APOE PHENOTYPE EXPRESSION AND ITS MODULATION BY CHLORPYRIFOS: NEW INSIGHTS INTO GENE - TOXIC INTERACTIONS

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Dipòsit Legal: T 198-2016

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Doctoral thesis

Thesis supervised by Prof. Maria Teresa Colomina Fosch,
and co-supervised by Prof. José Luis Domingo Roig.

Department of Psychology

Universitat Rovira i Virgili

Tarragona

2015

UNIVERSITAT ROVIRA I VIRGILI

Departament de Psicologia
Unitat de Psicobiologia
Centre d'Avaluació i Mesura de la Conducta (CRAMC)
Grup de Recerca en Neurocomportament i Salut (NEUROLAB)

Maria Teresa Colomina Fosch, Professor of Psychobiology at the Departament of Psychology of the Universitat Rovira i Virgili,

CERTIFY

That the present study, entitled "*ApoE phenotype expression and its modulation by chlorpyrifos: new insights into gene – toxic interactions*" and presented by Mrs. Fiona Peris Sampedro, has been performed under my supervision at the Department of Psychology of the Universitat Rovira i Virgili, in fulfilment of the requeriments for the degree of Doctor, and meets the requirements to qualify for International Mention.

Tarragona, 19 November 2015

The Supervisor of the Doctoral Thesis,

Prof. Maria Teresa Colomina Fosch

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Unitat de Toxicologia
Laboratori de Toxicologia i Salut Mediambiental

José Luis Domingo Roig, Professor of Toxicology and Environmental Health at the Department of Basic Medical Science of the Universitat Rovira i Virgili,

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That the present study, entitled "*ApoE phenotype expression and its modulation by chlorpyrifos: new insights into gene – toxic interactions*" and presented by Mrs. Fiona Peris Sampedro, has been performed under my supervision at the Department of Psychology of the Universitat Rovira i Virgili, in fulfilment of the requeriments for the degree of Doctor, and meets the requirements to qualify for International Mention.

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The Supervisor of the Doctoral Thesis,

Prof. José Luis Domingo Roig

All the experimental phases included in this doctoral dissertation were performed within the research group of Neurobehaviour and Health (NEUROLAB) of the Department of Psychology, the Research Center for Behaviour Assessment (CRAMC) and the Laboratory of Toxicology and Environmental Health of the Department of Basic Medical Science of the Universitat Rovira i Virgili, and were funded by:

· ***FI programme from the Agency for Administration of University and Research Grants*** *(AGAUR), Commission for Universities and Research of the Department of Innovation, Universities and Enterprise of the Catalan Government; grant number* ***2013FI_B 00170****. This programme is also supported by the European Social Fund (EFS).*

· ***National Programme for Fundamental Research Projects 2010*** *(subprogramme: non-guided fundamental research projects) from Ministry of Economy and Competitiveness of the Spanish Government; gran number* ***PSI2010-21743-C02-01****. This programme is also supported by the EFS.*

Estigues on estigues, güeli, demana't un *vaqueret* que anem a brindar!

A mi *pa* y a mi *ma*, por creer ciegamente en mí,

A Pol, perquè aquest treball també és seu.

ACKNOWLEDGEMENTS

Esta es la tercera vez que el vacío de este folio en blanco se me hace inmenso. Sí, tal cual. Cualquiera que lea esto pensará que estoy de atar, que cómo después de haber escrito un libro de 230 páginas se me pueden resistir un par de hojas más. Puede sonar ridículo, pero no sé ni cómo empezar, ni mucho menos cómo hacer para incluir en este limitado espacio a todas aquellas personas que me han ayudado a llegar hasta aquí; 13 de noviembre de 2015, despacho *puke rainbows*, de cara al ordenador, y ultimando los detalles de una tesis que ya huele a realidad. Lo más sencillo para mí sería no ahondar demasiado en detalles, pero no sería justo, y todos merecéis que haga un último esfuerzo.

En primer lloc, m'agradaria fer una menció especial a la meva directora de tesi i mentora, la Dra. Maria Teresa Colomina. Gràcies, Teresa. Gràcies per la teva paciència, sobretot al començament i al final d'aquest camí. Gràcies pel teu entusiasme, la teva passió, la teva dedicació, la teva originalitat i la teva fermesa. Gràcies per enlairar-me en aquest món, i per haver-me oferit la possibilitat de fer ciència amb tu, amb tot el què això implica. Gràcies de tot cor. La meva més sincera gratitud al meu també director de tesi, el Dr. José Luis Domingo Roig, per la seva ajuda i empenta, per les oportunitats que m'ha concedit, pels savis consells i la seva proximitat. Merci énormément.

Cómo no, a las mujeres valientes que formamos el núcleo duro de NEUROLAB: Dra. Maria Teresa Colomina, Dra. Maria Cabré, Dra. Íngrid Reverté, Pia, Laia y yo misma. Maria, gràcies per la teva franquesa, i per haver confiat sempre en mi encara que jo no ho fes. Íngrid... Per a tu no tinc paraules. *Com és possible que aguanti sense perdre el nord aquesta noia*, em deia a mi mateixa quan vaig arribar al labo. Ara ja ho sé. Perquè aquesta noia és valenta, tenaç, ambiciosa, responsable, i una enamorada de la seva feina. Per tot això, mil gràcies Íngrid; part de la culpa de la meva garra científica la téns tu. Pia... ¡Ay, Pia! ¡Qué torbellino, cuánto talento, y qué fortaleza (sobre)humana! Tú te mereces una plana aparte, sin más. I Laia, dona talentosa com poques, gràcies per haver arribat per quedar-te.

En general, gràcies a tots els membres del Departament de Psicologia, especialment a la Diana, al Joan, la Raquel, al Toni i la Esther; al CRAMC; i al Departament de Ciències Mèdiques Bàsiques de la Facultat de Medicina de Reus, especialment al personal del Laboratori de Toxicologia i Salut Mediambiental: Gemma, Is, Lolita i Anabel, gràcies. A Amparo, por su sabiduría y templanza que deberían ser ejemplo a seguir. A Bea, por ser sinónimo de generosidad suprema. A Juan y Esperanza, por las infinitas horas de estabulario. Y a Yoli, por ser tan bonita y arrancarme tantas sonrisas.

En estos cuatro años me he cruzado con personas maravillosas con las cuales he compartido las penas y alegrías de la ciencia.
A Iván, Jordi, Nohora, Celeste, Teresa Neri, Jenni y Phoebe por haberme ayudado tantísimo sin reclamar nada a cambio.
A mi querida familia *Random*, gracias por aquél 26 de Octubre de 2013, por haberme hecho sentir tan bien, por las cenas urgentes de desintoxicación laboral, por haberme hecho partícipe de vuestras realidades, por todos los bailes y carcajadas, por el apoyo incondicional de algunos y el aprendizaje vital al que me forzaron otros. Pero, sobretodo, gracias por haber sido mi oxígeno durante todo este tiempo. Pol, Iván, Nohora, Pablo, Teresa, Jordi, Pia, Erica, José Maria, Laura, Fadua, Martí, Simona, Marta, Anna, Gemma, Andrés y Cíntia... Mil gràcies.
Pia, gracias por creer que mi amistar podía valer la pena. Quiero aprovechar este momento para decirte que *ya me bajé de los tacones de vértigo, no porque no los aguantase, sino porque la fiesta acabó.* Eres una de las grandes; tu fuerza es titánica, *ctm*, y a mí me has convertido en hierro. Te doy las gracias por ello, por haberte quedado y por lo que nos queda.

Merci beaucoup au Professeur Christophe Magnan et à tout l'équipe de Régulation de la Glycémie par le Système Nerveux Central pour m'avoir accueilli si bien pendant mon séjour à Paris. Un remerciement tout spécial à Erwann Philippe et à Stéphanie Migrenne-Li pour tout ce qu'ils m'ont appris pendant ces trois mois, et pour avoir été si patients avec moi (ce qui n'est pas toujours évident). Merci à mes potes du labo pour m'avoir intégré parmis eux dès le premier jour: Antoine, Mika, Axelle, Julien Vérét, Florianne, Maxou, Julien Castel, Mélanie, Lara, Anne-So, Adrien, Anne, Chloé, Kelly, Élise, Marie... Merci également à Raphaël Denis pour son aide

désintéressée et les soirées au Dupont, et au Dr. Serge Luquet pour ses conseils. A ti también, Mar, por haber aparecido en mi vida.

A mis *xatis*, María y Alicia, Ana Miralles, Ali, Loli, Mirella y Ana Malo. Gracias por seguir ahí a pesar de todo, por quererme tanto en la distancia, y por respetarme. Volver a casa y ver que, diez años después, nada ha cambiado... No tiene precio.
A Montse, per haver estat sempre aquí malgrat aquests mesos de desconnexió.

