.6.1283

Getz, G.S., Reardon, C.A., 2009. Apoprotein E as a lipid transport and signaling protein in the blood, liver, and artery wall. J. Lipid Res. 50, S156–S161. https://doi.org/10.1194/jlr.R800058-JLR200

Ghi, P., Orsetti, M., Gamalero, S.R., Ferretti, C., 1999. Sex differences in memory performance in the object recognition test. Possible role of histamine receptors. Pharmacol. Biochem. Behav. 64, 761–766. https://doi.org/10.1016/S0091-3057(99)00143-4

Grayson, B., Leger, M., Piercy, C., Adamson, L., Harte, M., Neill, J.C., 2015. Assessment of disease-related cognitive impairments using the novel object recognition (NOR) task in rodents. Behav. Brain Res. 285, 176–193. https://doi.org/10.1016/j.bbr.2014.10.025

Grootendorst, J., Bour, A., Vogel, E., Kelche, C., Sullivan, P.M., Dodart, J.-C., Bales, K., Mathis, C., 2005. Human apoE targeted replacement mouse lines: h-apoE4 and h-apoE3 mice differ on spatial memory performance and avoidance behavior. Behav. Brain Res. 159, 1–14. https://doi.org/10.1016/j.bbr.2004.09.019

Guardia-Escote, L., Basaure, P., Blanco, J., Cabré, M., Pérez-Fernández, C., Sánchez-Santed, F., Domingo, J.L., Colomina, M.T., 2018. 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. Food Chem. Toxicol. 122, 1–10. https://doi.org/10.1016/j.fct.2018.09.069

Hrabovska, A., Krejci, E., 2014. Reassessment of the Role of the Central Cholinergic System. J. Mol. Neurosci. 53, 352–358. https://doi.org/10.1007/s12031-013-0164-8

Kendall, I., Slotten, H.A., Codony, X., Burgueño, J., Pauwels, P.J., Vela, J.M., Fone, K.C.F., 2011. E-6801, a 5-HT6 receptor agonist, improves recognition memory by combined modulation of cholinergic and glutamatergic neurotransmission in the rat. Psychopharmacology (Berl). 213, 413–430. https://doi.org/10.1007/s00213-010-1854-3

Klinkenberg, I., Blokland, A., 2010. The validity of scopolamine as a pharmacological model for cognitive impairment: A review of animal behavioral studies. Neurosci. Biobehav. Rev. 34, 1307–1350. https://doi.org/10.1016/j.neubiorev.2010.04.001

Lan, A., Kalimian, M., Amram, B., Kofman, O., 2017. Prenatal chlorpyrifos leads to autism-like deficits in C57Bl6/J mice. Environ. Heal. 16, 43. https://doi.org/10.1186/s12940-017-0251-3

Lane, R.M., Kivipelto, M., Greig, N.H., 2004. Acetylcholinesterase and its inhibition in Alzheimer disease. Clin. Neuropharmacol. 27, 141–149.

Levy, L.J., Astur, R.S., Frick, K.M., 2005. Men and women differ in object memory but not performance of a virtual radial maze. Behav. Neurosci. 119, 853–862. https://doi.org/10.1037/0735-7044.119.4.853

Li, Y., Sun, H., Chen, Z., Xu, H., Bu, G., Zheng, H., 2016. Implications of GABAergic neurotransmission in Alzheimer's disease. Front. Aging Neurosci. 8, 1–12. https://doi.org/10.3389/fnagi.2016.00031

López-Granero, C., Cardona, D., Giménez, E., Lozano, R., Barril, J., Aschner, M., Sánchez-Santed, F., Cañadas, F., 2014. Comparative study on short- and long-term behavioral consequences of organophosphate exposure: Relationship to AChE mRNA expression. Neurotoxicology 40, 57–64. https://doi.org/10.1016/j.neuro.2013.11.004

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, 41–49. https://doi.org/10.1016/j.tox.2013.03.009

López-Granero, C., Ruiz-Muñoz, A.M., Nieto-Escámez, F.A., Colomina, M.T., Aschner, M., Sánchez-Santed, F., 2016. Chronic dietary chlorpyrifos causes long-term spatial memory impairment and thigmotaxic behavior. Neurotoxicology 53, 85–92. https://doi.org/10.1016/j.neuro.2015.12.016

McLean, S.L., Grayson, B., Marsh, S., Zarroug, S.H.O., Harte, M.K., Neill, J.C., 2016. Nicotinic α7 and α4β2 agonists enhance the formation and retrieval of recognition memory: Potential mechanisms for cognitive performance enhancement in neurological and psychiatric disorders. Behav. Brain Res. 302, 73–80. https://doi.org/10.1016/j.bbr.2015.08.037

Melichercik, A.M., Elliott, K.S., Bianchi, C., Ernst, S.M., Winters, B.D., 2012. Nicotinic receptor activation in perirhinal cortex and hippocampus enhances object memory in rats. Neuropharmacology 62, 2096–2105. https://doi.org/10.1016/j.neuropharm.2012.01.008

Moser, V.C., Padilla, S., 1998. Age- and gender-related differences in the time course of behavioral and biochemical effects produced by oral chlorpyrifos in rats. Toxicol. Appl. Pharmacol. 149, 107–119.

Peris-Sampedro, F., Basaure, P., Reverte, I., Cabré, M., Domingo, J.L., Colomina, M.T., 2015. 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. https://doi.org/10.1016/j.physbeh.2015.03.006

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. https://doi.org/10.1016/j.fct.2016.03.029

Pichat, P., Bergis, O.E., Terranova, J.P., Urani, A., Duarte, C., Santucci, V., Gueudet, C., Voltz, C., Steinberg, R., Stemmelin, J., Oury-Donat, F., Avenet, P., Griebel, G., Scatton, B., 2007. SSR180711, a novel selective α7 nicotinic receptor partial agonist: (II) efficacy in experimental models predictive of activity against cognitive symptoms of schizophrenia. Neuropsychopharmacology 32, 17–34. https://doi.org/10.1038/sj.npp.1301188

Pope, C.N., Chakraborti, T.K., Chapman, M.L., Farrar, J.D., Arthun, D., 1991. Comparison of in vivo cholinesterase inhibition in neonatal and adult rats by three organophosphorothioate insecticides. Toxicology 68, 51–61. https://doi.org/10.1016/0300-483X(91)90061-5

Reiss, R., Chang, E.T., Richardson, R.J., Goodman, M., 2015. A review of epidemiologic studies of low-level exposures to organophosphorus insecticides in non-occupational populations. Crit. Rev. Toxicol. 45, 531–641. https://doi.org/10.3109/10408444.2015.1043976

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, 116–125. https://doi.org/10.1016/j.expneurol.2012.06.015

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. https://doi.org/10.1007/s00213-015-4113-9

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. https://doi.org/10.1016/S0041-008X(03)00229-1

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. https://doi.org/10.1093/toxsci/kfl032

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. https://doi.org/10.1093/toxsci/kfi081

Roses, A.D., 1996. Apolipoprotein E and Alzheimer's disease. A rapidly expanding field with medical and epidemiological consequences. Ann NY Acad Sci 802, 50–57.

Salomon-Zimri, S., Boehm-Cagan, A., Liraz, O., Michaelson, D.M., 2013. Hippocampus-Related Cognitive Impairments in Young apoE4 Targeted Replacement Mice. Neurodegener. Dis. 13, 86–92. https://doi.org/10.1159/000354777

Savy, C.Y., Fitchett, A.E., McQuade, R., Gartside, S.E., Morris, C.M., Blain, P.G., Judge, S.J., 2015. Low-level repeated exposure to diazinon and chlorpyrifos decrease anxiety-like behaviour in adult male rats as assessed by marble burying behaviour. Neurotoxicology 50, 149–156. https://doi.org/10.1016/j.neuro.2015.08.010

Siegel, J.A., Haley, G.E., Raber, J., 2012. Apolipoprotein E isoform-dependent effects on anxiety and cognition in female TR mice. Neurobiol. Aging 33, 345–358. https://doi.org/10.1016/j.neurobiolaging.2010.03.002

Slotkin, T. a., Skavicus, S., Levin, E.D., Seidler, F.J., 2015. Prenatal nicotine changes the response to postnatal chlorpyrifos: Interactions targeting serotonergic synaptic function and cognition. Brain Res. Bull. 111, 84–96. https://doi.org/10.1016/j.brainresbull.2015.01.003

Slotkin, T.A., Southard, M.C., Adam, S.J., Cousins, M.M., Seidler, F.J., 2004. α7 Nicotinic acetylcholine receptors targeted by cholinergic developmental neurotoxicants: nicotine and chlorpyrifos. Brain Res. Bull. 64, 227–235. https://doi.org/10.1016/j.brainresbull.2004.07.005

Soni, K., Parle, M., 2017. Trachyspermum ammi Seeds Supplementation Helps Reverse Scopolamine, Alprazolam and Electroshock Induced Amnesia. Neurochem. Res. 42, 1333–1344. https://doi.org/10.1007/s11064-017-2177-0

Soreq, H., Seidman, S., 2001. Acetylcholinesterase - new roles for an old actor. Nat. Rev. Neurosci. 2, 294–302. https://doi.org/10.1038/35067589

Sullivan, P.M., Mezdour, H., Aratanti, Y., Knouff, C., Najib, J., Reddick, R.L., Quarfordt, S.H., Maeda, N., 1997. Targeted Replacement of the Mouse Apolipoprotein E Gene with the Common Human APOE3 Allele Enhances Diet-induced Hypercholesterolemia and Atherosclerosis. J. Biol. Chem. 272, 17972–17980. https://doi.org/10.1074/jbc.272.29.17972

Sutcliffe, J.S., Marshall, K.M., Neill, J.C., 2007. Influence of gender on working and spatial memory in the novel object recognition task in the rat. Behav. Brain Res. 177, 117–125. https://doi.org/10.1016/j.bbr.2006.10.029

Terry, A. V., Gearhart, D.A., Beck, W.D., Truan, J.N., Middlemore, M.-L., 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. J. Pharmacol. Exp. Ther. 322, 1117–1128. https://doi.org/10.1124/jpet.107.125625

Thanapreedawat, P., Kobayashi, H., Inui, N., Sakamoto, K., Kim, M., Yoto, A., Yokogoshi, H., 2013. GABA Affects Novel Object Recognition Memory and Working Memory in Rats. J. Nutr. Sci. Vitaminol. (Tokyo). 59, 152–157. https://doi.org/10.3177/jnsv.59.152

van Goethem, N.P., Rutten, K., van der Staay, F.J., Jans, L. a W., Akkerman, S., Steinbusch, H.W.M., Blokland, A., van't Klooster, J., Prickaerts, J., 2012. Object recognition testing: Rodent species, strains, housing conditions, and estrous cycle. Behav. Brain Res. 232, 323–334. https://doi.org/10.1016/j.bbr.2012.03.023

Van Swearingen, A.E.D., Walker, Q.D., Kuhn, C.M., 2013. Sex differences in novelty- and psychostimulant-induced behaviors of C57BL/6 mice. Psychopharmacology (Berl). 225, 707–718. https://doi.org/10.1007/s00213-012-2860-4

Wang, R.H., Bejar, C., Weinstock, M., 2000. Gender differences in the effect of rivastigmine on brain cholinesterase activity and cognitive function in rats. Neuropharmacology 39, 497–506. https://doi.org/10.1016/S0028-3908(99)00157-4

3.3. Publication III

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

Laia Guardia-Escote, Pia Basaure, Jordi Blanco, Maria Cabré, Cristian Pérez-Fernández, Fernando Sánchez-Santed, José L. Domingo, Maria Teresa Colomina.

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Study III overview.	
What do we already know?	Spatial learning and memory are influenced by *APOE* genotype, which confers a different vulnerability to CPF effects. The cholinergic system underlies learning and memory and is modulated by *APOE* genotype.
What does this study add?	Analysis of specific differences between apoE4-TR and C57BL/6 mice, in a sex-dependent manner and of the long-term effects of postnatal exposure to CPF in middle-aged mice.
Highlights	ApoE4-TR mice presented the worst retention but CPF improved performance in apoE4 females. ApoE4 presented altered levels of cholinergic elements in both the frontal cortex and the hippocampus at 9 months. Long-term effects of CPF exposure were observed only with α7 nAChRs.

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UNIVERSITAT ROVIRA I VIRGILI
POSTNATAL CHLORPYRIFOS EXPOSURE INFLUENCES THE GUT MICROBIOTA AND THE EXPRESSION OF BIOLOGICAL AND NEUROBEHAVIORAL CHARACTERISTICS OF THE APOE GENOTYPE IN AN AGE-DEPENDENT MANNER
Laia Guardia Escoté

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.

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

	MWM		Gene Expression FC		Gene Expression HC	
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

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.

Table 2. Primers sequence used for the gene expression analysis.

Gene	Protein	Forward primer	Reverse primer	Reference
Bche	BChE	TAGCACAATGTG GCCTGTCT	ATTGCTCCAGCGA TGAAATC	(García-Gómez et al. 2015)
Chrm1	M1 mAChR	TGACAGGCAACC TGCTGGTGCT	AATCATCAGAGCT GCCCTGCGG	(Laspas et al. 2015)
Chrm2	M2 mAChR	CGGACCACAAAA ATGGCAGGCAT	CCATCACCACCAG GCATGTTGTTGT	(Laspas et al. 2015)
Chrna4	α4 nAChR	GTTCTATGACGG AAGGGTGCAGTG GACA	GGGATGACCAGCG AGGTGGACGGGAT GAT	(Léna et al. 1999)
Chrna7	α7 nAChR	GTGGAACATGTC TGAGTACCCCGG AGTGAA	GAGTCTGCAGGCA GCAAGAATACCAG CA	(Léna et al. 1999)
Ache	AChE-S	CTGAACCTGAAG CCCTTAGAG	CCGCCTCGTCCAG AGTAT	(Dori et al. 2011)
Ache	AChE-R	GAGCAGGGAATG CACAAG	GGGGAGGTGGAGA AGAGAG	(Dori et al. 2011)
Gapdh	GAPDH	ACAACTTTGGCA TTGTGGAA	GATGCAGGGATGA TGTTCTG	(Yao et al. 2016)

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

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).