A mi familia, a la que adoro, por ser tan auténtica. A mi abu, por habérmelo dado todo en estos 28 años. A mis tías, Begoña, Adri, Tati y Lorena... Gracias por mimarme tanto. También a mis tíos, Emilio, Sequín, Manolo y Paco. A mis soles, Carla, Sofía, Jordi, Catalina y Paco (¡Qué ganas de volver a cogerte!). A mis tetes, Eli, Natxo y Kike, y a Amparo. A mi *pastisset*, Mar, y a la guinda que está por venir, Quim. A mi sevillanita, Marina. A todos... Gracias por aguantar las comidas familiares al teléfono, las ausencias, y por los abrazos infinitos tras meses sin vernos. A Cori, Francesc pare i Francesc fill, i a Santi; gràcies per fer-me sentir com a casa.

Fa gairebé dos anys, la vida em va fer un regal immens. Sí; per casualitat (o no), vaig coïncidir amb la persona més meravellosa del planeta. Vaig tenir la sort de començar a conéixer-la i ara, passat un temps, m'emociono cada cop que penso en tot el camí que ens queda per recórrer plegats. Sóc incapaç d'agraïr-te, Pol, tot el què has fet per mi en un paràgraf; si més no hauria d'emplenar totes i cadascuna de les pàgines d'aquest manuscrit. Sé que no t'agraden aquestes coses, i per això no m'estendré gaire més. Gràcies per ser com ets, per treure el millor de mi i fer-me millor persona. Gràcies per la teva paciència, la teva serenitat, la teva bondat absoluta, la teva saviesa i maduresa. Per aixecar-te més d'hora cada matí i preparar-me l'esmorzar, només per deixar-me dormir una mica més. Per les abraçades de llit. Y por los saltitos que tanto me gustan. T'estimo.

Fa més de 10 anys que vaig arribar a Tarragona, amb cotxe, i una pena terrible. Echando la vista atrás, no hay ocasión que mi *ma* no haya aprovechado para *echarme* de casa, como dice ella. Siempre pensó que era lo mejor para mí, y que cualquier oportunidad era buena para que yo

saliese a *hacer mundo*. Mi *pa* no se salva; siempre ha sido cómplice. Y no sabéis cuánto os lo agradezco. Gracias, a los dos, por haber reprimido lágrimas, por las noches sin dormir, por haber aguantado la pena, por vuestra fuerza descomunal, y por haberme antepuesto a ninguna otra cosa. Todo lo que ahora soy; todo lo que siento, lo que pienso, lo que anhelo, lo que rechazo, lo que debato, lo que tolero y lo que no, lo que aspiro ser, lo que amo, por lo que lucho, lo que defiendo, lo que necesito, lo que me apasiona, lo que me mueve, lo que no soporto, lo que me desconcierta. Todo, os lo debo a vosotros. Por eso, os dedico este trocito de mí. Os dedico mi trabajo entero, para que os veáis reflejado en él.

Sé que estarás bien donde estés; ahí, dando guerra, criticando a Mariano y sacando dientes ante cualquiera que se meta con tu Madrid. Te echo de menos, más de lo que puedas imaginar. Sé que hoy estarías orgulloso de mí. Por eso, sólo te escribo para avisarte: demana't un vaqueret, i brindem per tu primer, i per mi després.

ABSTRACT

The cumulative impact of technological and social changes has prompted the epidemiological transition of the 20th century, in which the leading causes of death shifted from infectious diseases to chronic diseases, including an array of neuropsychiatric and metabolic disorders. In this context, pesticides have undeniably contributed to improve agricultural productivity. However, their extensive use and massive release into the environment has led some authors to wonder about the potential contribution of these substances to the emergence of such chronic diseases. Currently, organophosphates (OP) – and chlorpyrifos (CPF) in particular - are the most widely used insecticides in the world, due largely to their low environmental persistence and their high effectiveness. These substances exert they insecticidal activity by inhibiting acetylcholinesterase in central nervous system of insects, but the presence of this enzyme in many other organisms, including mammals, implies their neurotoxic effects extend to unintended targets. The ubiquity of CPF and the wide range of source of exposures entail not only crop dusters and farm workers are actually exposed, but also the general population. A great body of epidemiological and experimental data have reported neurobehavioural deficits upon acute and chronic exposures to CPF. However, most research focuses on learning and memory processes, while the assessment of more complex behaviours, such as inhibitory control and motivation has received scant attention. Similarly, there is some evidence on the contribution of OPs in boostering metabolic disorders, but this area of knowledege has been understudied. One of the critical issues is to establish whether there are subpopulations particularly susceptible to the detrimental effects of CPF. In regard to this, the three most common human apolipoprotein E (apoE) isoforms have been proved to confer varying vulnerabilities to metabolic diseases and neurodegeneration on their carriers. Further, there is evidence supporting their role in modulating cognition in the absence of a disease condition. In fact, while being carrier of the *ε4* allele stands as a genetic risk factor for Alzheimer's disease and poor cognitive outcome, the possession of the *ε2* has been related to increased longevity and better cognitive performance. In the meantime, the *APOE3* genotype has been associated to confer an intermediate healthy phenotype. To date, no single study exists inquiring about the response of these genetic polymorphisms to CPF. The main objective of this doctoral thesis was

to assess the behavioural and metabolic effects of both *APOE* genotype and the pesticide CPF, as well as to determine whether the interaction between both factors contributed to the expression of these effects. To test this, adult apoE targeted replacement (TR) mice, expressing human apoE2, apoE3 or apoE4 isoforms, were chronically or subchronically exposed to 2 mg/kg/day or 3.75 mg/kg/day CPF depending on the experimental phase. After exposure, male mice were behaviourally tested for spatial learning and memory abilities, while female mice were tested for attention, inhibitory control and motivation immediately after the exposure and following a CPF-free wash-out period. Aditionally, we determined the neurochemical and neuropharmacological bases of the potential behavioural differences among apoE TR female mice. Moreover, we designed a parallel experiment to respond to the increased weight observed in the first study, which focused on evaluating the metabolic changes induced by the pesticide in apoE3 male mice. In general, *APOE* genotype influenced spatial learning and memory, attention and inhibitory control during adulthood. Further, apoE TR female mice also show differences in brain neuromodulatory system and conditioned responses to GABAergic and cholinergic agents. On the other hand, CPF exposure altered overall metabolic functioning in male mice. Specifically, it elicited hyperglycaemia and hypercholesterolemia, enhanced food intake, increased insulin levels and impaired HOMA-IR index scores. Furthermore, the pesticide induced protracted attentional and motivational deficits in female mice. Besides mere treatment effects, the current results shed light into gene – toxic interactions. Thus, apoE3 mice were the most vulnerable to the metabolic-disruptor role of CPF, as they showed significant weight gain, and displayed higher insulin and leptin levels, as well as higher HOMA-IR index scores than their counterparts. In terms of behaviour, *APOE* genotype also modulated the effects of CPF. Indeed, the pesticide evoked mild memory impairment in apoE3 male mice, and reversed the lack of inhibitory control inherent to female apoE4 mice. Taken together, the results of this doctoral thesis expand the existing literature on the behavioural and metabolic effects of CPF, and provide valuable information on gene – toxic interactions hitherto unknown.

Keywords: Gene – toxic interaction, Apolipoprotein E, Pesticides, Chlorpyrifos, Obesity, Hormones, Diabetes, Behaviour, Learning, Attention, Impulsivity, Motivation.

ABBREVIATIONS

5-CSRTT, 5-Choice serial reaction time task

5-HT, Serotonin

ACh, Acetylcholine

AChE, Acetylcholinesterase

AD, Alzheimer's disease

ADHD, Attention deficit hyperactivity disorder

apoE, Apolipoprotein E

apoE TR, ApoE targeted replacement

ARC, Arcuate nucleus

BChE, Butyrylcholinesterase

BM, Barnes maze

BMI, Body mass index

CNS, Central nervous system

CPF, Chlorpyrifos

CYP, Cytochrome P-450

DA, Dopamine

DDT, Dichlorodiphenyltrichloroethane

DEP, Diethylphosphate

DETP, Diethylthiophosphate

EPA, Environmental Protection Agency

EU, European Union

FAO, Food and Agriculture Organization

FDA, Food and Drug Administration

HDL, High-density lipoprotein

IDF, International Diabetes Federation

LDL, Low-density lipoprotein

LDLR, LDL receptor

mAChRs, Muscarinic receptors

MTL, Medial temporal lobe

MWM, Morris water maze

nAChRs, Nicotinic receptors

OC, Organochlorine

OP, Organophosphate

PFC, Prefrontal cortex

PNS, Peripheral nervous system

PON1, Paraoxonase

PVN, Paraventricular nucleus

RBC AChE, Red blood cell AChE

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

TG, Triglycerides

VLDL, Very-low density lipoprotein

WHO, World Health Organization

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INTRODUCTION

1. INTRODUCTION

Throughout the 20th century, a great number of xenobiotics have been developed to meet a wide variety of agricultural, industrial, medical, and scientific needs. Despite the economic and social benefits, the extensive use of these products has led to the release of large amounts of waste into the environment, posing a huge threat to human health and natural ecosystems. Indeed, the World Health Organization (WHO) estimated that unintentional poisonings kill approximately 346 000 people each year *(WHO, 2004)*. Actually, about 70,000 chemical compounds are registered in the inventory of the US Environmental Protection Agency (EPA), a number that grows annually *(US EPA, 2015a)*. To date, however, less than 1% has been categorized as neurotoxic to humans *(Miodovnik, 2011)*. As a matter of fact, the ubiquity of these substances, which are ranged from heavy metals, solvents or pesticides, to food additives, cosmetics or drugs of abuse, along with the existence of multiple sources of exposure and different individual vulnerabilities hinder and delay the process of testing their neurotoxicity. Generally, a neurotoxic agent can be defined as any natural or synthetic product capable of causing a consistent pattern of adverse effects, whether transient or permanent, in central or peripheral nervous system (CNS, PNS), as well as in sensory organs *(Simonsen et al., 1994)*. Thus, the spectrum of possible functional changes is very broad and covers from cognitive disturbances, including learning, memory and attentional impairments, to dysfunctionality of more complex behavioural processes, such as inhibitory control and motivation.

In this recent context of globalization, we are witnessing the homogenization process of the behavioural, cultural, socio-economic and political determinants distinctive of a society. It is therefore no wonder that such diseases as obesity have become one of the greatest health public challenges of the 21st century *(Morris et al., 2014)*. According to the WHO, the worldwide prevalence of obesity nearly doubled since 1980, being 13% of the entire population obese in 2014 *(WHO, 2014)*. From the latest data concerning the European Union (EU), overweight affected over 50% of both men and women, while roughly 20% of the adult individuals were obese in 2008 *(WHO - Regional office for Europe, 2015)*. Thought there are well-recognized risk factors for obesity, such as sedentary lifestyle, excess caloric intake, or genetic inherited

predisposition, several studies claimed for a key role of hazardous chemicals to the aetiology of this health condition *(Arrebola et al., 2015; Jeon et al., 2015)*.

A gene-environment interaction refers to the differential effect of an environmental exposure, whatever its nature, on the risk of developing a disease depending on the gene pool of those exposed individuals. It has become generally accepted that it is the interplay of genetic and environmental factors that fosters differences in human cognitive and behavioural traits *(Lopizzo et al., 2015)*. Likewise, the determination of gene-environment interactions in obesity may stand as a promising area for future investigations. In point of fact, knowledge of potentially harmful agents and how different populations respond to their toxicity is crucial for public health: beyond shedding light on the biological bases of the disease, the identification of vulnerable genetics could improve the accuracy and precision of epidemiological risk models, thus allowing a substantial reduction of the social and economic costs that chemical compounds exposure entail *(Grandjean and Landrigan, 2014)*.

1. 1. PESTICIDES: HELPFUL OR HARMFUL AGENTS?

Unquestionably, pesticides have contributed to improve agricultural productivity. However, their use has been massive and their intentional release into the environment has alerted regulatory agencies and governments. Despite the growing eagerness to regulate their applicability, pesticide sales in the EU continue to grow steadily *(Eurostat, 2015)*. According to the Food and Agriculture Organization (FAO), nearly 2,000 tons per 1000 inhabitants of pesticides were used in Spain during the period 2000-2010 *(FAO Statistics Division, 2013)*.

1. 1. 1. Historical contextualization

About 10,000 years ago came the first form of agriculture, understood as the domestication of plants, in the region of the Fertile Crescent of Mesopotamia, current territorial extension covering Iraq, Turkey, Syria and Jordan *(Tanno and Willcox, 2006)*. However, until six centuries later, no hint of pesticide application was registered. From then until the 1940s, several plant and animal-derived compounds (e.g., smokes from burning straw or animal waste, pyrethrum

flowers powder, roots of flowering plants belonging to the *Tephrosia* genus, etc.) as well as available mineral and chemical products (e.g., arsenic, boric acid, cooper sulphate, lead, mercury, nitrophenols, petroleum oils, salt, sodium chlorate, sulphuric acid, etc.) were used to protect crops from pests and diseases *(Casida and Quistad, 1998; Jarman and Ballschmiter, 2012)*.

Due to the low specificity of most of these substances, the development of synthetic pesticides gained momentum. In 1939 Dr. Paul Müller discovered the broad-spectrum insecticidal properties of the organochlorine (OC) compound dichlorodiphenyltrichloroethane (DDT), earning him the Nobel Prize nine years later. Throughout its early years of commercialization, DDT mitigated epidemics of such infectious diseases as typhus, malaria and yellow fever, powered by the course of the World War II (WWII). Shortly later, from the second half of the 40s, DDT was gradually introduced in the market for controlling pest crops in developed countries *(Casida and Quistad, 1998)*. It became one of the most popular pesticides worldwide due to its reasonable price together with its apparent harmlessness.

In parallel, although organophosphates (OP) were discovered and synthetized for the first time across the 19th century, it was not until 1937 when Dr. Gerhard Schrader, based on the previous work of Lange and Krueger, documented the insecticide power of these substances *(Soltaninejad and Shadnia, 2013)*. About 2,000 OPs, including the pesticide parathion and several warfare nerve agents of the G series (i.e., tabun, sarin and soman) were developed by Schrader and collaborators in the mid-20th century *(Chambers et al., 2001)*. Advances in OPs chemistry were evident after the WWII, and allowed the synthesis of other compounds, such as malathion, in 1950, and chlorpyrifos (CPF), firstly introduced in 1965. The extensive use of OPs as pesticides was rapidly consolidated, boosted by the ban of most OC, including DDT, throughout the 70s *(Miodovnik, 2011)*.

Later, during the 80s, numerous and increasingly selective pesticides formulations including insecticides, herbicides and fungicides came to light. Indeed, the pesticide industry evolved significantly, which allowed to focus the use of these substances, thereby reducing their application.

1. 1. 2. Social awareness

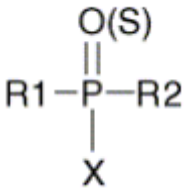
Over the golden age of synthetic pesticides (1940s and 1950s), both consumers and policymakers, overwhelmed by the lowering of food prices and promising productivity levels, were unaware of the risk posed by their massive use. However, the first data about the toxicity of DDT came quickly and triggered a progressive stigmatization of these substances *(Casida and Quistad, 1998)*. In 1962, the book *Silent* Spring by Rachel Carson stated the end of the DDT era, stirring the conscience of readers and sectors of the agricultural industry. From the 70s, regulatory agencies, such as the US EPA and the European Environment Agency (EEA) were thereupon created.

Over the last years, the rise of social awareness about the environmental pollution and toxicity of these compounds has prompted the search for other ways to control pests in agriculture. The development of genetically engineered crops able to cope by themselves the pest attack has grown notably, more and more formulations are environmentally friendly, and an increasing number of people are committed to organic farming *(Casida and Quistad, 1998; Dangour et al., 2009)*. Notwithstanding, the sale of pesticides has generally increased across the EU according to data from Eurostat Statistics for the period 1997-2008 *(Eurostat, 2015)*.

1. 1. 3. Organophosphates: overview and main uses

The OP family encompasses derivatives of phosphoric, phosphonic, phosphinic or thiophosphoric acids, all sharing a central phosphorus atom and a characteristic phosphoryl bond (P=O) or thiophosphoryl bond (P=S) (**Figure 1**).

Figure 1



Balali-Mood and Balali-Mood, 2008

Figure 1 General chemical structure of an organophosphate compound. R1 and R2 are alkyl-, alkoxy-, alkylthio-, or amido-groups. X is the acyl residue (labile fluorine-, cyano-, substituted- or branchedaliphatic, aromatic, or heterocyclic groups).