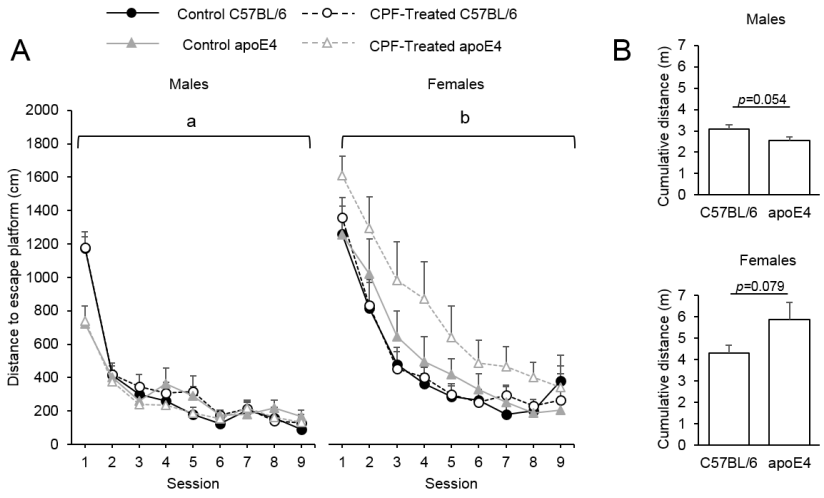

Fig. 1. Acquisition in the Morris Water Maze. Distance to escape platform traveled (A) by males and females over the nine sessions of training, and their cumulative distance depending on the genotype (B). Different letters (a,b) indicate a significant difference between groups at $p<0.05$.

Table 3. Summary table of results in the Morris Water Maze (MWM) and gene expression on 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

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.

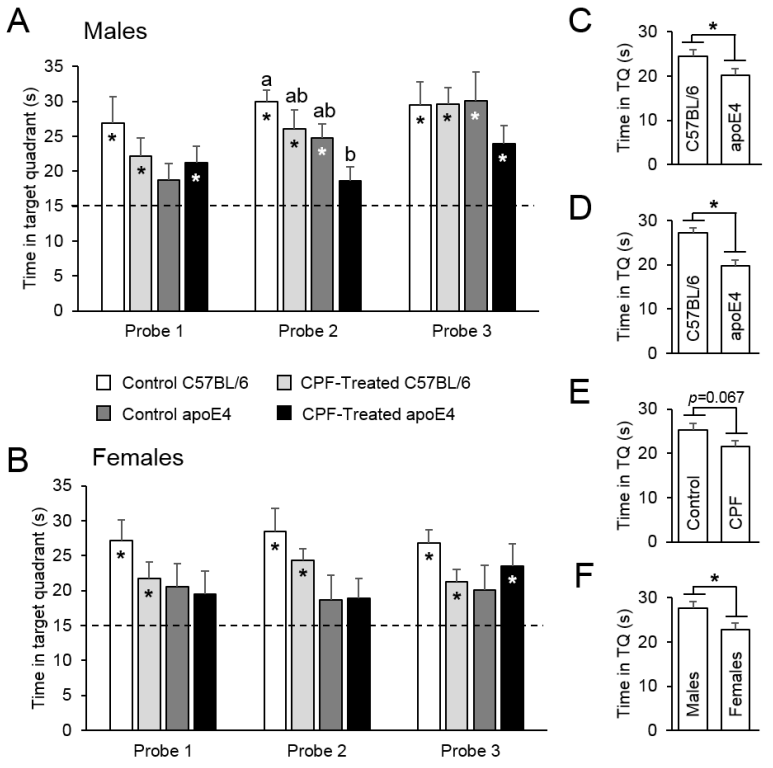

Fig. 2. Retention in the Morris Water Maze. Time in the target quadrant in males (A) and females (B) over the 3 retention sessions performed 24 hours after the third (Probe 1), the sixth (Probe 2) and the ninth (Probe 3) acquisition days. General effects of the genotype in the first (C) and the second 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 ($p<0.05$).

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).

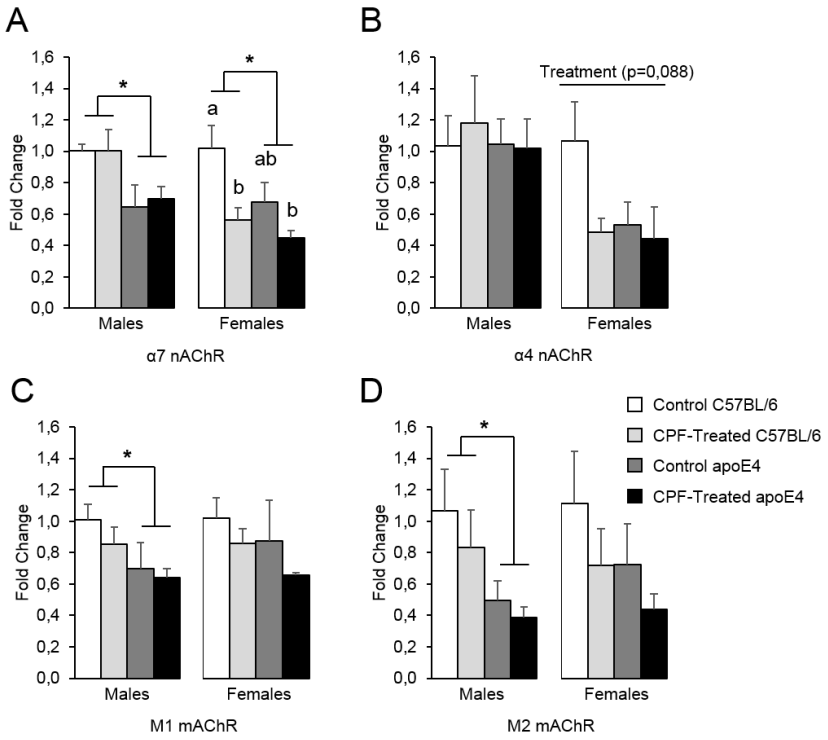

Fig. 3. Relative gene expression in the frontal cortex of α7 nAChR (A), α4 nAChR (B), M1 mAChR (C) and M2 mAChR (D). An asterisk indicates significant genotype differences at p<0.05. Different letters (a,b) indicate a significant difference between groups (p<0.05).

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).

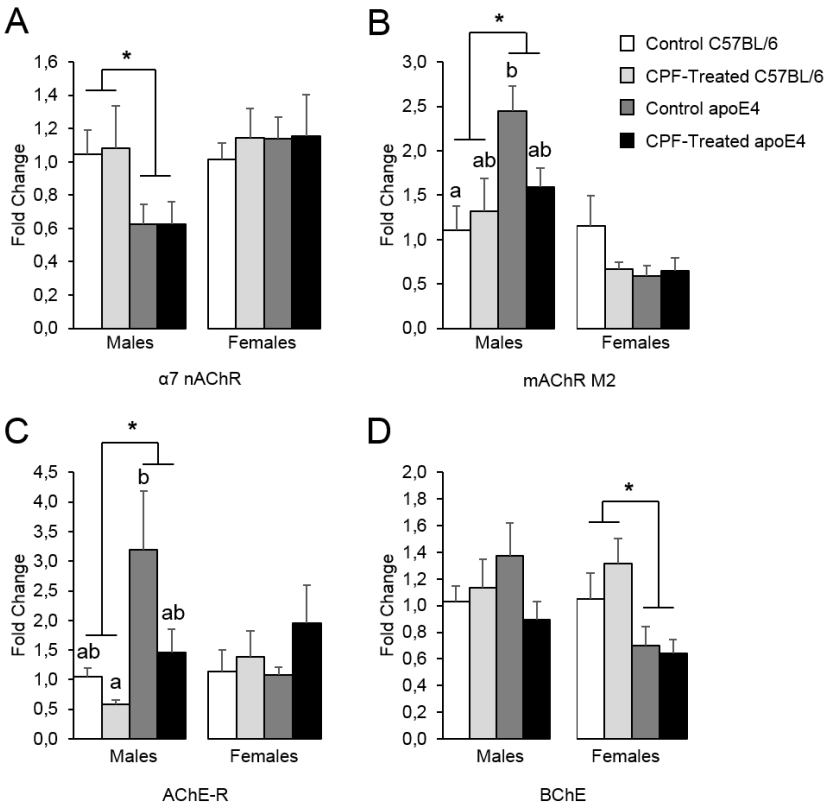

Fig. 4. Relative gene expression in the hippocampus of α7 nAChR (A), M2 mAChR (B), AChE-R (C) and BChE (D). An asterisk indicates significant genotype differences at $p<0.05$. Different letters (a,b) indicate a significant difference between groups ($p<0.05$).

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 receptors, mainly in α7 nAChR expression levels. Furthermore, C57BL/6 females displayed significantly different levels of α7 nAChR expression between treatment groups. In particular, the CPF-treated group expressed diminished levels of α7 nAChR, suggesting a long-term effect of the postnatal treatment with CPF. Slotkin et al. (2004) also detected a significant decrease in α7 nAChR binding in the forebrain and cerebellum after exposure to 5 mg/kg/day of CPF on postnatal days 11-14. Their findings, observed on postnatal days 15 and 20, indicated that α7 nAChR was a specific target for cholinergic neurotoxicants. In our study, the resulting levels of the C57BL/6 CPF-treated female group resemble those of the apoE4 group, which lends further support to the hypothesis of a basal difference in ACh signaling between apoE4-TR and C57BL/6 mice. Taken together, these results highlight

the importance of nicotinic receptors and the fact that they may be permanently altered by postnatal exposure to CPF. Considering that these differences in gene expression between treatment groups were still detectable after 9 months, they can be an underlying sign of future cognitive impairments. For this reason, it would be interesting to investigate whether they can lead to more evident deficits with age.

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

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

Allen, S.J., MacGowan, S.H., Tyler, S., Wilcock, G.K., Robertson, A.G.S., Holden, P.H., Smith, S.K.F., Dawbarn, D., 1997. Reduced cholinergic function in normal and Alzheimer's disease brain is associated with apolipoprotein E4 genotype. Neurosci. Lett. 239, 33–36. https://doi.org/10.1016/S0304-3940(97)00872-0.

Basaure, P., Guardia-Escote, L., Cabré, M., Peris-Sampedro, F., Sánchez-Santed, F., Domingo, J.L., Colomina, M.T., 2018. Postnatal chlorpyrifos exposure and apolipoprotein E (APOE) genotype differentially affect cholinergic expression and developmental

parameters in transgenic mice. Food Chem. Toxicol. 118, 42–52. https://doi.org/10.1016/j.fct.2018.04.065.

Basaure, P., Peris-Sampedro, F., Cabré, 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. https://doi.org/10.1016/j.bbr.2016.10.014.

Berson, A., Knobloch, M., Hanan, M., Diamant, S., Sharoni, M., Schuppli, D., Geyer, B.C., Ravid, R., Mor, T.S., Nitsch, R.M., Soreq, H., 2008. Changes in readthrough acetylcholinesterase expression modulate amyloid-beta pathology. Brain 131, 109–119. https://doi.org/10.1093/brain/awm276.

Blake, M.G., Krawczyk, M.C., Baratti, C.M., Boccia, M.M., 2014. Neuropharmacology of memory consolidation and reconsolidation: insights on central cholinergic mechanisms. J. Physiol. Paris 108, 286–291. https://doi.org/10.1016/j.jphysparis.2014.04. 005.

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. https://doi.org/10.1016/j.atherosclerosis.2010.02. 027.

Bour, A., Grootendorst, J., Vogel, E., Kelche, C., Dodart, J.C., Bales, K., Moreau, P.H., Sullivan, P.M., Mathis, C., 2008. Middle-aged human apoE4 targeted-replacement mice show retention deficits on a wide range of spatial memory tasks. Behav. Brain Res. 193, 174–182. https://doi.org/10.1016/j.bbr.2008.05.008.

Buratti, F.M., De Angelis, G., Ricceri, L., Venerosi, A., Calamandrei, G., Testai, E., 2011. Foetal and neonatal exposure to chlorpyrifos: biochemical and metabolic alterations in the mouse liver at different developmental stages. Toxicology 280, 98–108. https://doi.org/10.1016/j.tox.2010.11.013.

Campanari, M.L., Navarrete, F., Ginsberg, S.D., Manzanares, J., Sáez-Valero, J., García-Ayllón, M.S., 2016. Increased expression of readthrough acetylcholinesterase variants in the brains of Alzheimer's disease patients. J. Alzheim. Dis. 53, 831–841. https://doi.org/10.3233/JAD-160220.

Darreh-Shori, T., Brimijoin, S., Kadir, A., Almkvist, O., Nordberg, A., 2006. Differential CSF butyrylcholinesterase levels in Alzheimer's disease patients with the ApoE ε4 allele, in

relation to cognitive function and cerebral glucose metabolism. Neurobiol. Dis. 24, 326–333. https://doi.org/10.1016/j.nbd.2006.07.013.

Darreh-Shori, T., Modiri, N., Blennow, K., Baza, S., Kamil, C., Ahmed, H., Andreasen, N., Nordberg, A., 2011. The apolipoprotein E ε4 allele plays pathological roles in AD through high protein expression and interaction with butyrylcholinesterase. Neurobiol. Aging 32, 1236–1248. https://doi.org/10.1016/j.neurobiolaging.2009. 07.015.

Darvesh, S., Hopkins, D.A., Geula, C., 2003. Neurobiology of butyrylcholinesterase. Nat. Rev. Neurosci. 4, 131–138. https://doi.org/10.1038/nrn1035.

De Angelis, S., Tassinari, R., Maranghi, F., Eusepi, A., Di Virgilio, A., Chiarotti, F., Ricceri, L., Venerosi Pesciolini, A., Gilardi, E., Moracci, G., Calamandrei, G., Olivieri, A., Mantovani, A., 2009. Developmental exposure to chlorpyrifos induces alterations in thyroid and thyroid hormone levels without other toxicity signs in CD-1 mice. Toxicol. Sci. 108, 311–319. https://doi.org/10.1093/toxsci/kfp017.

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. https://doi.org/10.1111/jnc.13417.

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. https://doi.org/10.1016/j.neuroscience.2011.03.044.

Douglas, C.L., Baghdoyan, H.A., Lydic, R., 2001. M2 muscarinic autoreceptors modulate acetylcholine release in prefrontal cortex of C57BL/6J mouse. J. Pharmacol. Exp. Therapeut. 299, 960–966.

Eaton, D.L., Daroff, R.B., Autrup, H., Bridges, J., Buffler, P., Costa, L.G., Coyle, J., McKhann, G., Mobley, W.C., Nadel, L., Neubert, D., Schulte-Hermann, R., Spencer, P.S., 2008. Review of the toxicology of chlorpyrifos with an emphasis on human exposure and neurodevelopment. Crit. Rev. Toxicol. 38, 1–125. https://doi.org/10.1080/10408440802272158.