Organophosphate compounds

Although OPs are being still mainly employed as agricultural pesticides, their uses have been very diverse. In their most controversial aspect, OP compounds have been used for military purposes. Over the course of the WWII, the potential toxicity of the G series agents drawn the attention of the German Ministry of Defence which ended up producing several thousand tons of tabun and sarin, and slightly less of soman *(Watson et al., 2011)*. Throughout the Cold War, both the Soviet Union and the US joined the marketing of these substances, and stored large quantities of chemical warfare agents, which continue to accumulate to date in stockpile sites *(Watson et al., 2011)*. Chronically in time, the Iraq-Iran war left a record on the use of these chemical weapons between 1984 and 1988 *(Balali-Mood and Balali-Mood, 2008)*, while the Gulf war did it in the early 90s *(Chao et al., 2010)*. Besides, albeit most countries are members since 1993 of the Organization for the Prohibition of Chemical Weapons, and despite the existing regulations on the production, stockpiling and use of these substances, there is tangible evidence pointing to terrorist purposes. The first report was in 1994, when a sarin attack was conducted in a residential area of the city of Matsumoto (Japan). One year later, the same substance was released by members of the Aum Shinrikyo religious cult in different trains of the Tokyo subway. Both attacks took the lives of 18 people, but about 6,000 were poisoned *(Soltaninejad and Shadnia, 2013; Tokuda et al., 2006)*.

Nevertheless OPs have been instrumental in the development of medicine and industry. Within the scope of medicine, they have been exploited to treat Alzheimer's disease (AD), myasthenia gravis and glaucoma; in veterinary medicine to treat parasitic diseases of farm and domestic animals; and in the industry they have been used as oil additives, solvents, varnishes, artificial leather, flame retardants and plastic softeners *(Adams, 2013; Gupta, 2011)*.

Organophosphate pesticides

As stated above, the use of OPs is nowadays focused on agriculture, where they are used as pesticides. Lower environmental persistence, compared to such OC compounds as DDT and a high effectiveness against different insect species are qualities that place them among the most widely used insecticides in the world *(De Silva et al., 2006)*. Among over 100 different substances, the most commonly applied are CPF, parathion, methyl parathion, malathion, diazinon and dichlorvos. They exert their insecticidal activity by inhibiting acetylcholinesterase (AChE) in CNS of insects, thus causing neurotoxicity *(Fukuto, 1990)*. The presence of this enzyme in many other organisms, including mammals, implies the neurotoxic effect of these agents extends to unintended targets. However, mammals are more efficient in detoxifying OPs, and consequently are less sensitive than insects to acute intoxication. Nonetheless, OP pesticides are responsible for several cases of poisoning worldwide, including intentional and non-intentional accidents, especially in developing countries where adequate preventive and protective measures are lacking *(Chowdhary et al., 2014; London et al., 2005)*.

In developed countries, farmers and their families, distributors of plant protection products, crop dusters, and even the general population are daily exposed to these substances in a very diverse range of doses *(Eaton et al., 2008)*. The current absence of safe driving practices, as well as consulting and certification programs; the improper use of protective equipment; deficient regulations on transport, storage and waste management, have contributed to an alarming situation. Even today, the misuse of pesticides causes acute occupational poisoning requiring medical attention *(Faiz et al., 2011; Roldán-Tapia et al., 2005)*. Nonetheless, other milder exposures

that do not initially require health care go unnoticed but can lead to the development of a chronic exposure.

In recent years, a large body of epidemiological studies have linked occupational OP exposure with an increased risk of developing several diseases, such as different types of cancer (i.e., Brest, thyroid, ovarian, colorectal or lung cancers, as well as non-Hodgkin lymphoma, or glioma) *(Lee et al., 2005, 2004; Lerro et al., 2015)*, vein thrombosis *(Lim et al., 2015)*, cardiovascular disease *(Hung et al., 2015)*, type 2 diabetes *(Saldana et al., 2007; Starling et al., 2014)*, or neurodegenerative diseases *(Moretto and Colosio, 2013; Sánchez-Santed et al., 2015; Zaganas et al., 2013)*. In addition, a constellation of neuropsychiatric and neuropsychological disorders has been reported after both acute and chronic exposures to OP pesticides *(London et al., 2005; Mackenzie Ross et al., 2010; Povey et al., 2014; Roldán-Tapia et al., 2006)*.

1. 2. CHLORPYRIFOS

1. 2. 1. Generalities and current main uses

Chlorpyrifos (CPF) belongs to the OP family of pesticides, and actually serves as an insecticide and acaricide. The WHO classifies it within the moderately hazardous compounds (Class II), with an LD50 in rats of 135 mg/kg *(WHO, 2009)*. Since its introduction in the marketplace in 1965, it was extensively used in agricultural areas and for public health maintenance, and even for residential purposes. In the home, it was used to control insect pests caused by termites, cockroaches and other insects, while it also stood as the active component in tick and flea collars for pets. However, following the reconsideration of its neurotoxic potential, the EPA banned its homeowner uses in 2001, except baits for ants and cockroaches packaged childproof. In the EU, CPF non-agricultural uses are being phased-out, but the situation is fairly complex. Despite the evidence supporting its deleterious effects to human health and the efforts of many non-governmental organizations, such as the Pesticide Action Network along with those of some governments, the European authorities were still debating in 2009 banning CPF residential use, alleging the lack of conclusive evidence *(Saunders et al., 2012)*.

Currently, CPF applications are largely limited to crop protection in both rural and urban areas. Nevertheless, it is still used worldwide for mosquito and fire ants control, professional care of golf courses, cattle ear tags, non-structural wood treatments (e.g., utility poles), and in green houses *(US EPA, 2015b)*, thus implying a pervasive pattern of exposure. Overall, more than 400 commercial products contain CPF as an active component, but the most popular are those marketed by Dow Agro Sciences, named Dursban and Lorsban *(Eaton et al., 2008)*.

In Spain, the use of CPF in agriculture is ubiquitous: cereal and tubers crops; fruit, olive and nut trees; vineyards, etc. *(Ministerio de Agricultura Alimentación y Medio Ambiente, 2015)*. According to the plant protection products inventory of the Ministry of Agriculture, Food and Environment of Spain, CPF is present in varying concentrations in 8 formulations from different suppliers, resulting in 40 commercially available products. Although they are mostly intended for an agricultural purpose, some of them are used for the fumigation of gardens and parks in urban areas, as well as for home gardening.

1. 2. 2. Structure and physicochemical properties

The chemical structure of CPF is depicted in **Figure 2**. The molecule has a central tetracoordinated pentavalent phosphorus atom, covalently bonded to sulphur. Additionally, phosphorus presents two more stable unions with ethyl groups. The other part of the molecule is represented by a more complex and unstable aromatic structure that can easily be released during the biotransformation process. It is worth pointing out that phosphorothioates (P=S) have little or no anticholinesterase activity, and thus require prior activation to the oxon form (P=O) to exert their toxic effect *(Gupta, 2011)* (**Figure 2**).

According to its physicochemical properties, summarized in **Table 1**, CPF is a white crystalline solid with a slight mercaptan odour. Its high partition coefficient reflects its hydrophobic character. In fact, the highest concentrations of CPF in the body are found in the fat and fatty tissues, an issue that will be further discussed in ***section 1.2.4***.

Table 1 Most relevant physicochemical properties of chlorpyrifos

Chemical name	*O*,*O*-diethyl *O*-3,5,6-trichloropyridin-2-yl phosphorothioate
Chemical formula	$C_9H_{11}Cl_3NO_3PS$
CAS number	2921-88-2
Molecular weight	350.57 g/mol
Melting point	41-42ºC
Boiling point	Decomposes at approximately 160ºC
Solubility	
Water, 20ºC	0.7 mg/L
Water, 25ºC	2.0 mg/L
Isooctane	79% w/w
Methanol	43% w/w
Partition coefficients	
Log K_{OW}	4.82
Log K_{OC}	3.73
Conversion factors	1 ppm = 14.3 mg/m^3
	1 mg/m^3 = 0.07 ppm

Adapted from National Center for Biotechnology Information, 2015

1. 2. 3. Sources of environmental exposure

Due to its low solubility in water (**Table 1**), CPF rapidly binds to soil components and plants once released into the environment, where it undergoes a progressive degradation. The half-life of the pesticide and its metabolites can vary from 6 h to 3 days, always depending on the sunlight availability and the presence of potential degrader microorganisms. In indoor environments CPF residues may therefore remain for longer periods of time *(Eaton et al., 2008)*. Strikingly, CPF

residues have been recently found in such remote areas as the Arctic environment, suggesting long-range transport and bioaccumulation may also occur in the case of OP pesticides, and not only for OC *(Vorkamp and Rigét, 2014)*.

As previously discussed, the ubiquity of CPF implies the risk of exposure to the pesticide is not exclusively restricted to the applicators and farm workers, but also to the general population, although to a lesser extent. Toxic exposure can occur through the most common ways: ingestion, inhalation, and dermal absorption. Dietary exposure to trace levels of CPF appears to be the main source of non-occupational exposures *(Lu et al., 2008)*. Nevertheless, secondary ingestion of contaminated house dust/soils, and even hand-to-mouth contact may also account for total exposures in residential settings of rural areas *(Eaton et al., 2008)*. On the other hand, both the inhalation of vapours or aerosols following application, and dermal absorption upon contact with skin are the most predominant pathways in occupational exposures. All those individuals in direct contact with the exposed workers are included within this pattern of exposure *(Eaton et al., 2008)*.

Considering the aforementioned, it seems difficult to estimate a daily reference dose for a typical exposure to CPF *(Saunders et al., 2012)*. In the meantime, the US Department of Agriculture along with the Food and Drug Administration (FDA) have established a reference value for CPF daily intake that varies depending on age (i.e., 0.005 μg/kg body weight/day in adults, 0.014 μg/kg body weight/day in toddlers, and 0.009 μg/kg body weight/day in infants*)* *(Eaton et al., 2008)*.

In addition to the different exposure pathways, other variables may influence the toxicity of CPF. Some directly concern the compound (e.g., concentrations applied, formulation stability, presence of additives, etc.), while others take into account characteristics of the exposed individual (e.g., work practices, metabolism, gender, age, diseases, drug treatments, etc.).
However, it is the study of the interaction of both (i.e., gene-environment interactions), the ecogenetics, that is gaining ground in recent years *(Costa, 2006; Costa et al., 2013)* ***(see section 1.2.8)***.

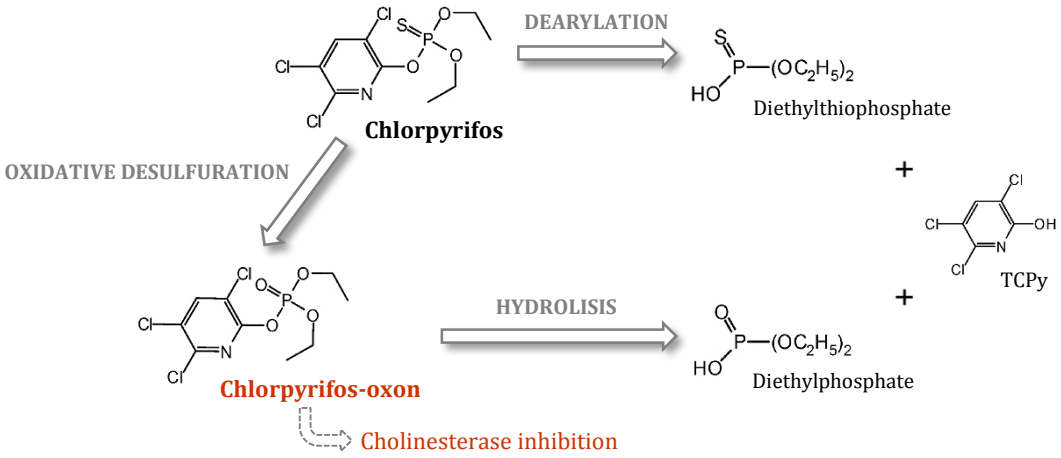
1. 2. 4. Absorption, distribution, biotransformation and excretion

CPF can be rapidly and efficiently absorbed through the intestine *(Nolan et al., 1984; Timchalk et al., 2002)* and lungs *(Geer, 2004)*, and to a lesser extent through the skin *(Meuling et al., 2005)*. Its free blood levels are low because it binds to various proteins, such as plasma albumin *(Tarhoni et al., 2008)*. Due to its lipophilic nature, CPF accumulates mainly in fatty tissues and in the brain. Likewise, breast milk also stands as an efficient storage compartment due to its high fat content, thus resulting in an additional source of potentially hazardous exposure to the infant. Ultimately, CPF reaches the liver and kidneys, where the biotransformation process takes place *(Chambers et al., 2001)*.

The biotransformation pathways of CPF are shown in **Figure 2**. As mentioned earlier, these stages are crucial for the expression of CPF-related neurotoxicity. Once entered the body, CPF can undergo a cytochrome P-450 (CYP)-dependent oxidative desulfuration to its active oxygen analogue, which is a potent ChE inhibitor. The inactivation of the oxon form occurs mainly by hydrolysis, primarily mediated by such oxonases as paraoxonase (PON1), and leads to the formation of diethylphosphate (DEP) and 3,5,6-trichloro-2-pyridinol (TCPy). Besides, CPF can be directly converted to diethylthiophosphate (DETP) and TCPy through CYP-mediated dearylation. In either case, both reactions contribute to the formation of TCPy, which is the main CPF metabolite found in urine *(Timchalk et al., 2007)*.

In addition to urine, which stands as the major excretion pathway, CPF metabolites can be eliminated through other fluids. Indeed, some experimental studies attested their biliary and faecal elimination, while measurable levels of CPF were detected in breast milk samples from nursing mothers *(Sanghi et al., 2003)*.

Figure 2



Adapted from Eaton et al., 2008

Figure 2 Biotransformation of chlorpyrifos

1. 2. 5. Biomonitoring chlorpyrifos exposure

Blood biomarkers, such as the ChE enzymes AChE and butyrylcholinesterase (BChE) have been traditionally used for monitoring CPF exposure, as they were its first described molecular targets. Because BChE is more sensitive than AChE to the inhibitor effect of the oxon metabolite, plasma activity of BChE has long served to assess the physiological course of CPF exposure *(Farahat et al., 2011)*. In turn, the AChE expressed in red blood cells (RBC AChE) is more reflective of the AChE status in CNS and PNS *(Eaton et al., 2008)*.

The Ellman colorimetric assay *(Ellman et al., 1961)*, aimed at determining enzymatic activities of BChE and RBC AChE, is still the most widely used method for biomonitoring OP exposures. However, in recent years, a more robust approach has gained ground. This technique, further detailed in ***section 2.5.2***, relies on the ability of CPF-oxon to create stable, covalently bound

adducts upon the inhibition of BChE. Therefore, the CPF-adducted enzyme has longer half-periods than most CPF metabolites, thus allowing to extent the detection window for biomonitoring the pesticide *(Carter et al., 2007; Marsillach et al., 2013)*. The formation of adducts extends to other protein capable of interacting with CPF (e.g., other serine hydrolases, albumin, etc.).

On the other hand, the determination of urinary TCPy has been commonly used for CPF exposure assessment. Nevertheless, there is enough evidence to suggest that it is an inadequate biomarker because it is not exclusively a product of CPF degradation, as it can also be found by itself in dietary samples *(Eaton et al., 2008)*.

1. 2. 6. Clinical picture upon chlorpyrifos poisoning

Overview on the cholinergic system

The cholinergic system is based on the neurotransmitter acetylcholine (ACh), which is widely distributed in both CNS and PNS. Briefly, the synthesis of ACh is catalysed by the enzyme choline acetyltransferase (ChAT) that transfers the acetyl group from the acetyl-CoA to a molecule of choline. The resulting neurotransmitter is stored in vesicles that end up being released into the synaptic cleft by exocytosis upon the opening of voltage-dependent Ca^{2+} channels, and the consequent entry of Ca^{2+} into the neuron. ACh exerts its action on two types of receptors: nicotinic (nAChRs) and muscarinic (mAChRs). The nAChRs are ionotropic, thereby presenting fast and short-acting mode not requiring second messengers. They are directly linked to an ion channel through which the ion transfer (Na^{+}, K^{+}, Ca^{2+}, and Cl^{-}) allows the modulation of the depolarization or hyperpolarization of the neuron upon ACh binding. Meanwhile, mAChRs are metabotropic, so that their action is slower and long-lasting. They are coupled to G proteins, and following activation they trigger a cascade of responses, including the formation of a second messenger that acts on other molecules and channels. The intervention of ChE enzymes brings the cycle to a close: ChE are responsible for the breakdown of the ACh into choline and acetic acid, and thus allow the restauration of the cholinergic neuron *(Karczmar, 2007)*.

As already mentioned, AChE (EC 3.1.1.7) is the key target for OP compounds in both insects and mammals *(Fukuto, 1990)*. This enzyme is mainly located in the synapses of central and peripheral nerves, where it terminates the cholinergic transmission, but it could also be found on RBC membranes.