Ferreira-Vieira, T.H., Guimaraes, I.M., Silva, F.R., Ribeiro, F.M., 2016. Alzheimer's disease: targeting the cholinergic system. Curr. Neuropharmacol. 14, 101–115. https://doi.org/10.2174/1570159X13666150716165726.

Flaskos, J., 2012. The developmental neurotoxicity of organophosphorus insecticides: a direct role for the oxon metabolites. Toxicol. Lett. 209, 86–93. https://doi.org/10.1016/j.toxlet.2011.11.026.

García-Gómez, B.E., Fernández-Gómez, F.J., Muñoz-Delgado, E., Buée, L., Blum, D., Vidal, C.J., 2016. 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. 58, 411–415. https://doi.org/10.1007/s12031-015-0699-y.

Gómez-Giménez, B., Llansola, M., Hernández-Rabaza, V., Cabrera-Pastor, A., Malaguarnera, M., Agusti, A., Felipo, V., 2017. Sex-dependent effects of developmental exposure to different pesticides on spatial learning. The role of induced neuroinflammation in the hippocampus. Food Chem. Toxicol. 99, 135–148. https://doi.org/10.1016/j.fct.2016.11.028.

González-Alzaga, B., Lacasaña, M., Aguilar-Garduño, C., Rodríguez-Barranco, M., Ballester, F., Rebagliato, M., Hernández, a. F., 2014. A systematic review of neurodevelopmental effects of prenatal and postnatal organophosphate pesticide exposure. Toxicol. Lett. 230, 104–121. https://doi.org/10.1016/j.toxlet.2013.11.019.

Gotti, C., Moretti, M., Gaimarri, A., Zanardi, A., Clementi, F., Zoli, M., 2007. Heterogeneity and complexity of native brain nicotinic receptors. Biochem. Pharmacol. 74, 1102–1111. https://doi.org/10.1016/j.bcp.2007.05.023.

Grisaru, D., Sternfeld, M., Eldor, A., Glick, D., Soreq, H., 1999. Structural roles of acetylcholinesterase variants in biology and pathology. Eur. J. Biochem. 264, 672–686. https://doi.org/10.1046/j.1432-1327.1999.00693.x.

Grootendorst, J., Bour, A., Vogel, E., Kelche, C., Sullivan, P.M., Dodart, J.-C., Bales, K., Mathis, C., 2005. Human apoE targeted replacement mouse lines: h-apoE4 and h-apoE3 mice differ on spatial memory performance and avoidance behavior. Behav. Brain Res. 159, 1–14. https://doi.org/10.1016/j.bbr.2004.09.019.

Hartmann, J., Kiewert, C., Duysen, E.G., Lockridge, O., Greig, N.H., Klein, J., 2007. Excessive hippocampal acetylcholine levels in acetylcholinesterase-deficient mice are moderated by butyrylcholinesterase activity. J. Neurochem. 100, 1421–1429. https://doi.org/10.1111/j.1471-4159.2006.04347.x.

Hrabovska, A., Krejci, E., 2014. Reassessment of the role of the central cholinergic system. J. Mol. Neurosci. 53, 352–358. https://doi.org/10.1007/s12031-013-0164-8.

Ito, H.T., 2018. Prefrontal–hippocampal interactions for spatial navigation. Neurosci. Res. 129, 2–7. https://doi.org/10.1016/j.neures.2017.04.016.

Jokanović, M., 2001. Biotransformation of organophosphorus compounds. Toxicology 166, 139–160. https://doi.org/10.1016/S0300-483X(01)00463-2.

Kitaichi, K., Hori, T., Srivastava, L.K., Quirion, R., 1999. Antisense oligodeoxynucleotides against the muscarinic m2, but not m4, receptor supports its role as autoreceptors in the rat hippocampus. Mol. Brain Res. 67, 98–106. https://doi.org/10.1016/S0169-328X(99)00047-9.

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. https://doi.org/10.1007/s12031-015-0524-7.

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, 12126–12131. https://doi.org/10.1073/pnas.96.21.12126.

López-Granero, C., Cardona, D., Giménez, E., Lozano, R., Barril, J., Aschner, M., Sánchez-Santed, F., Cañadas, F., 2014. Comparative study on short- and long-term behavioral consequences of organophosphate exposure: relationship to AChE mRNA expression. Neurotoxicology 40, 57–64. https://doi.org/10.1016/j.neuro.2013.11.004.

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, 41–49. https://doi.org/10.1016/j.tox.2013.03.009.

Mahley, R.W., 1988. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. Science 80 (240), 622–630. https://doi.org/10.1126/science. 3283935.

Mahley, R.W., Rall, S.C., 2000. Apolipoprotein E: far more than a lipid transport protein. Annu. Rev. Genom. Hum. Genet. 1, 507–537. https://doi.org/10.1146/annurev.genom.1.1.507.

Monfort, P., Gomez-Gimenez, B., Llansola, M., Felipo, V., 2015. Gender differences in spatial learning, synaptic activity, and long-term potentiation in the Hippocampus in rats: molecular mechanisms. ACS Chem. Neurosci. 6, 1420–1427. https://doi.org/10.1021/acschemneuro.5b00096.

Montes de Oca, L., Moreno, M., Cardona, D., Campa, L., Suñol, C., Galofré, M., Flores, P., Sánchez-Santed, F., 2013. Long term compulsivity on the 5-choice serial reaction time task after acute Chlorpyrifos exposure. Toxicol. Lett. 216, 73–85. https://doi.org/10.1016/j.toxlet.2012.11.012.

Moser, V.C., Padilla, S., 1998. Age-and gender-related differences in the time course of behavioral and biochemical effects produced by oral chlorpyrifos in rats. Toxicol. Appl. Pharmacol. 149, 107–119.

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. https://doi.org/10.1016/j.physbeh.2015. 03.006.

Peris-Sampedro, F., Blanco, J., Cabré, M., Basaure, P., Guardia-Escote, L., Domingo, J.L., Sánchez, D.J., Colomina, M.T., 2018. New mechanistic insights on the metabolic- disruptor role of chlorpyrifos in apoE mice: a focus on insulin- and leptin-signalling pathways. Arch. Toxicol. 92, 1717–1728. https://doi.org/10.1007/s00204-018- 2174-3.

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. https://doi.org/10.1016/j.envres.2015.06.036.

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. https://doi.org/10.1016/j.fct.2016.03. 029.

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. 72, 289–294. https://doi.org/10.1016/j.fct.2014.07.036.

Perrier, N.A., Salani, M., Falasca, C., Bon, S., Augusti-Tocco, G., Massoulié, J., 2005. The readthrough variant of acetylcholinesterase remains very minor after heat shock, organophosphate inhibition and stress, in cell culture and in vivo. J. Neurochem. 94, 629–638. https://doi.org/10.1111/j.1471-4159.2005.03140.x.

Piber, D., Nowacki, J., Mueller, S.C., Wingenfeld, K., Otte, C., 2018. Sex effects on spatial learning but not on spatial memory retrieval in healthy young adults. Behav. Brain Res. 336, 44–50. https://doi.org/10.1016/j.bbr.2017.08.034.

Raber, J., Wong, D., Buttini, M., Orth, M., Bellosta, S., Pitas, R.E., Mahley, R.W., Mucke, L., 1998. Isoform-specificeffects of human apolipoprotein E on brain function revealed in ApoE knockout mice: increased susceptibility of females. Proc. Natl. Acad. Sci. U.S.A. 95, 10914–10919.

Rauh, V.A., Garfinkel, R., Perera, F.P., Andrews, H.F., Hoepner, L., Barr, D.B., Whitehead, R., Tang, D., Whyatt, R.W., 2006. Impact of prenatal chlorpyrifos exposure on neurodevelopment in the first 3 Years of life among inner-city children. Pediatrics 118, e1845–e1859. https://doi.org/10.1542/peds.2006-0338.

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 trans- genic mice. Exp. Neurol. 237, 116–125. https://doi.org/10.1016/j.expneurol.2012. 06.015.

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 (Berlin) 233, 295–308. https://doi.org/10.1007/s00213-015-4113-9.

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. https://doi.org/10.1016/S0041-008X(03)00229-1.

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. https://doi.org/10.1093/toxsci/ kfl032.

Roldán-Tapia, L., Parrón, T., Sánchez-Santed, F., 2005. Neuropsychological effects of long-term exposure to organophosphate pesticides. Neurotoxicol. Teratol. 27, 259–266. https://doi.org/10.1016/j.ntt.2004.12.002.

Roses, A.D., 1996. Apolipoprotein E and Alzheimer's disease. A rapidly expanding field with medical and epidemiological consequences. Ann. N. Y. Acad. Sci. 802, 50–57.

Saucier, D.M., Shultz, S.R., Keller, A.J., Cook, C.M., Binsted, G., 2008. Sex differences in object location memory and spatial navigation in Long-Evans rats. Anim. Cognit. 11, 129–137. https://doi.org/10.1007/s10071-007-0096-1.

Schliebs, R., Arendt, T., 2011. The cholinergic system in aging and neuronal degeneration. Behav. Brain Res. 221, 555–563. https://doi.org/10.1016/j.bbr.2010.11.058.

Schliebs, R., Arendt, T., 2006. The significance of the cholinergic system in the brain during aging and in Alzheimer's disease. J. Neural. Transm. 113, 1625–1644. https://doi.org/10.1007/s00702-006-0579-2.

Slotkin, T.A., Brown, K.K., Seidler, F.J., 2005. Developmental exposures of rats to chlorpyrifos elicits sex-selective hyperlipidemia and hyperinsulinemia in adulthood. Environ. Health Perspect. 113, 1291–1294. https://doi.org/10.1289/ehp.8133.

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. https://doi.org/10.1016/S0006-8993(01)02387-3.

Slotkin, T.A., Southard, M.C., Adam, S.J., Cousins, M.M., Seidler, F.J., 2004. α7 Nicotinic acetylcholine receptors targeted by cholinergic developmental neurotoxicants: nicotine and chlorpyrifos. Brain Res. Bull. 64, 227–235. https://doi.org/10.1016/j.brainresbull.2004.07.005.

Soreq, H., Seidman, S., 2001. Acetylcholinesterase - new roles for an old actor. Nat. Rev. Neurosci. 2, 294–302. https://doi.org/10.1038/35067589.

Sullivan, P.M., Mezdour, H., Aratanti, Y., Knouff, C., Najib, J., Reddick, R.L., Quarfordt, S.H., Maeda, N., 1997. Targeted replacement of the mouse apolipoprotein E gene with the common human. J. Biol. Chem. 272, 17972–17980. https://doi.org/10.1074/jbc.272.29.17972.

Tsatsakis, A.M., Docea, A.O., Tsitsimpikou, C., 2016. New challenges in risk assessment of chemicals when simulating real exposure scenarios; simultaneous multi-chemicals' low

dose exposure. Food Chem. Toxicol. 96, 174–176. https://doi.org/10.1016/j.fct.2016.08.011.

Tsatsakis, A.M., Kouretas, D., Tzatzarakis, M.N., Stivaktakis, P., Tsarouhas, K., Golokhvast, K.S., Rakitskii, V.N., Tutelyan, V.A., Hernandez, A.F., Rezaee, R., Chung, G., Fenga, C., Engin, A.B., Neagu, M., Arsene, A.L., Docea, A.O., Gofita, E., Calina, D., Taitzoglou, I., Liesivuori, J., Hayes, A.W., Gutnikov, S., Tsitsimpikou, C., 2017. Simulating real-life exposures to uncover possible risks to human health: a proposed consensus for a novel methodological approach. Hum. Exp. Toxicol. 36, 554–564. https://doi.org/10.1177/0960327116681652.

Ungar, L., Altmann, A., Greicius, M.D., 2014. Apolipoprotein E, gender, and Alzheimer's disease: an overlooked, but potent and promising interaction. Brain Imag. Behav. 8, 262–273. https://doi.org/10.1007/s11682-013-9272-x.

Venerosi, A., Cutuli, D., Colonnello, V., Cardona, D., Ricceri, L., Calamandrei, G., 2008. Neonatal exposure to chlorpyrifos affects maternal responses and maternal aggression of female mice in adulthood. Neurotoxicol. Teratol. 30, 468–474. https://doi.org/10.1016/j.ntt.2008.07.002.

Whitney, K.D., Seidler, F.J., Slotkin, T.A., 1995. Developmental neurotoxicity of chlorpyrifos: cellular mechanisms. Toxicol. Appl. Pharmacol. 134, 53–62. https://doi.org/ 10.1006/taap.1995.1168.

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, 1–10. https://doi.org/10.1038/srep25023.

Zhang, W., Basile, A.S., Gomeza, J., Volpicelli, L.A., Levey, A.I., Wess, J., 2002. Characterization of central inhibitory muscarinic autoreceptors by the use of muscarinic acetylcholine receptor knock-out mice. J. Neurosci. 22, 1709–1717. https://doi.org/10.1523/jneurosci.22-05-01709.2002.

4. DISCUSSION

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4. DISCUSSION

This thesis was designed to investigate the short-, middle- and long-term effects of a postnatal exposure to the pesticide CPF in a humanized transgenic mouse model carrying the most frequent allelic forms of the *APOE* gene in humans, while evaluating sex differences in adults. The present study covers the effects of developmental exposure to CPF in three main stages of life. First, we evaluated the acute effects of CPF exposure on gut microbiota composition and cerebral SCFA concentration in infant male mice. Second, we assessed the long-term effects of CPF exposure on recognition memory in young adults and the involvement of the cholinergic and GABAergic systems. Both male and female apoE3-TR, apoE4-TR and C57BL/6 mice were included in the study. Third, we analyzed the long-term effects on spatial learning and memory and the integrity of the cholinergic system in both male and female apoE4-TR and C57BL/6 mice in middle age. This chapter discusses the results in an integrated manner and provides a longitudinal perspective for the effects of a limited postnatal exposure to CPF and how these are modulated by *APOE* genotype, age and/or sex. Also discussed are the limitations of the study and the future perspectives.