In turn, BChE (EC 3.1.1.8), often referred to as pseudocholinesterase or plasma ChE, is synthesized in the liver and secreted in plasma. Although BChE could also contribute to the hydrolysis of ACh, its specific physiological function has not yet been elucidated. However, it is well-established that the enzyme exerts a protective role against several exogenous substances (e.g., cocaine, acetylsalicylic acid, and procaine) *(Duysen et al., 2008; Kolarich et al., 2008; Yuan et al., 2007)*. Indeed BChE is crucial to cushion the CPF-related neurotoxic effects, since it prevents or at least minimizes CPF-oxon binding to its primary brain target, AChE *(Costa, 2006)*.
Beyond the above, De Vriese and collaborators demonstrated eleven years ago the critical role of BChE in regulating ghrelin levels *(De Vriese et al., 2004)* ***(see section 1.5.1)***.

Acute neurotoxicity

Following CPF exposure, AChE inhibition elicits the accumulation of ACh at cholinergic synapses, thus leading to the overstimulation of both nAChRs and mAChRs. When inhibition exceeds 70%, the cholinergic acute syndrome ensues *(Savolainen, 2001)*. Generally, the first signs to appear stem from the mAChRs stimulation: the contraction of the pupil (i.e., miosis) emerges in 80% of cases, being sometimes the only apparent symptom of toxicity *(Lotti, 2001)*. Lately, the clinical picture includes excessive sweating and salivation, bronchoconstriction, tremors, increased intestinal motility and subsequent diarrhoea. Ultimately, death may occur, primarily due to multifactorial respiratory failure *(Lotti, 2001)*.

From a molecular point of view, AChE inhibition occurs when the oxon form phosphorylates a hydroxyl group, which is located on a serine residue in the active site of the enzyme, thereby preventing AChE action on its physiological substrate. Thereafter the loss of one of the alkyl groups, the enzyme-oxon complex (i.e., adduct) is irreversibly bounded. In this case, it is

considered that the AChE is irreversibly inhibited and can only be replaced by a new molecule *(Marsillach et al., 2013)*.

Intermediate syndrome and delayed polyneuropathy

In half of acute poisoning cases, an intermediate syndrome arises approximately 24 h after the most severe clinical manifestations. Despite the certainty that the link between the occurrence of this syndrome and the AChE inhibition is not causal, evidence suggests that it may be indirect. In fact, this characteristic syndrome involves weakness of neck and respiratory muscles, combined with paralysis of the proximal members, pointing to a possible desensitization of nAChRs upon OP exposure *(Lotti, 2001)*.

The OP-induced delayed polyneuropathy (OPIDP) usually occurs after severe exposures to some OPs, when both acute and intermediate episodes have subsided. It is not a direct consequence of cholinergic toxicity, but rather appears to be related to the ability of few OP compounds to inhibit the neuropathy target esterase (NTE). Indeed, the degree of NTE inhibition is strongly correlated with the severity of the clinical picture. Typically, OPIDP is characterized by distal axonal degeneration which leads to progressive motor weakness that can be recovered in varying degrees *(Yang and Deng, 2007)*. In addition, it is also common to report some peripheral sensory disturbances that may persist even years after exposure.

1. 2. 7. Chlorpyrifos and disease

Both, acute and relatively short-term effects caused by OPs, largely attributed to AChE inhibition, have been broadly investigated and are currently well-defined. However, the OP-related long-term health impacts are still subject to large uncertainties and discrepancies *(Rohlman et al., 2011)*. A mounting body of experimental data suggest that CPF impacts on other neurotransmitter systems, such as the serotoninergic, dopaminergic, and GABAergic *(Pung et al., 2006; Slotkin and Seidler, 2007; Torres-Altoro et al., 2011)*. Considering the number of potential targets, CPF exposure would be expected to have a wide range of effects. Nowadays, most of the population is almost permanently exposed to low doses of CPF. Thus, it is important to clarify to

what extent this kind of cumulative exposures is harmful to human health *(Ross et al., 2013)*. Chronic low-level exposure to OPs could be defined as such prolonged exposure to doses which do not produce recognized clinical symptoms of acute toxicity requiring medical evaluation or intervention *(Ross et al., 2013)*.

1. 2. 7. 1. Cognitive and mood disorders

Epidemiological evidence

Following the Japanese terrorist attacks with sarin in the mid-90s, a great deal of epidemiological research emerged to closely monitor the victims' health evolution. Interestingly, most of these studies confirm the persistence of behavioural alterations not only in victims, but also in both medical and security staff. These long-lasting sequelae were evident even 7 years after the accident, thereby indicating long-term effects on the CNS *(Miyaki et al., 2005; Nishiwaki et al., 2001)*. Similarly, the Gulf War veterans showed psychological and psychiatric symptoms that persisted even 15 years after returning from combat *(Chao et al., 2010; Iversen et al., 2007)*. Additionally, reduced hippocampal volumes were found in victims of both events, thus implying sarin induced irreversible structural changes in the CNS *(Chao et al., 2010; Yamasue et al., 2007)*.

From the second half of the 50s, shortly after OP pesticides came into use, early indications of neurobehavioural deficits in field workers were reported, including memory impairments, anxiety, confusion, fatigue and irritability *(Holmes and Gaon, 1956; Tabershaw and Cooper, 1966)*. Thenceforth, the impact of these substances on human health has been assessed from two perspectives: resulting impairments as a consequence of acute poisoning or as consequence of prolonged exposure. Research focusing on chronic low-level CPF exposures has identified deficits in neurobehavioural performance, including an array of cognitive and motivational disturbances *(Farahat et al., 2010; Mackenzie Ross et al., 2010; Rohlman et al., 2015; Ross et al., 2013; Stephens and Sreenivasan, 2004)*. These findings, unlike those found in victims of war or terrorist attacks, may not be due to traumatic experiences.

In spite of the complexity of standardized psychometric tests used to assess neuropsychological functioning of exposed individuals, the emerging cognitive deficits can be classified into eight major functional domains: motor speed and coordination; reaction time; information processing speed and executive function; verbal comprehension; sustained attention; attention and short-term memory; memory; and perception *(Rohlman et al., 2011)*. In this regard, the implementation of this battery of tests on English sheep farmers exposed to low-level OPs for 24 years concluded the presence of memory deficits, reduced response speed, impaired fine motor control and poor cognitive flexibility *(Mackenzie Ross et al., 2010)*. In Spain, greenhouse workers in the region of Almeria, exposed throughout their working lives to such OP as CPF showed lower performances on both verbal and visual memories. In addition, those who were exposed for more than 10 years also displayed worse scores on tests of visuospatial ability *(Roldán-Tapia et al., 2005)*.

It is worth noting that, even if most of the data issued from these investigations concerned general pesticide applicators, crop dusters, greenhouse workers, or livestock breeders, some authors agree on the risk posed by this kind of exposure for people living with them, or whose residence is in surrounding agricultural areas *(Coronado et al., 2011; Muñoz-Quezada et al., 2013; Valcke et al., 2006)*.

Although more discreetly, the scientific literature also reflects the onset of affective disorders (i.e., anxiety, depression, aggressive behaviour, and social withdrawal) in OP pesticide applicators *(London et al., 2005; Mackenzie Ross et al., 2010; Roldán-Tapia et al., 2006, 2005; Steenland et al., 2000)*. For example, increased symptoms of psychological distress, including depression, anxiety, obsessive-compulsiveness, interpersonal sensitivity and suicidal thoughts, were found in Costa Rican banana workers with previous OP poisoning *(Wesseling et al., 2010)*.

Evidence from experimental animals

Consistently, data issued from animal models of acute or repeated CPF intoxication evidenced the development of cognitive and behavioural deficits. To date, however, much of the current

scientific literature pays particular attention to the neurodevelopmental effects elicited by CPF, being the studies concerning adult individuals less common.

Focusing on adulthood exposures, it has been confirmed that CPF alters spatial learning and memory processes when administered both acutely *(Cañadas et al., 2005; Peris-Sampedro et al., 2014; Sánchez-Santed et al., 2004)* and chronically *(López-Granero et al., 2013b; Moser et al., 2005; Yan et al., 2012)*. These changes may persist or develop long after exposure, as was described in a work carried out by our group *(Peris-Sampedro et al., 2014)* in which acutely CPF-treated male mice showed retention shortfalls in a Morris water maze (MWM) 6 months after exposure. Similarly, male rats exposed to CPF every other day for 30 days exhibited difficulty in acquire a MWM almost 5 months after exposure *(Terry et al., 2012)*. Besides, Moser and collaborators observed that dietary administration of subtoxic CPF doses for 1 year led to long-term spatial learning deficits in male rats evaluated in a MWM *(Moser et al., 2005)*. Likewise, male rats exhibited impaired spatial memory in the same task immediately after a 4-week intragastric exposure to low doses of CPF *(Yan et al., 2012)*.