4.1. General discussion

In all experimental sections of this thesis, the *APOE* genotype has emerged as a consistent factor behind some of the differences observed between groups. These differences were observed both during development and in adulthood, though the contributions were different depending on the time point studied. Basal differences between genotypes in the composition of gut microbiota were observed as early as 15 days after birth. These included different levels of some of the species belonging to the phylum *Verrucomicrobia*. ApoE4 males presented a higher relative abundance of this species than apoE3 and C57BL/6 mice, with *A. muciniphila* being the most representative. On the other hand, apoE3 mice presented higher levels of the three most abundant SCFAs in the brain at the same early age. Young adults showed differences between genotypes in

recognition memory that were also influenced by the sex. At this stage of life, apoE4 mice performed best, while apoE3 mice failed to fulfill the task. ApoE4 mice also responded differently to the cholinergic and GABAergic drugs administered before the ORT task in a sex-dependent manner. In light of the above, we further analyzed the specific contribution of the *APOE4* genotype in a new study in which we compared middle-aged apoE4-TR and C57BL/6 mice. When we studied spatial learning and memory, we found that apoE4 mice, especially females, performed worse than wild type mice. Differences between apoE4 and C57BL/6 mice were observed in the gene expression of several cholinergic elements in a sex-dependent manner. These elements, studied in the frontal cortex and the hippocampus, included nicotinic and muscarinic receptors, the expression of BChE and the levels of AChE-R.

Differences in gut microbiota composition between *APOE* genotypes have recently been reported (Tran et al., 2019). However, to the best of our knowledge, this is the first study at such a young age. Numerous studies suggest that alterations in gut colonization at an early age can greatly affect health status (Borre et al., 2014; Zhuang et al., 2019). Moreover, a new role for gut microbiota in the regulation of behavior, especially during development, has been gaining ground. In fact, previous results have shown that early-life gut colonization can impact the programming of the brain circuits involved in the development of the brain and influence subsequent behavior (Desbonnet et al., 2015; Diaz Heijtz, 2016). Behaviors modulated by the composition of gut microbiota include social behaviors, motor activity and anxiety-like behaviors (Diaz Heijtz et al., 2011; Neufeld et al., 2011; Sudo et al., 2004). Diaz Heijtz et al. (2011), for example, reported differences in motor activity and anxiety-like behavior in germ-free (GF) mice compared to mice with normal microbiota. However, when the GF mice were exposed to gut microbiota early in life, they observed behaviors resembling those of the control mice (Diaz Heijtz et al., 2011). Recent studies have also reported the effects of gut microbiota on cognitive functions. Fröhlich et al. (2016), for example, observed a worse performance in the ORT by young

adults exposed to antibiotics. This disruption was associated with changes in the gene expression of the BDNF and NPY signaling pathways in the brain (Fröhlich et al., 2016). We can therefore hypothesize that differences in the composition of core microbiota between genotypes during development can have an impact on brain development and function later in life.

Similarly, differences in SCFA levels during development may also shape brain function at later ages. Although SCFAs levels in the gut are well defined, information about the levels of SCFA in the brain is still scarce. A correlation has been found between the amount of certain microorganisms in the gut and SCFAs in the brain (Li et al., 2018; Sun et al., 2016). Our study found several correlations between the composition of gut microbiota and cerebral SCFA levels. However, those correlations did not explain the differences between genotypes and treatment groups in the levels of SCFAs observed in the brain, which suggests that other sources or regulating mechanisms are involved. Other studies found direct correlation between gut microbiota and SCFA levels in the brain. For example, treatment with *Clostridium butyricum* increased the levels of butyrate in the brain, which is involved in the modulation of several cerebral functions (Li et al., 2018; Sun et al., 2016). More specifically, higher levels of butyrate in the brain conferred greater neuroprotection in a mouse model of traumatic brain injury (Li et al., 2018). In a similar study, higher butyrate attenuated cerebral ischemia/reperfusion injury in mice (Sun et al., 2016). The higher levels of SCFA observed in apoE3 mice in the present study may therefore indicate long-term neuroprotection against cognitive decline and AD later in life. Butyrate is also an inhibitor of histone deacetylase enzymes, which give this molecule an important role as an epigenetic regulator (Bourassa et al., 2016). Early and persistent differences between genotypes may therefore also be related to differences in butyrate levels.

Both the pharmacological challenge in young adults and the determination of gene expression in aged mice attempted to shed more light on the differences in the cholinergic system between genotypes. The different response to cholinergic drugs observed during the ORT can partly be explained by basal differences in certain cholinergic elements, such as those we observed in middle age. For instance, the lower sensitivity to scopolamine observed in apoE4 males can be explained by the fact that scopolamine is a cholinergic antagonist that targets the muscarinic receptors (Renner et al., 2005). Nine-month-old apoE4-TR males presented lower expression of these receptors in the frontal cortex and hippocampus, which suggests a possible causal explanation for the lower sensitivity. On the other hand, a different response to rivastigmine was observed depending on genotype and sex, with improved performance in males but a worse performance in apoE4 females. This effect can also be explained by differences in the cholinergic system functioning between groups. In fact, rivastigmine is a cholinergic agonist that may inhibit both AChE and BChE (Lane et al., 2004). In this context, we observed a lower expression of BChE in apoE4 females than in C57BL/6 females, which may explain the worse response to rivastigmine by this group. On the other hand, altered levels of cholinergic elements observed in the *APOE4* genotype corresponded to a worse performance on the MWM in middle age, thus demonstrating how important the cholinergic system is for cognition.

Deficits in the cholinergic system have previously been observed in the brain of patients suffering from dementia and AD. For example, reduced levels of ChAT were observed in the amygdala, hippocampus and cortex (Davies and Maloney, 1976) as well as in the parietal cortex and caudate nucleus (Perry et al., 1977). Lower levels of AChE (Davies and Maloney, 1976) and fewer cholinergic neurons in the nucleus basalis (Whitehouse et al., 1981) were also found in the brains of AD patients. Based on these observations, hypofunction of the cholinergic system was linked to age-related cognitive impairments. In fact, it was this association that led to the cholinergic hypothesis of AD in the 1970s,

which postulates that a dysfunction of cholinergic neurons in the brain is associated with cognitive decline in advanced age and AD (Bartus, 2000; Terry, 2003). Several pharmacological approaches were based on this hypothesis, all of which aimed to block the synaptic degradation of ACh by inhibiting the AChE (Hampel et al., 2018). The three most common drugs used for this purpose are donepezil, rivastigmine and galantamine (Khoury et al., 2018).

Hypofunction in the cholinergic system was also linked to ε4 carriers. For example, a decrease in metabolic activity of the cholinergic neurons of the nucleus basalis was observed in AD patients carrying at least one ε4 allele (Salehi et al., 1998). Another study observed altered distribution volumes of M2 mAChR in aging apoE4 subjects, which suggests that synaptic ACh has a lower concentration in those subjects (Cohen et al. 2003). The results of the present study are in line with these observations, since apoE4-TR mice presented lower levels of α7 nAChR in the frontal cortex and hippocampus in both males and females and lower levels of M1 and M2 mAChR in the frontal cortex of apoE4 males than C57BL/6. Lower levels of BChE were observed in the hippocampus of apoE4 females. These results support earlier observations regarding the cholinergic hypofunction on the brain of apoE4 subjects. However, apoE4 males presented enhanced levels of M2 mAChR and AChE-R in the hippocampus. M2 is normally found as a presynaptic autoreceptor that inhibits the release of ACh by negative feedback (Zhang et al., 2002), whereas AChE-R expression is rare and normally induced in response to multiple stress stimuli (Perrier et al., 2005).

Sex was not included in the first study. However, in the other two studies it emerged as an important factor. Note that the sex is not always considered in toxicological studies. Some authors avoid including females due to the variability induced by the estrous cycle. However, in the ORT we demonstrated that it was not a determinant factor influencing recognition memory. On the

other hand, throughout this thesis we have reported significant differences between males and females in several behavioral and biochemical aspects, so it is important to take into account this parameter. Especially important is the response of apoE4-TR females to rivastigmine, which we mentioned earlier. The fact that treatment led to a worsening in this group raises important questions, since rivastigmine is currently used to treat AD.

Females presented enhanced activity during habituation of the ORT and better task retention than their male counterparts. On the other hand, males performed better in the MWM. This is in line with previous results that reported that females had better recognition memory (Ghi et al., 1999; Sutcliffe et al., 2007) whereas males had better spatial memory (Monfort et al., 2015; Saucier et al., 2008). The worse performance of middle-aged apoE4 females is also in line with the higher incidence of AD in females (Raber et al., 1998; Ungar et al., 2014). In our study, we observed a better performance of young adult apoE4 females on ORT but a worse performance in middle age. This difference may be related to an age-dependent memory decline in apoE4-TR females (Bour et al., 2008). Impairments in both spatial and visual recognition memory have also been proposed as markers of early AD diagnosis (Grayson et al., 2015).

Developmental exposure to CPF has generated great interest in the last few decades (De Angelis et al., 2009; Ricceri et al., 2006, 2003; Slotkin and Seidler, 2007; Venerosi et al., 2008). Earlier research by our group has already demonstrated that exposure to low doses of CPF during the postnatal developmental period can affect normal development (Basaure et al., 2018) and behavioral processes later in life (Basaure et al., 2019). Differences observed between mice carrying the different isoforms of the *APOE* gene and between males and females collectively confer different vulnerabilities to the toxic effects of CPF (Basaure et al., 2019, 2018). Following this line of investigation, in this thesis we have assessed the effects of this complex interaction on other

behavioral and molecular aspects at different stages of life. The effects of postnatal exposure to CPF on the composition of microbiota four hours after the final dose showed that CPF can induce dysbiosis. ApoE4-TR mice were the most sensitive to its toxic effect since they presented greater alterations in microbiota composition than the other two genotypes. Postnatal exposure to CPF also tended to decrease the previously described basal differences between genotypes for *A. muciniphila* species by reducing its relative abundance levels. On the other hand, CPF exposure tended to alter the cerebral levels of certain SCFA in C57BL/6 and apoE3 but not in apoE4 mice at the same young age. In young adults, CPF impaired recognition memory in an ORT task but the apoE4 genotype was more susceptible to its detrimental effects. Middle-aged CPF-treated apoE4 females accounted for the worst acquisition in the MWM. CPF-treated apoE4 males presented worse retention than their controls whereas CPF led to an improvement in females.

In the present study, all animals were exposed to the pesticide from PND 10 to PND 15, which is an important period for murine brain maturation that in humans would correspond approximately to the last trimester of gestation and the first few months of life (Watson et al., 2006). Important processes such as neurogenesis, myelination and synaptogenesis take place during this period (Semple et al., 2013). Exposure to CPF during the second postnatal week was previously shown to induce long-term effects (Aldridge et al., 2004; Navarro et al., 2001; Slotkin et al., 2004, 2001). All these studies administered a dose of 5 mg/kg/day of CPF from PND 11 to PND 14. In comparison, the dose we have used in this study (1 mg/kg/day) is significantly lower, though it is still several times higher than the estimated average dose for infants, which is 0.009 µg/kg/day (Eaton et al., 2008).

The ability of CPF to induce dysbiosis has previously been described (Condette et al., 2015, 2014; Fang et al., 2018; Joly et al., 2013; Liang et al., 2019; Réquilé

et al., 2018; Reygner et al., 2016; Zhao et al., 2016). As we discussed above, early life alterations in gut composition and cerebral SCFAs can lead to a disruption in the brain's normal development. CPF-induced changes may determine the metabolic or cognitive disparities observed during the subject's lifespan. Note that the mucin-degrading species *A. muciniphila* (Derrien et al., 2004) is not only one of the hallmark differences between genotypes but it also represents one of the species most affected by the toxic effect of CPF. *A. muciniphila* has previously been linked to a healthier metabolic status (Dao et al., 2016). In this case, the higher relative abundance found in apoE4-TR mice would correspond to the leaner phenotype normally observed in apoE4 compared to apoE3 (Huebbe et al., 2015; Kypreos et al., 2009). Moreover, CPF is reported to trigger metabolic impairments (Slotkin, 2011; Slotkin et al., 2005) while apoE3 is reported to show greater vulnerability to those detrimental effects in adulthood (Peris-Sampedro et al., 2015a, 2015b). *A. muciniphila* has also been linked to pathologies such as autism (De Angelis et al., 2013) and Parkinson disease (Bedarf et al., 2017), since it has been found at high levels in the samples of patients. We can therefore hypothesize that *A. muciniphila* may influence some of the differences that are observed later in the lifes of apoE4 mice in comparison with apoE3 and C57BL/6 mice.

Interestingly, CPF reduced the levels of *A. muciniphila* only in apoE4 mice, which originally presented different basal levels of this species from those of the other genotypes. Our current results suggest that CPF was able to match the levels of the three genotypes. This is not the first time that CPF has normalized the abnormal apoE4 basal levels to make them resemble more those of the wild type or apoE3 mice. The same effect was observed, for instance, during the acquisition of the ORT task, where apoE4 females presented low basal levels of exploration but in the CPF-treated group these were increased to the same level as the others. Another example is found in the retention of MWM, where CPF-treated apoE4 females performed better than the non-treated group, which showed no preference for the target quadrant in any of the trials. These results

suggest that postnatal CPF exposure slightly improved the deteriorated condition of apoE4 females. This beneficial effect observed only in apoE4 mice may be linked to the cholinergic hypofunction we suggested earlier for the *APOE4* genotype. In fact, we suggest that exposure to CPF may inhibit the AChE enzyme during a critical developmental period, boost the cholinergic system, and compensate for some innate deficiencies in apoE4 mice.

In line with previous studies, CPF disrupted the behavioral tasks and impaired learning and memory. The general effects of the treatment were observed in the ORT task, where performance during retention was impaired. In the MWM, a tendency towards worse retention in the CPF-treated mice was also observed. With regard to gene expression, the differences induced by postnatal exposure to CPF nine months later were subtle. The most affected elements were the nAChRs, since differences in α7 nAChR and a tendency in α4 nAChR were observed. Previous studies reported CPF-induced alterations to the nAChR levels (Slotkin et al., 2004). In the present study, we found that only C57BL/6 females presented lower levels of α nAChRs in the frontal cortex nine month after the final dose of CPF. Our results show that CPF can lead to long-term effects on gene expression but also that postnatal exposure to moderate doses of CPF for a short time is not enough to cause important long-term differences after nine months.

From our current results, we can affirm that the *APOE* genotype confers significant gender-dependent differences on their carriers. Both the *APOE* genotype and sex modulate response to postnatal exposure to CPF in the various parameters studied. These results shed more light on the mechanisms behind the vulnerabilities described in this thesis. Interestingly, the *APOE4* genotype confers greater vulnerability to the effects of postnatal CPF on microbiota composition and learning and memory. These results highlight the impact of developmental exposure to CPF as well as the importance of considering the

genetic background and sex. This opens up future possibilities for a more personalized approach.

4.2. Limitations and future perspectives

The results of this thesis imply the need to address several limitations and future perspectives. Firstly, during the thesis the importance of sex in response to the pesticide became clear. Indeed, previous studies had already reported sex-dependent differences in, for example, learning and memory, social behaviors and anxiety (An et al., 2011; Jonasson, 2005; Saucier et al., 2008). One limitation of our first study is that only males were included in the assessment of gut microbiota and cerebral SCFAs. An early-life study of females would provide further insight into the sex-dependent maturation of gut microbiota and SCFA composition in the brain.

Secondly, the final part of this thesis included only apoE4-TR and C57BL/6 mice. Although we selected these two genotypes on the basis of previous results, significant information about apoE3-TR mice was excluded. Throughout the discussion of this thesis, however, the importance of genotype became clear. The final part of this study evaluated spatial learning and memory and assessed the gene expression of several cholinergic elements. We are therefore missing important information on behavioral performance and cholinergic integrity in apoE3-TR mice in comparison with the other two genotypes, which may be behind the previously observed behavioral differences.

We would like to make the following observations based on our results:

- We found differences in gut microbiota composition and cerebral SCFA levels at early ages that depend on *APOE4* genotype and postnatal treatment. As discussed earlier, it would be interesting to analyze these differences in a study

that includes females. It would also be interesting to assess how these differences evolve in order to determine whether they are maintained with age. Also, as the CPF-induced effects were evaluated four hours after exposure, it would be interesting to analyze whether these effects are acute or whether they are maintained permanently through life.

- Since we observed differences in *A. muciniphila,* which has been broadly linked to metabolic impairments, we intend to further study metabolic status by introducing a dietary challenge with a high-fat diet. We will also study feeding behavior in order to present a broad approach to energy homeostasis.

- We studied the effects of postnatal exposure to CPF and modulation by genotype and sex on spatial and recognition learning and memory. Changes in the expression of cholinergic components in both the frontal and hippocampal brain regions were also observed. We suggest including other behavioral tasks in order to assess behavioral flexibility and its possible relationship to the changes observed in the brain.

- We observed changes depending on age. However the oldest mice included in this study were nine months old. We therefore suggest including older age groups in order to further analyze the age-dependent effects of postnatal CPF exposure.

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5. CONCLUSIONS

Short-term effects.

1. The *APOE* genotype affects the composition of gut microbiota at early ages. ApoE4 male mice present greater differences in their core microbial communities than apoE3 and C57BL/6 male mice.

2. The relative abundance of *Akkermansia muciniphila* is influenced by *APOE* genotype and postnatal exposure to CPF. Specifically, the higher levels inherent in apoE4 mice are reduced in CPF-treated apoE4 mice, whose values resemble those obtained with other genotypes.

3. The concentration of SCFA in the brain is influenced by *APOE* genotype and postnatal exposure to CPF. Specifically, apoE3 mice present higher levels of acetic acid and butyric acid, while exposure to CPF increases the general concentration of isovaleric acid and 4-methylvaleric acid.

Mid-term effects.

4. Sex influences recognition memory in young adults. More specifically, females present higher activity during habituation and better discrimination during task retention than their male counterparts.

5. Basal differences between genotypes outline performance in the ORT task. ApoE3 mice fail to recognize the novel object during retention and none of the pharmacological conditions leads to any improvement.

6. Postnatal CPF exposure influences recognition memory in adulthood. CPF-treated mice present better exploratory behavior during acquisition and worse discrimination performance during retention in the ORT than controls.

7. The response to cholinergic agonist and antagonist drugs strongly depends on genotype and sex. ApoE4 mice are less sensitive to the effects of scopolamine. Rivastigmine improves performance during retention in apoE4 and C57BL/6 males but worsens performance in apoE4 females.

8. The administration of alprazolam affects recognition memory in a sex- and genotype-dependent manner. Alprazolam worsens retention in females, especially those in the apoE4 control group.

Long-term effects.

9. Spatial learning and memory are influenced by genotype and sex at nine months of age. Males present better learning than females, being especially evident in apoE4 mice, and both male and female C57BL/6 mice exhibited better performance in the retention part of the MWM.

10. Postnatal CPF exposure leads to long-term effects on spatial memory that depend on genotype and sex. Specifically, CPF exposure induces a worse performance in apoE4 males during retention but improves retention in apoE4 females in comparison with their apoE4 control counterparts.

11. The *APOE4* genotype presents lower levels of several elements of the cholinergic system in the frontal cortex in a sex-dependent manner. Both male and female apoE4 mice present lower expression levels of α7 nAChR, while apoE4 males present lower levels of M1 and M2 mAChR.

12. The *APOE4* genotype effects the gene expression of several elements of the cholinergic system in the hippocampus in a sex-dependent manner. Lower expression levels are observed in apoE4 males in relation to α7

nAChR and in apoE4 females in relation to BChE. ApoE4 males showed higher levels of M2 mAChR and AChE-R than their C57BL/6 counterparts.

13. Lower levels of α7 nAChR were observed in the frontal cortex of C57BL/6 females nine months after postnatal exposure to the pesticide CPF.

General conclusion.

Both *APOE* genotype and postnatal CPF exposure modulate early gut microbiota composition and cerebral SCFAs levels and produce long-lasting effects on learning and memory and the expression of cholinergic elements.

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6. REFERENCES

Abdel-Rahman AA, Blumenthal GM, Abou-Donia SA, et al (2002) Pharmacokinetic profile and placental transfer of a single intravenous injection of [14C] chlorpyrifos in pregnant rats. Arch Toxicol 76:452–459.

Abolaji AO, Ojo M, Afolabi TT, et al (2017) Protective properties of 6-gingerol-rich fraction from *Zingiber officinale* (Ginger) on chlorpyrifos-induced oxidative damage and inflammation in the brain, ovary and uterus of rats. Chem Biol Interact 270:15–23.

Abrahamsson TR, Jakobsson HE, Andersson AF, et al (2014) Low gut microbiota diversity in early infancy precedes asthma at school age. Clin Exp Allergy 44:842–850.

Abreu-Villaça Y, Filgueiras CC, Manhães AC (2011) Developmental aspects of the cholinergic system. Behav Brain Res 221:367–378.

Acker CI, Nogueira CW (2012) Chlorpyrifos acute exposure induces hyperglycemia and hyperlipidemia in rats. Chemosphere 89:602–608.

Ahn J, Sinha R, Pei Z, et al (2013) Human Gut Microbiome and Risk for Colorectal Cancer. J Natl Cancer Inst 105:1907–1911.

Al-Lahham SH, Peppelenbosch MP, Roelofsen H, et al (2010) Biological effects of propionic acid in humans; metabolism, potential applications and underlying mechanisms. Biochim Biophys Acta - Mol Cell Biol Lipids 1801:1175–1183.

Aldridge JE, Seidler FJ, Slotkin TA (2004) Developmental exposure to chlorpyrifos elicits sex-selective alterations of serotonergic synaptic function in adulthood: Critical periods and regional selectivity for effects on the serotinin transporter, receptor subtypes, and cell signaling. Environ Health Perspect 112:148–155.

Allen SJ, MacGowan SH, Tyler S, et al (1997) Reduced cholinergic function in normal and Alzheimer's disease brain is associated with apolipoprotein E4 genotype. Neurosci Lett 239:33–36.

An XL, Zou JX, Wu RY, et al (2011) Strain and sex Differences in Anxiety-Like and Social Behaviors in C57Bl/6J and BALB/cJ Mice. Exp Anim 60:111–123.

Arbones-Mainar JM, Johnson LA, Altenburg MK, 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.

Bäckhed F, Manchester JK, Semenkovich CF, Gordon JI (2007) Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci 104:979–984.

Balali-Mood M, Saber H (2012) Recent advances in the treatment of organophosphorous poisonings. Iran J Med Sci 37:74–91.

Bartus RT (2000) On neurodegenerative diseases, models, and treatment strategies: Lessons learned and lessons forgotten a generation following the cholinergic hypothesis. Exp Neurol 163:495–529.

REFERENCES

Basaure P, Guardia-Escote L, Cabré M, et al (2018) Postnatal chlorpyrifos exposure and apolipoprotein E *(APOE)* genotype differentially affect cholinergic expression and developmental parameters in transgenic mice. Food Chem Toxicol 118:42–52.

Basaure P, Guardia-Escote L, Cabré M, et al (2019) Learning, memory and the expression of cholinergic components in mice are modulated by the pesticide chlorpyrifos depending upon age at exposure and apolipoprotein E *(APOE)* genotype. Arch Toxicol 93:693–707.

Basu SK, Brown MS, Ho YK, et al (1981) Mouse macrophages synthesize and secrete a protein resembling apolipoprotein E. Proc Natl Acad Sci 78:7545–7549.

Bedarf JR, Hildebrand F, Coelho LP, et al (2017) Functional implications of microbial and viral gut metagenome changes in early stage L-DOPA-naïve Parkinson's disease patients. Genome Med 9:1–13.

Bekris LM, Yu C-E, Bird TD, Tsuang DW (2010) Genetics of Alzheimer Disease. J Geriatr Psychiatry Neurol 23:213–227.

Berteau-Pavy F, Park B, Raber J (2007) Effects of sex and *APOE* ε4 on object recognition and spatial navigation in the elderly. Neuroscience 147:6–17.

Boets E, Gomand S V., Deroover L, et al (2017) Systemic availability and metabolism of colonic-derived short-chain fatty acids in healthy subjects: a stable isotope study. J Physiol 595:541–555.

Borre YE, O'Keeffe GW, Clarke G, et al (2014) Microbiota and neurodevelopmental windows: implications for brain disorders. Trends Mol Med 20:509–518.

Bour A, Grootendorst J, Vogel E, et al (2008) Middle-aged human apoE4 targeted-replacement mice show retention deficits on a wide range of spatial memory tasks. Behav Brain Res 193:174–182.

Bourassa MW, Alim I, Bultman SJ, Ratan RR (2016) Butyrate, neuroepigenetics and the gut microbiome: Can a high fiber diet improve brain health? Neurosci Lett 625:56–63.

Brecht WJ, Harris FM, Chang S, et al (2004) Neuron-Specific Apolipoprotein E4 Proteolysis Is Associated with Increased Tau Phosphorylation in Brains of Transgenic Mice. J Neurosci 24:2527–2534.

Bronfman FC, Tesseur I, Hofker MH, et al (2000) No evidence for cholinergic problems in apolipoprotein E knockout and apolipoprotein E4 transgenic mice. Neuroscience 97:411–418.

Burke RD, Todd SW, Lumsden E, et al (2017) Developmental neurotoxicity of the organophosphorus insecticide chlorpyrifos: from clinical findings to preclinical models and potential mechanisms. J Neurochem 142:162–177.

Canfora EE, Jocken JW, Blaak EE (2015) Short-chain fatty acids in control of body weight and insulin sensitivity. Nat Rev Endocrinol 11:577–591.

Cardona D, López-Crespo G, Sánchez-Amate MC, et al (2011) Impulsivity as long-term sequelae after chlorpyrifos intoxication: Time course and individual differences. Neurotox Res 19:128–137.

Carr RL, Armstrong NH, Buchanan AT, et al (2017) Decreased anxiety in juvenile rats following exposure to low levels of chlorpyrifos during development. Neurotoxicology 59:183–190.

Carroll IM, Ringel-Kulka T, Siddle JP, Ringel Y (2012) Alterations in composition and diversity of the intestinal microbiota in patients with diarrhea-predominant irritable bowel syndrome. Neurogastroenterol Motil 24:521-e248.

Casida JE, Quistad GB (1998) Golden Age of Insecticide Research: Past, Present, or Future? Annu Rev Entomol 43:1–16.

Castellano JM, Kim J, Stewart FR, et al (2011) Human apoE Isoforms Differentially Regulate Brain Amyloid- Peptide Clearance. Sci Transl Med 3:89ra57.

Chen J, Li Q, Wang J (2011) Topology of human apolipoprotein E3 uniquely regulates its diverse biological functions. Proc Natl Acad Sci 108:14813–14818.

Chen WQ, Zhang YZ, Yuan L, et al (2014) Neurobehavioral evaluation of adolescent male rats following repeated exposure to chlorpyrifos. Neurosci Lett 570:76–80.

Chow J, Lee SM, Shen Y, et al (2010) Host-bacterial symbiosis in health and disease. Adv Immunol 107:243–274.

Clemente JC, Ursell LK, Parfrey LW, Knight R (2012) The impact of the gut microbiota on human health: An integrative view. Cell 148:1258–1270.

Cohen RM, Podruchny TA, Bokde ALW, et al (2003) Higher in vivo muscarinic-2 receptor distribution volumes in aging subjects with an apolipoprotein E-ε4 allele. Synapse 49:150–156.

Condette CJ, Bach V, Mayeur C, et al (2015) Chlorpyrifos Exposure During Perinatal Period Affects Intestinal Microbiota Associated with Delay of Maturation of Digestive Tract in Rats. J Pediatr Gastroenterol Nutr 61:30–40.

Condette CJ, Khorsi-Cauet H, Morlière P, et al (2014) Increased gut permeability and bacterial translocation after chronic chlorpyrifos exposure in rats. PLoS One 9:e102217.

Conejero-Goldberg C, Gomar JJ, Bobes-Bascaran T, et al (2014) APOE2 enhances neuroprotection against Alzheimer's disease through multiple molecular mechanisms. Mol Psychiatry 19:1243–1250.

Corder EH, Ghebremedhin E, Taylor MG, et al (2004) The Biphasic Relationship between Regional Brain Senile Plaque and Neurofibrillary Tangle Distributions: Modification by Age, Sex, and APOE Polymorphism. Ann N Y Acad Sci 1019:24–28.

Corder EH, Saunders AM, Risch NJ, et al (1994) Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer disease. Nat Genet 7:180–184.

Corder EH, Saunders AM, Strittmatter WJ, et al (1993) Gene Dose of Apolipoprotein E Type 4 Allele and the Risk of Alzheimer's Disease in Late Onset Families. Science 261:921–923.

Cosentino S, Scarmeas N, Helzner E, et al (2008) APOE Ε4 allele predicts faster cognitive decline in mild Alzheimer's disease. Neurology 70:1842–1849.

Costa LG (2006) Current issues in organophosphate toxicology. Clin Chim Acta 366:1–13.

Costa LG, Giordano G, Cole TB, et al (2013) Paraoxonase 1 (PON1) as a genetic determinant of susceptibility to organophosphate toxicity. Toxicology 307:115–122.

Croom EL, Wallace AD, Hodgson E (2010) Human variation in CYP-specific chlorpyrifos metabolism. Toxicology 276:184–191.

Dafnis I, Argyri L, Sagnou M, et al (2016) The ability of apolipoprotein E fragments to promote intraneuronal accumulation of amyloid beta peptide 42 is both isoform and size-specific. Sci Rep 6:1–15.