Furthermore, CPF has also been related to induce deficits in sustained attention. In this regard, male rats given an acute CPF dose were less attentive to signals in a visual signal detection task *(Bushnell et al., 2001)*. On the other hand, Middlemore-Risher et al. used the 5-choice serial reaction time task (5-CSRTT) to demonstrate empirically detrimental effects on sustained attention in male rats subjected to a 4-week CPF challenge, alterations that were still evident over a period of drug withdrawal *(Middlemore-Risher et al., 2010)*.

With regard to inhibitory control, some studies revealed impulsiveness in male rats tested in a delay discounting task 10 weeks, 6 months and 1 year after a single high CPF dose *(Cardona et al., 2011, 2006; López-Granero et al., 2014)* or one week after a 31-week dietary exposure to the pesticide *(López-Granero et al., 2013b)*. In addition, this lack of inhibitory behaviour has also been assessed using the 5-CSRTT under a pattern of repeated exposure, which led to similar results *(Middlemore-Risher et al., 2010; Montes de Oca et al., 2013)*.

Although occasionally contradictorily, CPF-related anxiety-like behaviours in animal models have been well-documented by using an array of tests *(Braquenier et al., 2010; López-Crespo et al., 2009, 2007; Peris-Sampedro et al., 2014; Sánchez-Amate et al., 2001)*. By cons, the exploration of other affective or mood disorders is still lacking. Few empirical data suggested that CPF affects motivation, both maternal and social behaviour, and ultrasonic vocalizations *(Aldridge et al., 2005; De Felice et al., 2014; Venerosi et al., 2015, 2010)*.

1. 2. 7. 2. Metabolic diseases

Epidemiological evidence

As previously discussed, the current lifestyle has prompted the worldwide prevalence of obesity and type 2 diabetes increases at an unprecedented rate. In the light of this trend, the risk factors commonly studied appeared to be insufficient to account for the progress of both diseases. Hence, "non-traditional" risk factors have been reconsidered *(Thayer et al., 2012)*, and research addressing the role of environmental hazardous agents in both diseases outcomes has rapidly expanded in the past several years.

While most investigations traditionally focused on deciphering the contribution of OC pesticides in triggering type 2 diabetes and related metabolic dysfunctions *(Arrebola et al., 2013; Jaacks and Staimez, 2015)*, some epidemiological approaches have pointed to a neglected role of OPs *(Jaacks and Staimez, 2015; Montgomery et al., 2008; Saldana et al., 2007; Starling et al., 2014)*.
Few clinical studies, mostly case reports including those of Japanese terrorist attacks, collected evidence on blood glucose increases after exposure to several OP compounds *(Raafat et al., 2012; Yanagisawa et al., 2006)*. A prospective agricultural health study of incident diabetes, consisting in a 5-year follow-up interview (1999-2003), revealed that OP applicators had increased odds of diabetes *(Montgomery et al., 2008)*. In another study, non-diabetic Egyptian farmers tended to develop insulin resistance after exposure to malathion *(Rafaat et al., 2012)*.

Nowadays, there is a gap of knowledge on the obesogenic effect induced by OP pesticides. In fact, only 20 articles were found upon matching in PubMed "organophosphate" and "obesity" as keywords and limiting the results to "humans". From these, only 6 investigations explicitly refer to the search terms *(This search was conducted on September 13, 2015.)* *(Camps et al., 2009; Gonzalez et al., 2012; Huen et al., 2013; Lee et al., 2014; Meggs and Brewer, 2010; Slotkin, 2011)*. Strikingly, however, only the study by Gonzalez and co-workers provides a direct epidemiological approach on pesticide exposure and increased prevalence of obesity *(Gonzalez et al., 2012)*. Furthermore, some of them, designed as brief communications, are limited to collect and gather evidence to convince public opinion of the need to explore the contribution of OP pesticides to the current epidemic of obesity *(Meggs and Brewer, 2010; Slotkin, 2011)*.

Evidence from experimental animals

Following the same trend, there is a general lack of empirical research assessing the impact of OP pesticides on the development of obesity, type 2 diabetes and related metabolic disturbances. If already references are not abundant, when focusing solely on CPF, results are even scarcer. Furthermore, the bulk of existing CPF investigations refer to the developing brain *(Lassiter and Brimijoin, 2008; Slotkin et al., 2005)*, while adulthood assessment largely stays in the background. Current knowledge on the latter is limited to four studies carried out in male rats. From these, two described a weight gain in treated subjects *(Ehrich et al., 2004; Meggs and Brewer, 2007)* and the other two pointed to disturbances of both glucose and lipid homeostasis in exposed animals *(Acker and Nogueira, 2012; Elsharkawy et al., 2013)*. In general, these protocols were based on high CPF doses.

While hyperglycaemia emerged as an unavoidable consequence following acute doses of malathion *(Lasram et al., 2009)* and diazinon *(Teimouri et al., 2006)*, as well as a consequence of prolonged highly-dosed exposures to both OP compounds *(Mostafalou et al., 2012; Pournourmohammadi et al., 2005)*, other studies reported no differences at this level *(Sadeghi-Hashjin et al., 2008)*.

In addition to increased blood glucose, the study conducted by Pournourmohammadi et al. revealed hyperinsulinemia in male rats following a 28-day dietary challenge with malathion *(Pournourmohammadi et al., 2005)*. In this sense, previous research suggested that some OP can induce insulin resistance by promoting the dysfunction of pancreatic β cells, or the decrease of insulin action in target tissues *(Pournourmohammadi et al., 2005)*.

On the other hand, a number of experimental studies have endorsed the disruptor role of OPs in lipid homeostasis. Thus, increased plasma levels of triglycerides (TG), total cholesterol and low-density lipoprotein (LDL) were observed in male rats chronically treated with malathion *(Kalender et al., 2010)*. Similarly, Ogutcu and co-workers reported increases in cholesterol levels after repeated dichlorvos administration *(Ogutcu et al., 2008)*.

1. 2. 8. Genetic susceptibility

Through this dissertation, it has been recurrently emphasized the importance of identifying genetic variations that may condition the risk for adverse health outcomes upon exposure to a hazardous agent. In the case of CPF, genetic polymorphisms in biotransformation enzymes or target molecules are critical in modulating its toxicity.

Different CYPs (i.e., 1A2, 2B6, 2C9, 2C19, and 3A4) mediate each major pathway for CPF metabolism. Thus, while CYP2B6 is the main enzyme responsible for the formation of oxon, CYP2C19 is primarily responsible for its detoxification. CYP2B6 is a highly polymorphic enzyme and its genetic variants may account for the interindividual differences in toxicity emerged after exposure to CPF *(Crane et al., 2012)*.

Concerning PON1, the several allelic variants attributed to its gene differently affect the catalytic efficiency of the enzyme and its expression level *(Furlong et al., 2005)*. Hence, the intrinsic ability of an individual to detoxify CPF-oxon will largely depend on the polymorphism he carries.

In parallel, a large number of genetic polymorphisms have also been described for BChE, of which at least 39 present nucleotide alterations in the coding region, entailing lesser enzyme activities. While 76% people is homozygous for wild-type BChE, and thus present a normal scavenging rate against CPF-oxon, the remaining 24% carry at least one of these genetic variants, and thereby are predicted to be more susceptible to CPF toxicity *(Lockridge and Masson, 2000)*.

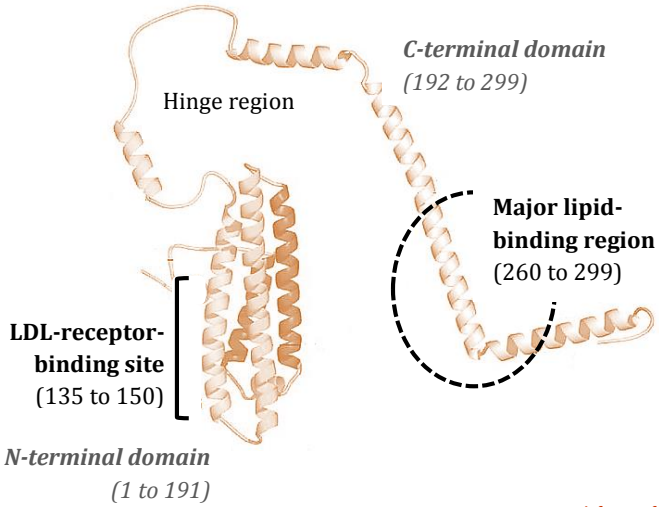
1. 3. APOLIPOPROTEIN E

1. 3. 1. Generalities and structural organization

Apolipoprotein E (apoE) is a prominent constituent of plasma and brain lipoproteins, which mainly exerts an anti-atherogenic function primarily upon interaction with members of the LDL receptor (LDLR) family. Though apoE synthesis is largely hepatic, its production can be readily detected in several tissues, such as the adrenal gland, testis, skin, kidney, spleen, adipose tissue and brain *(Mahley, 1988)*. Indeed, beyond its well-established role in regulating lipid homeostasis, it has been recurrently speculated that apoE is involved in additional biological processes.