Dalile B, Van Oudenhove L, Vervliet B, Verbeke K (2019) The role of short-chain fatty acids in microbiota–gut–brain communication. Nat Rev Gastroenterol Hepatol 16:461–478.

Dao MC, Everard A, Aron-Wisnewsky J, et al (2016) *Akkermansia muciniphila* and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology. Gut 65:426–436.

Davies P, Maloney AJF (1976) Selective Loss of Central Cholinergic Neurons in Alzheimer's Disease. Lancet 308:1403.

De Angelis M, Piccolo M, Vannini L, et al (2013) Fecal Microbiota and Metabolome of Children with Autism and Pervasive Developmental Disorder Not Otherwise Specified. PLoS One 8:e76993.

De Angelis S, Tassinari R, Maranghi F, et al (2009) Developmental exposure to chlorpyrifos induces alterations in thyroid and thyroid hormone levels without other toxicity signs in Cd-1 mice. Toxicol Sci 108:311–319.

De Felice A, Venerosi A, Ricceri L, et al (2014) Sex-dimorphic effects of gestational exposure to the organophosphate insecticide chlorpyrifos on social investigation in mice. Neurotoxicol Teratol 46:32–39.

del Pino J, Moyano P, Anadon MJ, et al (2015) Acute and long-term exposure to chlorpyrifos induces cell death of basal forebrain cholinergic neurons through AChE variants alteration. Toxicology 336:1–9.

Derrien M, Vaughan EE, Plugge CM, de Vos WM (2004) *Akkermansia municiphila* gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. Int J Syst Evol Microbiol 54:1469–1476.

Desbonnet L, Clarke G, Traplin A, et al (2015) Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour. Brain Behav Immun 48:165–173.

Diaz Heijtz R (2016) Fetal, neonatal, and infant microbiome: Perturbations and subsequent effects on brain development and behavior. Semin Fetal Neonatal Med 21:410–417.

Diaz Heijtz R, Wang S, Anuar F, et al (2011) Normal gut microbiota modulates brain development and behavior. Proc Natl Acad Sci USA 108:3047–52.

Dolejší E, Liraz O, Rudajev V, et al (2016) Apolipoprotein E4 reduces evoked hippocampal acetylcholine release in adult mice. J Neurochem 136:503–509.

Dolgin E (2017) The greatest hits of the human genome. Nature 551:427–431.

Dong L, Weisgraber KH (1996) Human apolipoprotein E4 domain interaction. Arginine 61 and glutamic acid 255 interact to direct the preference for very low density lipoproteins. J Biol Chem 271:19053–19057.

Donohoe DR, Garge N, Zhang X, et al (2011) The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. Cell Metab 13:517–526.

Eaton DL, Daroff RB, Autrup H, et al (2008) Review of the Toxicology of Chlorpyrifos With an Emphasis on Human Exposure and Neurodevelopment. Crit Rev Toxicol 38:1–125.

Eckburg PB, M. Bik E, Bernstein C, et al (2005) Diversity of the Human Intestinal Microbial Flora. Science 308:1635–1638.

EFSA - European Food Safety Authority (2014) Conclusion on the peer review of the pesticide human health risk assessment of the active substance chlorpyrifos. EFSA J 12:3640, 34pp.

Eftekhari A, Ahmadian E, Azami A, et al (2018) Protective effects of coenzyme Q10 nanoparticles on dichlorvos-induced hepatotoxicity and mitochondrial/lysosomal injury. Environ Toxicol 33:167–177.

Eisenberg DTA, Kuzawa CW, Hayes MG (2010) Worldwide allele frequencies of the human apolipoprotein E gene: Climate, local adaptations, and evolutionary history. Am J Phys Anthropol 143:100–111.

Ellis JA, Ponsonby AL, Pezic A, et al (2011) *APOE* genotype and Cardio-Respiratory fitness interact to determine adiposity in 8-Year-Old children from the Tasmanian infant health survey. PLoS One 6:e26679.

Ellman GL, Courtney KD, Andres V, Featherstone RM (1961) A new and Rapid Colorimetric Determination of Acetylcholinesterase Activity. Biochem Pharmacol 7:88–95.

Elsharkawy EE, Yahia D, El-Nisr NA (2013) Sub-chronic exposure to chlorpyrifos induces hematological, metabolic disorders and oxidative stress in rat: Attenuation by glutathione. Environ Toxicol Pharmacol 35:218–227.

Elshourbagy NA, Liao WS, Mahley RW, Taylor JM (1985) Apolipoprotein E mRNA is abundant in the brain and adrenals, as well as in the liver, and is present in other peripheral tissues of rats and marmosets. Proc Natl Acad Sci USA 82:203–7

Engstrom AK, Snyder JM, Maeda N, Xia Z (2017) Gene-environment interaction between lead and Apolipoprotein E4 causes cognitive behavior deficits in mice. Mol Neurodegener 12:1–23.

Ezzi L, Belhadj Salah I, Haouas Z, et al (2016) Histopathological and genotoxic effects of chlorpyrifos in rats. Environ Sci Pollut Res 23:4859–4867.

Fang B, Li JW, Zhang M, et al (2018) Chronic chlorpyrifos exposure elicits diet-specific effects on metabolism and the gut microbiome in rats. Food Chem Toxicol 111:144–152.

FAO Statistics Division, 2015. Pesticides use in agricultural use in tonnes by country at <http://www.fao.org/faostat/en/#data/RP> (accessed 22.07.19)

Ferreira-Vieira TH, Guimaraes IM, Silva FR, Ribeiro FM (2016) Alzheimer's disease: Targeting the Cholinergic System. Curr Neuropharmacol 14:101–115.

Finch CE, Sapolsky RM (1999) The evolution of Alzheimer disease, the reproductive schedule, and apoE isoforms. Neurobiol Aging 20:407–428

Flaskos J (2012) The developmental neurotoxicity of organophosphorus insecticides: A direct role for the oxon metabolites. Toxicol Lett 209:86–93.

Fleisher A, Grundman M, Clifford R. J, et al (2005) Sex, Apolipoprotein E ε4 Status, and Hippocampal Volume in Mild Cognitive Impairment. Arch Neurol 62:953-957.

Fröhlich EE, Farzi A, Mayerhofer R, et al (2016) Cognitive impairment by antibiotic-induced gut dysbiosis: Analysis of gut microbiota-brain communication. Brain Behav Immun 56:140–155.

Frost G, Sleeth ML, Sahuri-Arisoylu M, et al (2014) The short-chain fatty acid acetate reduces appetite via a central homeostatic mechanism. Nat Commun 5:3611.

Furlong CE, Cole TB, Jarvik GP, et al (2005) Role of paraoxonase (PON1) status in pesticide sensitivity: Genetic and temporal determinants. Neurotoxicology 26:651–659.

Ghi P, Orsetti M, Gamalero SR, Ferretti C (1999) Sex differences in memory performance in the object recognition test. Possible role of histamine receptors. Pharmacol Biochem Behav 64:761–766.

Golubeva A V., Crampton S, Desbonnet L, et al (2015) Prenatal stress-induced alterations in major physiological systems correlate with gut microbiota composition in adulthood. Psychoneuroendocrinology 60:58–74.

Gómez-Giménez B, Felipo V, Cabrera-Pastor A, et al (2018) Developmental Exposure to Pesticides Alters Motor Activity and Coordination in Rats: Sex Differences and Underlying Mechanisms. Neurotox Res 33:247–258.

Gómez-Giménez B, Llansola M, Hernández-Rabaza V, et al (2017) Sex-dependent effects of developmental exposure to different pesticides on spatial learning. The role of induced neuroinflammation in the hippocampus. Food Chem Toxicol 99:135–148.

Gorecki L, Korabecny J, Musilek K, et al (2016) SAR study to find optimal cholinesterase reactivator against organophosphorous nerve agents and pesticides. Arch Toxicol 90:2831–2859.

Gottlieb DJ, DeStefano AL, Foley DJ, et al (2004) *APOE* ε4 is associated with obstructive sleep apnea/hypopnea: The Sleep Heart Health Study. Neurology 63:664–668.

Graybeal JJ, Bozzelli PL, Graybeal LL, et al (2015) Human ApoE ε4 alters circadian rhythm activity, IL-1β, and GFAP in CRND8 mice. J Alzheimer's Dis 43:823–834.

Grayson B, Leger M, Piercy C, et al (2015) Assessment of disease-related cognitive impairments using the novel object recognition (NOR) task in rodents. Behav Brain Res 285:176–193.

Griffin P, Mason H, Heywood K, Cocker J (1999) Oral and dermal absorption of chlorpyrifos: a human volunteer study. Occup Environ Med 56:10–13.

Grisaru D, Sternfeld M, Eldor A, et al (1999) Structural roles of acetylcholinesterase variants in biology and pathology. Eur J Biochem 264:672–686.

Grootendorst J, Bour A, Vogel E, et al (2005) Human apoE targeted replacement mouse lines: h-apoE4 and h-apoE3 mice differ on spatial memory performance and avoidance behavior. Behav Brain Res 159:1–14.

Guo-Ross SX, Chambers JE, Meek EC, Carr RL (2007) Altered Muscarinic Acetylcholine Receptor Subtype Binding in Neonatal Rat Brain following Exposure to Chlorpyrifos or Methyl Parathion. Toxicol Sci 100:118–127.

Guo J, Zhang J, Wu C, et al (2019) Associations of prenatal and childhood chlorpyrifos exposure with Neurodevelopment of 3-year-old children. Environ Pollut 251:538–546.

Hampel H, Mesulam MM, Cuello AC, et al (2018) The cholinergic system in the pathophysiology and treatment of Alzheimer's disease. Brain 141:1917–1933.

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

Hartman RE, Wozniak DF, Nardi A, et al (2001) Behavioral phenotyping of GFAP-ApoE3 and -ApoE4 transgenic mice: ApoE4 mice show profound working memory impairments in the absence of Alzheimer's-like neuropathology. Exp Neurol 170:326–344.

Hawrelak JA, Myers SP (2004) The causes of intestinal dysbiosis: a review. Altern Med Rev 9:180–97

Holtzman DM, Bales KR, Wu S, et al (1999) Expression of human apolipoprotein E reduces amyloid-β deposition in a mouse model of Alzheimer's disease. J Clin Invest 103:15–21

Howell GE, Kondakala S, Holdridge J, et al (2018) Inhibition of cholinergic and non-cholinergic targets following subacute exposure to chlorpyrifos in normal and high fat fed male C57BL/6J mice. Food Chem Toxicol 118:821–829.

Hrabovska A, Krejci E (2014) Reassessment of the Role of the Central Cholinergic System. J Mol Neurosci 53:352–358.

Huang Y, Liu XQ, Wyss-Coray T, et al (2001) Apolipoprotein E fragments present in Alzheimer's disease brains induce neurofibrillary tangle-like intracellular inclusions in neurons. Proc Natl Acad Sci 98:8838–8843.

Huang Y, Mahley RW (2014) Apolipoprotein E: Structure and function in lipid metabolism, neurobiology, and Alzheimer's diseases. Neurobiol Dis 72:3–12.

Huebbe P, Dose J, Schloesser A, et al (2015) Apolipoprotein E *(APOE)* genotype regulates body weight and fatty acid utilization - Studies in gene-targeted replacement mice. Mol Nutr Food Res 59:334–343.

Huff R a., Abu-Qare AW, Abou-Donia MB (2001) Effects of sub-chronic in vivo chlorpyrifos exposure on muscarinic receptors and adenylate cyclase of rat striatum. Arch Toxicol 75:480–486.

Jiang S, Li Y, Zhang C, et al (2014) M1 muscarinic acetylcholine receptor in Alzheimer's disease. Neurosci Bull 30:295–307.

Joly C, Gay-Quéheillard J, Léké A, et al (2013) Impact of chronic exposure to low doses of chlorpyrifos on the intestinal microbiota in the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) and in the rat. Environ Sci Pollut Res 20:2726–2734.

Jonasson Z (2005) Meta-analysis of sex differences in rodent models of learning and memory: a review of behavioral and biological data. Neurosci Biobehav Rev 28:811–825.

Kang DW, Park JG, Ilhan ZE, et al (2013) Reduced Incidence of *Prevotella* and Other Fermenters in Intestinal Microflora of Autistic Children. PLoS One 8:e68322.

Karagiannides I, Abdou R, Tzortzopoulou A, et al (2008) Apolipoprotein E predisposes to obesity and related metabolic dysfunctions in mice. FEBS J 275:4796–4809.

Karalliedde LD, Edwards P, Marrs TC (2003) Variables influencing the toxic response to organophosphates in humans. Food Chem Toxicol 41:1–13.

Karanth S, Liu J, Mirajkar N, Pope C (2006) Effects of acute chlorpyrifos exposure on in vivo acetylcholine accumulation in rat striatum. Toxicol Appl Pharmacol 216:150–156.

Katsimichas T, Antonopoulos AS, Katsimichas A, et al (2019) The intestinal microbiota and cardiovascular disease. Cardiovasc Res 115:1471-1486.

Khoury R, Rajamanickam J, Grossberg GT (2018) An update on the safety of current therapies for Alzheimer's disease: focus on rivastigmine. Ther Adv Drug Saf 9:171–178.

Kim J, Yoon H, Basak J, Kim J (2014) Apolipoprotein E in Synaptic Plasticity and Alzheimer's Disease: Potential Cellular and Molecular Mechanisms. Mol Cells 37:767–776.

Koch H, Haas S, Jurgens T (2005) On the physiological relevance of muscarinic acetylcholine receptors in Alzheimer's disease. Curr Med Chem 12:2915–2921

Kulminski AM, Loika Y, Culminskaya I, et al (2019) Independent associations of *TOMM40* and *APOE* variants with body mass index. Aging Cell 18: e12869.

Kypreos KE, Karagiannides I, Fotiadou EH, et al (2009) Mechanisms of obesity and related pathologies: Role of apolipoprotein E in the development of obesity. FEBS J 276:5720–5728.

Lan A, Stein D, Portillo M, et al (2019) Impaired innate and conditioned social behavior in adult C57Bl6/J mice prenatally exposed to chlorpyrifos. Behav Brain Funct 15:1–9.

Lane RM, Kivipelto M, Greig NH (2004) Acetylcholinesterase and its inhibition in Alzheimer disease. Clin Neuropharmacol 27:141–149

Lasrich D, Bartelt A, Grewal T, Heeren J (2015) Apolipoprotein E promotes lipid accumulation and differentiation in human adipocytes. Exp Cell Res 337:94–102.

Lassiter TL, Brimijoin S (2008) Rats gain excess weight after developmental exposure to the organophosphorothionate pesticide, chlorpyrifos. Neurotoxicol Teratol 30:125–130.

Lee RS, Pirooznia M, Guintivano J, et al (2015) Search for common targets of lithium and valproic acid identifies novel epigenetic effects of lithium on the rat leptin receptor gene. Transl Psychiatry 5:1–10.

Lee YS, Lewis JA, Ippolito DL, et al (2016) Repeated exposure to neurotoxic levels of chlorpyrifos alters hippocampal expression of neurotrophins and neuropeptides. Toxicology 340:53–62.

Levin ED, Addy N, Nakajima A, et al (2001) Persistent behavioral consequences of neonatal chlorpyrifos exposure in rats. Dev Brain Res 130:83–89.

Li H, Sun J, Du J, et al (2018) *Clostridium butyricum* exerts a neuroprotective effect in a mouse model of traumatic brain injury via the gut-brain axis. Neurogastroenterol Motil 30:e13260.

Liang Y, Zhan J, Liu D, et al (2019) Organophosphorus pesticide chlorpyrifos intake promotes obesity and insulin resistance through impacting gut and gut microbiota. Microbiome 7:19.

Libeu CP, Lund-Katz S, Phillips MC, et al (2001) New Insights into the Heparan Sulfate Proteoglycan-binding Activity of Apolipoprotein E. J Biol Chem 276:39138–39144.

Liu J, Sun J, Wang F, et al (2015) Neuroprotective Effects of *Clostridium butyricum* against Vascular Dementia in Mice via Metabolic Butyrate. Biomed Res Int 2015: 412946.

Liu M, Kuhel DG, Shen L, et al (2012) Apolipoprotein E does not cross the blood-cerebrospinal fluid barrier, as revealed by an improved technique for sampling CSF from mice. Am J Physiol Integr Comp Physiol 303:903–908.

López-Crespo GA, Carvajal F, Flores P, et al (2007) Time course of biochemical and behavioural effects of a single high dose of chlorpyrifos. Neurotoxicology 28:541–547.

López-Granero C, Cardona D, Giménez E, et al (2013) Chronic dietary exposure to chlorpyrifos causes behavioral impairments, low activity of brain membrane-bound acetylcholinesterase, and increased brain acetylcholinesterase-R mRNA. Toxicology 308:41–49.

López-Granero C, Cardona D, Giménez E, et al (2014) Comparative study on short- and long-term behavioral consequences of organophosphate exposure: Relationship to AChE mRNA expression. Neurotoxicology 40:57-64

López-Granero C, Ruiz-Muñoz AM, Nieto-Escámez FA, et al (2016) Chronic dietary chlorpyrifos causes long-term spatial memory impairment and thigmotaxic behavior. Neurotoxicology 53:85–92.

MacFabe DF (2015) Enteric short-chain fatty acids: microbial messengers of metabolism, mitochondria, and mind: implications in autism spectrum disorders. Microb Ecol Heal Dis 26:1–14.

Mackay D, Giesy JP, Solomon KR (2014) Fate in the Environment and Long-Range Atmospheric Transport of the Organophosphorus Insecticide, Chlorpyrifos and Its Oxon, in: Giesy JP and Solomon KR (eds), Ecological Risk Assessment for Chlorpyrifos in Terrestrial and Aquatic Systems in the United States. Reviews of Environmental Contamination and Toxicology 231, pp 35–76

Maeda N (2011) Development of Apolipoprotein E–Deficient Mice. Arterioscler Thromb Vasc Biol 31:1957–1962.

Mahley RW (1988) Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. Science 240:622–630.

Mahley RW (2017) Apolipoprotein E: Remarkable protein sheds light on cardiovascular and neurological diseases. Clin Chem 63:14–20.

Mahley RW (2016) Central Nervous System Lipoproteins: ApoE and Regulation of Cholesterol Metabolism. Arterioscler Thromb Vasc Biol 36:1305–1315.

Mahley RW, Huang Y (2012) Apolipoprotein E Sets the Stage: Response to Injury Triggers Neuropathology. Neuron 76:871–885.

Mahley RW, Huang Y (2007) Atherogenic remnant lipoproteins: role for proteoglycans in trapping, transferring, and internalizing. J Clin Invest 117:94–98.

Mahley RW, Huang Y, Rall SC (1999) Pathogenesis of type III hyperlipoproteinemia (dysbetalipoproteinemia): questions, quandaries, and paradoxes. J Lipid Res 40:1933–1949

Mahley RW, Innerarity TL, Rall SC, Weisgraber KH (1984) Plasma lipoproteins: apolipoprotein structure and function. J Lipid Res 25:1277–1294

Mahley RW, Rall Jr. SC (1999) Is epsilon4 the ancestral human apoE allele? Neurobiol Aging 20:429–430

Manichanh C, Rigottier-Gois L, Bonnaud E, et al (2006) Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut 55:205–211.

Mansour SA, Mossa AH (2010) Adverse effects of lactational exposure to chlorpyrifos in suckling rats. Hum Exp Toxicol 29:77–92.

Marizzoni M, Provasi S, Cattaneo A, Frisoni GB (2017) Microbiota and neurodegenerative diseases. Curr Opin Neurol 30:630–638.

Marsillach J, Costa LG, Furlong CE (2013) Protein adducts as biomarkers of exposure to organophosphorus compounds. Toxicology 307:46–54.

Marsillach J, Costa LG, Furlong CE (2016) Paraoxonase-1 and Early-Life Environmental Exposures. Ann Glob Heal 82:100–110.

Maslowski KM (2019) Metabolism at the centre of the host–microbe relationship. Clin Exp Immunol 197:193-204.

Mayeux R, Small SA, Tang M-X, et al (2001) Memory performance in healthy elderly without Alzheimer's disease: effects of time and apolipoprotein-E. Neurobiol Aging 22:683–689.

Meggs WJ, Brewer KL (2007) Weight gain associated with chronic exposure to chlorpyrifos in rats. J Med Toxicol 3:89–93.

Meng F-TT, Zhao J, Fang H, et al (2017) Upregulation of Mineralocorticoid Receptor in the Hypothalamus Associated with a High Anxiety-like Level in Apolipoprotein E4 Transgenic Mice. Behav Genet 47:416–424.

Middlemore-Risher ML, Buccafusco JJ, Terry A V. (2010) Repeated exposures to low-level chlorpyrifos results in impairments in sustained attention and increased impulsivity in rats. Neurotoxicol Teratol 32:415–424.

Miller AW, Orr T, Dearing D, Monga M (2019) Loss of function dysbiosis associated with antibiotics and high fat, high sugar diet. ISME J 13:1379–1390.

Ministerio de agricultura, pesca y alimentación, 2019. Registro de productos fitosanitarios at <https://www.mapa.gob.es/es/agricultura/temas/sanidad-vegetal/productos-fitosanitarios/registro/productos/consusact.asp> (accessed 23.07.19)

Mohammadzadeh L, Hosseinzadeh H, Abnous K, Razavi BM (2018) Neuroprotective potential of crocin against malathion-induced motor deficit and neurochemical alterations in rats. Environ Sci Pollut Res 25:4904–4914.

Monfort P, Gomez-Gimenez B, Llansola M, Felipo V (2015) Gender Differences in Spatial Learning, Synaptic Activity, and Long-Term Potentiation in the Hippocampus in Rats: Molecular Mechanisms. ACS Chem Neurosci 6:1420–1427.

Montes de Oca L, Moreno M, Cardona D, et al (2013) Long term compulsivity on the 5-choice serial reaction time task after acute Chlorpyrifos exposure. Toxicol Lett 216:73–85.

Morgan MK, Sheldon LS, Jones PA, et al (2011) The reliability of using urinary biomarkers to estimate children's exposures to chlorpyrifos and diazinon. J Expo Sci Environ Epidemiol 21:280–290.

Morrison DJ, Preston T (2016) Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes 7:189–200.

Morrow JA, Arnold KS, Dong J, et al (2000a) Effect of Arginine 172 on the Binding of Apolipoprotein E to the Low Density Lipoprotein Receptor. J Biol Chem 275:2576–2580.

Morrow JA, Segall ML, Lund-Katz S, et al (2000b) Differences in Stability among the Human Apolipoprotein E Isoforms Determined by the Amino-Terminal Domain. Biochemistry 39:11657–11666.

Moser VC (1998) Age- and Gender-Related Differences in Sensitivity to Chlorpyrifos in the Rat Reflect Developmental Profiles of Esterase Activities. Toxicol Sci 46:211–222.

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

Moser VC, Padilla S (1998) Age- and gender-related differences in the time course of behavioral and biochemical effects produced by oral chlorpyrifos in rats. Toxicol Appl Pharmacol 149:107–119

Mostafalou S, Abdollahi M (2018) The link of organophosphorus pesticides with neurodegenerative and neurodevelopmental diseases based on evidence and mechanisms. Toxicology 409:44–52.

Muir JL (1997) Acetylcholine, Aging, and Alzheimer's Disease. Pharmacol Biochem Behav 56:687–696.

Nair A, Jacob S (2016) A simple practice guide for dose conversion between animals and human. J Basic Clin Pharm 7:27-31.

National Center for Biotechnology Information, 2019. PubChem Compound database at <https://pubchem.ncbi.nlm.nih.gov/compound/2730> (accessed 25.07.19)

Naughton SX, Terry AV (2018) Neurotoxicity in acute and repeated organophosphate exposure. Toxicology 408:101–112.

Navarro HA, Basta PV, Seidler FJ, Slotkin TA (2001) Neonatal chlorpyrifos administration elicits deficits in immune function in adulthood: A neural effect? Dev Brain Res 130:249–252.

Neufeld KM, Kang N, Bienenstock J, Foster JA (2011) Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterol Motil 23:255–e119.

Nicholson JK, Holmes E, Kinross J, et al (2012) Host-Gut Microbiota Metabolic Interactions. 336:1262–1267.

Nishitsuji K, Hosono T, Nakamura T, et al (2011) Apolipoprotein E regulates the integrity of tight junctions in an isoform-dependent manner in an *in vitro* blood-brain barrier model. J Biol Chem 286:17536–17542.

Nostrandt AC, Padilla S, Moser VC (1997) The relationship of oral chlorpyrifos effects on behavior, cholinesterase inhibition, and muscarinic receptor density in rat. Pharmacol Biochem Behav 58:15–23.

Pallotta MM, Ronca R, Carotenuto R, et al (2017) Specific effects of chronic dietary exposure to chlorpyrifos on brain gene expression - A mouse study. Int J Mol Sci 18:2467.

Palmer C, Bik EM, DiGiulio DB, et al (2007) Development of the human infant intestinal microbiota. PLoS Biol 5:1556–1573.

Peris-Sampedro F, Basaure P, Reverte I, et al (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, Blanco J, Cabré M, et al (2018) New mechanistic insights on the metabolic-disruptor role of chlorpyrifos in apoE mice: a focus on insulin- and leptin-signalling pathways. Arch Toxicol 92:1717–1728.

Peris-Sampedro F, Cabré M, Basaure P, et al (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, et al (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 JG, Cabré M, et al (2014) Impaired retention in AβPP Swedish mice six months after oral exposure to chlorpyrifos. Food Chem Toxicol 72:289–294.

Perrier NA, Salani M, Falasca C, et al (2005) The readthrough variant of acetylcholinesterase remains very minor after heat shock, organophosphate inhibition and stress, in cell culture and in vivo. J Neurochem 94:629–638.

Perry E, Perry R, Blessed G, Tomlinson B (1977) Necropsy evidence of central cholinergic deficits in senile dementia. Lancet 309:189.

Petersen C, Round JL (2014) Defining dysbiosis and its influence on host immunity and disease. Cell Microbiol 16:1024–1033.

Phillips MC (2014) Apolipoprotein E isoforms and lipoprotein metabolism. IUBMB Life 66:616–623.

Pitas RE, Boyles JK, Lee SH, et al (1987) Astrocytes synthesize apolipoprotein E and metabolize apolipoprotein E-containing lipoproteins. Biochim Biophys Acta 917:148–161.

Poirier J, Delisle MC, Quirion R, et al (1995) Apolipoprotein E4 allele as a predictor of cholinergic deficits and treatment outcome in Alzheimer disease. Proc Natl Acad Sci 92:12260–12264.

Pontifex M, Vauzour D, Minihane AM (2018) The effect of *APOE* genotype on Alzheimer's disease risk is influenced by sex and docosahexaenoic acid status. Neurobiol Aging 69:209–220.

Pope CN (1999) Organophosphorus pesticides: do they all have the same mechanism of toxicity? J Toxicol Environ Heal Part B: Critical Reviews 2:161–181.

Pope CN, Chakraborti TK, Chapman ML, et al (1991) Comparison of in vivo cholinesterase inhibition in neonatal and adult rats by three organophosphorothioate insecticides. Toxicology 68:51–61.

Pouteau E, Nguyen P, Ballevre O, Krempf M (2003) Production rates and metabolism of short-chain fatty acids in the colon and whole body using stable isotopes. Proc Nutr Soc 62:87–93.

Prada D, Colicino E, Power MC, et al (2016) *APOE* ε4 allele modifies the association of lead exposure with age-related cognitive decline in older individuals. Environ Res 151:101–105.

Prado VF, Roy A, Kolisnyk B, et al (2013) Regulation of cholinergic activity by the vesicular acetylcholine transporter. Biochem J 450:265-274.

Raber J, Wong D, Buttini M, et al (1998) Isoform-specific effects of human apolipoprotein E on brain function revealed in *ApoE* knockout mice: Increased susceptibility of females. Proc Natl Acad Sci USA 95:10914–10919.

Rall SC, Weisgraber KH, Mahley W (1982) Human Apolipoprotein E. The complete amino acid sequence. J Biol Chem 257:4171–4178.

Rauh V, Arunajadai S, Horton M, et al (2015) Seven-year neurodevelopmental scores and prenatal exposure to chlorpyrifos, a common agricultural pesticide. Environ Health Perspect 119:1196-201.

Rauh VA, Garfinkel R, Perera FP, et al (2006) Impact of Prenatal Chlorpyrifos Exposure on Neurodevelopment in the First 3 Years of Life Among Inner-City Children. Pediatrics 118:e1845–e1859.

Reiss R, Chang ET, Richardson RJ, Goodman M (2015) A review of epidemiologic studies of low-level exposures to organophosphorus insecticides in non-occupational populations. Crit Rev Toxicol 45:531–641.

Reitz C, Mayeux R (2014) Alzheimer disease: Epidemiology, diagnostic criteria, risk factors and biomarkers. Biochem Pharmacol 88:640–651.

Renner UD, Oertel R, Kirch W (2005) Pharmacokinetics and pharmacodynamics in clinical use of scopolamine. Ther Drug Monit 27:655–665.

Réquilé M, Gonzàlez Alvarez DO, Delanaud S, et al (2018) Use of a combination of in vitro models to investigate the impact of chlorpyrifos and inulin on the intestinal microbiota and the permeability of the intestinal mucosa. Environ Sci Pollut Res 25:22529–22540.

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

Reverte I, Klein AB, Domingo JL, Colomina MT (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 AB, Ratner C, et al (2012) Behavioral phenotype and BDNF differences related to apoE isoforms and sex in young transgenic mice. Exp Neurol 237:116–125.

Reverte I, Peris-Sampedro F, Basaure P, et al (2016) Attentional performance, impulsivity, and related neurotransmitter systems in apoE2, apoE3, and apoE4 female transgenic mice. Psychopharmacology 233:295–308.

Reverte I, Pujol A, Domingo JL, Colomina MT (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.

Reygner J, Condette CJ, Bruneau A, et al (2016a) Changes in composition and function of human intestinal microbiota exposed to chlorpyrifos in oil as assessed by the SHIME® model. Int J Environ Res Public Health 13:1088.

Reygner J, Lichtenberger L, Elmhiri G, et al (2016b) Inulin supplementation lowered the metabolic defects of prolonged exposure to chlorpyrifos from gestation to young adult stage in offspring rats. PLoS One 11: e0164614.

Reynolds CA, Prince JA, Feuk L, et al (2006) Longitudinal memory performance during normal aging: Twin association models of APOE and other Alzheimer candidate genes. Behav Genet 36:185–194.

Ricceri L, Markina N, Valanzano A, et al (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, et al (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 JR, Chambers JE (2005) Effects of repeated oral postnatal exposure to chlorpyrifos on cholinergic neurochemistry in developing rats. Toxicol Sci 84:352–359.

Richardson JR, Chambers JE (2003) Effects of gestational exposure to chlorpyrifos on postnatal central and peripheral cholinergic neurochemistry. J Toxicol Environ Heal - Part A 66:275–289.

Richardson JR, Chambers JE (2004) Neurochemical effects of repeated gestational exposure to chlorpyrifos in developing rats. Toxicol Sci 77:83–90.

Roses AD (1996) Apolipoprotein E and Alzheimer's disease. A rapidly expanding field with medical and epidemiological consequences. Ann NY Acad Sci 802:50–57

Rothschild D, Weissbrod O, Barkan E, et al (2018) Environment dominates over host genetics in shaping human gut microbiota. Nature 555:210–215.

Salehi A, Dubelaar EJG, Mulder M, Swaab DF (1998) Aggravated decrease in the activity of nucleus basalis neurons in Alzheimer's disease is apolipoprotein E-type dependent. Proc Natl Acad Sci 95:11445–11449.

Sánchez-Amate MC, Flores P, Sánchez-Santed F (2001) Effects of chlorpyrifos in the plus-maze model of anxiety. Behav Pharmacol 12:285–292.

Sanghi R, Pillai MKK, Jayalekshmi TR, Nair A (2003) Organochlorine and organophosphorus pesticide residues in breast milk from Bhopal, Madhya Pradesh, India. Hum Exp Toxicol 22:73–76.

Saucier DM, Shultz SR, Keller AJ, et al (2008) Sex differences in object location memory and spatial navigation in Long-Evans rats. Anim Cogn 11:129–137.

Saunders M, Magnanti BL, Correia Carreira S, et al (2012) Chlorpyrifos and neurodevelopmental effects: a literature review and expert elicitation on research and policy. Environ Heal 11:S5.

Savy CY, Fitchett AE, McQuade R, et al (2015) Low-level repeated exposure to diazinon and chlorpyrifos decrease anxiety-like behaviour in adult male rats as assessed by marble burying behaviour. Neurotoxicology 50:149–156.

Schliebs R, Arendt T (2011) The cholinergic system in aging and neuronal degeneration. Behav Brain Res 221:555–563.

Schliebs R, Arendt T (2006) The significance of the cholinergic system in the brain during aging and in Alzheimer's disease. J Neural Transm 113:1625–1644.

Segev Y, Livne A, Mints M, Rosenblum K (2016) Concurrence of High Fat Diet and APOE Gene Induces Allele Specific Metabolic and Mental Stress Changes in a Mouse Model of Alzheimer's Disease. Front Behav Neurosci 10:1–11.

Sekirov I, Russell SL, Antunes LCM, Finlay BB (2010) Gut Microbiota in Health and Disease. Physiol Rev 90:859–904.

Semple BD, Blomgren K, Gimlin K, et al (2013) Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. Prog Neurobiol 106–107:1–16.

Shi Y, Yamada K, Liddelow SA, et al (2017) ApoE4 markedly exacerbates tau-mediated neurodegeneration in a mouse model of tauopathy. Nature 549:523–527.

Shih DM, Gu L, Xia YR, et al (1998) Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. Nature 394:284–287.

Slotkin TA (2004) Cholinergic systems in brain development and disruption by neurotoxicants: Nicotine, environmental tobacco smoke, organophosphates. Toxicol Appl Pharmacol 198:132–151.

Slotkin TA (2011) Does early-life exposure to organophosphate insecticides lead to prediabetes and obesity? Reprod Toxicol 31:297–301.

Slotkin TA, Brown KK, Seidler FJ (2005) Developmental exposures of rats to chlorpyrifos elicits sex-selective hyperlipidemia and hyperinsulinemia in adulthood. Environ Health Perspect 113:1291–1294.

Slotkin TA, Cousins MM, Tate CA, Seidler FJ (2001) Persistent cholinergic presynaptic deficits after neonatal chlorpyrifos exposure. Brain Res 902:229–243.

Slotkin TA, Seidler FJ (2007) Comparative developmental neurotoxicity of organophosphates in vivo: Transcriptional responses of pathways for brain cell development, cell signaling, cytotoxicity and neurotransmitter systems. Brain Res Bull 72:232–274.

Slotkin TA, Southard MC, Adam SJ, et al (2004) α7 Nicotinic acetylcholine receptors targeted by cholinergic developmental neurotoxicants: nicotine and chlorpyrifos. Brain Res Bull 64:227–235.

Tratnik J, Falnoga I, Trdin A, et al (2017) Prenatal mercury exposure, neurodevelopment and apolipoprotein E genetic polymorphism. Environ Res 152:375–385.

Solomon KR, Williams WM, Mackay D, et al (2014) Properties and Uses of Chlorpyrifos in the United States, in: Giesy JP and Solomon KR (eds), Ecological Risk Assessment for Chlorpyrifos in Terrestrial and Aquatic Systems in the United States. Reviews of Environmental Contamination and Toxicology 231, pp 13–34

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

Stilling RM, van de Wouw M, Clarke G, et al (2016) The neuropharmacology of butyrate: The bread and butter of the microbiota-gut-brain axis? Neurochem Int 99:110–132.

Sudo N, Chida Y, Aiba Y, et al (2004) Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. J Physiol 558:263–275.

Sullivan PM, Mace BE, Maeda N, Schmechel DE (2004) Marked regional differences of brain human apolipoprotein E expression in targeted replacement mice. Neuroscience 124:725–733.

Sullivan PM, Mezdour H, Aratanti Y, et al (1997) Targeted Replacement of the Mouse Apolipoprotein E Gene with the Common Human *APOE3* Allele Enhances Diet-induced Hypercholesterolemia and Atherosclerosis. J Biol Chem 272:17972–17980.

Sun J, Ling Z, Wang F, et al (2016) *Clostridium butyricum* pretreatment attenuates cerebral ischemia/reperfusion injury in mice via anti-oxidation and anti-apoptosis. Neurosci Lett 613:30–35.

Suratman S, Edwards JW, Babina K (2015) Organophosphate pesticides exposure among farmworkers: Pathways and risk of adverse health effects. Rev Environ Health 30:65–79.

Sutcliffe JS, Marshall KM, Neill JC (2007) Influence of gender on working and spatial memory in the novel object recognition task in the rat. Behav Brain Res 177:117–125.

Tamburini S, Shen N, Wu HC, Clemente JC (2016) The microbiome in early life: implications for health outcomes. Nat Med 22:713–22.

Tan J, McKenzie C, Potamitis M, et al (2014) The Role of Short-Chain Fatty Acids in Health and Disease. Advances in Immunology 121:91-119.

Tejedor MT, Garcia-Sobreviela MP, Ledesma M, Arbones-Mainar JM (2014) The apolipoprotein E polymorphism rs7412 associates with body fatness independently of plasma lipids in middle aged men. PLoS One 9:e108605.

Terry AV, Buccafusco JJ (2003) The Cholinergic Hypothesis of Age and Alzheimer's Disease-Related Cognitive Deficits: Recent Challenges and Their Implications for Novel Drug Development. J Pharmacol Exp Ther 306:821–827.

Terry AV, Beck WD, Warner S, et al (2012) Chronic impairments in spatial learning and memory in rats previously exposed to chlorpyrfos or diisopropylfluorophosphate. Neurotoxicol Teratol 34:1–8.

Terry AV, Gearhart DA, Beck WD, et al (2007) Chronic, Intermittent Exposure to Chlorpyrifos in Rats: Protracted Effects on Axonal Transport, Neurotrophin Receptors, Cholinergic Markers, and Information Processing. J Pharmacol Exp Ther 322:1117–1128.

Timchalk C, Busby A, Campbell JA, et al (2007) Comparative pharmacokinetics of the organophosphorus insecticide chlorpyrifos and its major metabolites diethylphosphate, diethylthiophosphate and 3,5,6-trichloro-2-pyridinol in the rat. Toxicology 237:145–157.

Toro CA, Zhang L, Cao J, Cai D (2019) Sex Differences in Alzheimer's Disease: Understanding the Molecular Impact. Brain Res 1719:194–207.

Tran TTT, Corsini S, Kellingray L, et al (2019) *APOE* genotype influences the gut microbiome structure and function in humans and mice: relevance for Alzheimer's disease pathophysiology. FASEB J 33:8221–8231.

Turnbaugh PJ, Hamady M, Yatsunenko T, et al (2009) A core gut microbiome in obese and lean twins. Nature 457:480–484.

Turnbaugh PJ, Ley RE, Mahowald MA, et al (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444:1027–1031.

Turpin W, Espin-Garcia O, Xu W, et al (2016) Association of host genome with intestinal microbial composition in a large healthy cohort. Nat Genet 48:1413–1417.

Ungar L, Altmann A, Greicius MD (2014) Apolipoprotein E, Gender, and Alzheimer's Disease: An Overlooked, but Potent and Promising Interaction. Brain Imaging Behav 8:262–273.

Venerosi A, Cutuli D, Colonnello V, et al (2008) Neonatal exposure to chlorpyrifos affects maternal responses and maternal aggression of female mice in adulthood. Neurotoxicol Teratol 30:468–474.

Verma RS, Mehta A, Srivastava N (2007) In vivo chlorpyrifos induced oxidative stress: Attenuation by antioxidant vitamins. Pestic Biochem Physiol 88:191–196.

Volcik KA, Barkley RA, Hutchinson RG, et al (2006) Apolipoprotein E polymorphisms predict low density lipoprotein cholesterol levels and carotid artery wall thickness but not incident coronary heart disease in 12,491 ARIC study participants. Am J Epidemiol 164:342–348.

Von Ehrenstein OS, Ling C, Cui X, et al (2019) Prenatal and infant exposure to ambient pesticides and autism spectrum disorder in children: Population based case-control study. BMJ 364:1–10.

Wang JJ, Qin J, Li Y, et al (2012) A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature 490:55–60.

Watson RE, DeSesso JM, Hurtt ME, Cappon GD (2006) Postnatal growth and morphological development of the brain: a species comparison. Birth Defects Res Part B Dev Reprod Toxicol 77:471–484.

Weisgraber KH (1990) Apolipoprotein E distribution among human plasma lipoproteins: role of the cysteine-arginine interchange at residue 112. J Lipid Res 31:1503–1511

Weisgraber KH, Rall SC, Mahley RW (1981) Human E apoprotein heterogeneity. Cysteine-arginine interchanges in the amino acid sequence of the apo-E isoforms. J Biol Chem 256:9077–83

Whitehouse PJ, Price DL, Clark AW, et al (1981) Alzheimer disease: Evidence for selective loss of cholinergic neurons in the nucleus basalis. Ann Neurol 10:122–126.

Wisdom NM, Callahan JL, Hawkins KA (2011) The effects of apolipoprotein E on non-impaired cognitive functioning: a meta-analysis. Neurobiol Aging 32:63–74.

Xu Q, Bernardo A, Walker D, et al (2006) Profile and Regulation of Apolipoprotein E (ApoE) Expression in the CNS in Mice with Targeting of Green Fluorescent Protein Gene to the ApoE Locus. J Neurosci 26:4985–4994.

Yadav M, Verma MK, Chauhan NS (2018) A review of metabolic potential of human gut microbiome in human nutrition. Arch Microbiol 200:203–217.

Yan C, Jiao L, Zhao J, et al (2012) Repeated exposures to chlorpyrifos lead to spatial memory retrieval impairment and motor activity alteration. Neurotoxicol Teratol 34:442–449.

Yuan T, Chu C, Shi R, et al (2019) ApoE-Dependent Protective Effects of Sesamol on High-Fat Diet-Induced Behavioral Disorders: Regulation of the Microbiome-Gut–Brain Axis. J Agric Food Chem 67:6190–6201.

Zhang H, Wu L-M, Wu J (2011) Cross-Talk between Apolipoprotein E and Cytokines. Mediators Inflamm 2011:1–10.

Zhang S, Reddick R, Piedrahita J, Maeda N (1992) Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. Science 258:468–471.

Zhang W, Basile AS, Gomeza J, et al (2002) Characterization of central inhibitory muscarinic autoreceptors by the use of muscarinic acetylcholine receptor knock-out mice. J Neurosci 22:1709–1717.

Zhao Y, Zhang Y, Wang G, et al (2016) Effects of chlorpyrifos on the gut microbiome and urine metabolome in mouse *(Mus musculus)*. Chemosphere 153:287–293.

Zhong N, Weisgraber KH (2009) Understanding the association of apolipoprotein E4 with Alzheimer disease: Clues from its structure. J Biol Chem 284:6027–6031.

Zhuang L, Chen H, Zhang S, et al (2019) Intestinal Microbiota in Early Life and Its Implications on Childhood Health. Genomics, Proteomics Bioinforma 17:13–25.

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