Human apoE is a 299-residue soluble glycoprotein structurally complex. According to crystallographic studies, the N-terminal region forms an anti-parallel four-helix bundle containing the LDLR-binding site. For its part, the C-terminal residues shape a separately folded domain consisting in three amphipathic α-helices that initiate binding of the protein to lipid surfaces. Both terminal domains are connected through a hinge region, essential for the full LDLR-binding effectiveness, and interact by formation of hydrogen-bonds and salt-bridges *(Hatters et al., 2006)* (**Figure 3**).

Figure 3



Adapted from Hatters al., 2006

Figure 3 Model of the apolipoprotein E structure

1. 3. 2. *Apolipoprotein E* polymorphisms

The human apoE-encoding gene is localized on chromosome 19 at locus q13.31 *(Das et al., 1985)*. It is polymorphic with two major SNPs (rs429358C>T, rs7412C>T) in the coding region of the exon 4. Upon their combination at the *APOE* locus, three allelic variants emerge: ε*2*, ε*3* and ε*4* *(Seripa et al., 2007)*, accounting for more than 95% of the total genetic variants of the *APOE* gene in Caucasians *(Nickerson et al., 2000)*. At the mature protein level, these nucleotide exchanges are reflected by amino acid substitution, Arg per Cys, at positions 112 and 158, resulting in three main protein isoforms: apoE2, E3 and E4. ApoE3 contains Cys at position 112 and Arg at 158, whereas apoE2 and apoE4 contain Cys and Arg, respectively, at both positions *(Weisgraber, 1994)* (**Table 2**).

ApoE3 isoform displays lipid-binding ability and high affinity for the LDLR. Consequently, it operates optimally in promoting clearance of TG-rich lipoprotein and is associated with normal plasma lipid levels. Indeed, apoE3 has been traditionally referred as the healthy phenotype. Nonetheless, recent evidence suggests ε*3* carriers are more sensitive to diet-induced obesity *(Arbones-Mainar et al., 2008; Huebbe et al., 2015; Karagiannides et al., 2008)* ***(see section 1.3.4.3).***

The amino acid substitution at position 158 in apoE2 is close to the LDLR binding site, which makes their interaction notably weaker. As a result, ε*2* carriers poorly remove TG-rich lipoproteins, and thereby tend to develop type III hyperlipidaemia, a lipid disorder characterized by increased plasma levels of cholesterol and TG, and premature atherosclerosis *(Mahley and Rall, 2000)*.

Although apoE4 shows high affinity for the LDLR, this isoform is also associated with dyslipidemia. In point of fact, the presence of Arg 112 in its protein sequence facilitates a salt bridge interaction between its both domains, resulting in a differential C-terminal organization and a subsequent enhancing of the lipoprotein-binding affinity. Consequently, apoE4 prefers binding to very-low density lipoproteins (VLDL) or LDL, while apoE2 and apoE3 commonly bind cholesterol-rich high-density lipoproteins (HDL) (**Table 2**) *(Dong et al., 1994)*. Therefore, the *APOE4* genotype is associated with a more pro-atherogenic lipoprotein-cholesterol distribution. Furthermore, it has been identified as a major risk factor for AD, by means of an array of mechanisms that will be further contemplated in ***section 1.3.4.1.***

Allelic frequency and geographic distribution

Allelic variation attributed to *APOE* genotype is inherent to humans. Sequence comparisons between species revealed that non-human primate *apoE* and human *APOE4* are perfectly matched at positions 112 and 158, indicating the ε*4* is the ancestral allele *(Hanlon and Rubinsztein, 1995)*. After both lineages split, successive single mutations arose and gave rise first to the ε*3* allele and later the ε*2*. In general terms, the ε*3* variant is the most abundant in all human population, followed by ε*4* and ε*2* *(Corbo and Scacchi, 1999)* (**Table 2**).

Table 2 Human apoE isoforms prevalence and key differences

	ApoE isoform		
	ApoE2	**ApoE3**	**ApoE4**
Amino acid variation			
112	Cys	Cys	Arg
158	Cys	Arg	Arg
Allelic frequency range (*) (%)	0-14.5	55.3-91.1	5.2-40.7
Functional differences			
LDL receptor affinity	Low	High	High
Lipoprotein-binding preference	HDL	HDL	VLDL/LDL
Structural differences			
Domain interaction	No	No	Yes

(*) Allelic frequencies are from Corbo and Scacchi, 1999

Interestingly, however, their distribution varies by geographical areas. Accordingly, there is a north-to-south gradient of ε*3* and ε*4* notably in Europe. The ε*3* is more frequent in populations with a long-established agricultural economy like those of Mediterranean areas, while ε*4* abounds in Northern regions *(Egert et al., 2012)*. Therefore, it has been recently suggested that the *APOE4* genotype could compensate the lack of Ultraviolet radiation by displaying a better vitamin D status *(Huebbe et al., 2011)*. On the other hand, carriers of the ε*3* allele exploit more efficiently nutrients from the diet, and tend to accumulate fat in adipose tissue, factors that could have considerably contributed to its worldwide prevalence *(Huebbe et al., 2015)*.

1. 3. 3. Apolipoprotein E functions

ApoE operates as part of an anchoring mechanism that aids the transport and deliver of TG, phospholipid, cholesteryl esters, and cholesterol into cells and tissues *(Mahley and Rall, 2000)*. To

accomplish it, apoE must be biologically active and thus requires prior association to lipoproteins (e.g., VLDL, LDL, HDL, and chylomicron remnants) before interacting with members of the LDLR family. The interaction between apoE and the LDLR elicits the removal of apoE-containing lipoproteins from the circulation, thereby ensuring the maintenance of the lipid homeostasis in the peripheral system *(Hatters et al., 2006)*.

Neurobiology

By the mid-80s, early clues pointing to a pivotal role of apoE in neurobiology surfaced. On the one hand, apoE was found to be produced in abundance in the brain, notably by astrocytes and neurons, and served as the principal lipid transport vehicle in cerebrospinal fluid. On the other hand, increasing evidence suggested apoE was involved in neuronal repair: its synthesis was strongly fostered upon peripheral nerve injury, where it mediated the redistribution of lipids, vital for the structural and functional integrity of cell membranes, to regenerating axons and to Schwann cells during remyelinization *(Mahley and Rall, 2000)*. At present, it is well-known that apoE is crucial for neuronal plasticity, neurite outgrowth and synaptogenesis, among others *(Hauser et al., 2011)*.

Other functions

In recent years, a considerable amount of literature has inquired about other potential functions attributable to apoE *(Alata et al., 2014; Huang and Mahley, 2014; Levy et al., 2015; Vance and Hayashi, 2010)*. These studies evidenced its role in regulating both neuronal and astrocyte performance, including preventing excitotoxicity, promoting neuron survival, protecting neurons against oxidative stress, and modulating innate and adaptive immune responses *(Huang et al., 2004; Shen et al., 2008)*.

Within the brain, apoE mRNA is found in such areas as the hypothalamus and the olfactory bulb, indicating involvement in appetite and regulation of food intake *(Nathan et al., 2007; Shen et al., 2011, 2008)*. Interestingly, the work conducted by Shen and collaborators revealed that intracerebroventricular apoE, but not systemically-administered apoE, significantly decreased

food intake in rats. Nevertheless, other studies noted no differences at this level *(Chiba, 2003; Gao et al., 2007)*.

In 1991, Zechner and co-workers reported for the first time that adipocyte expressed high abundance of apoE *(Zechner et al., 1991)*. Thenceforth, an increasing number of both *in vitro* and animal-based studies supported its key role in inducing adipocyte differentiation and lipid storage in adipose tissue *(Huebbe et al., 2015; Kypreos et al., 2009; Lasrich et al., 2015)*, and thus in promoting diet-induced obesity and related metabolic dysfunctions. Corroborating these ideas, apoE$^{-/-}$ mice have been shown to have less body fat content and to be resistant to diet-induced obesity *(Huang et al., 2006; Karagiannides et al., 2008)*.

1. 3. 4. Apolipoprotein E and disease

1. 3. 4. 1. Alzheimer's disease

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases worldwide, accounting for more than 80% of dementia cases in the elderly *(Anand et al., 2014)*. It is characterized by a progressive and irreversible deterioration of cognitive capacity. At the anatomical-pathological level, AD patients exhibit an atypical intracellular accumulation of hyperphosphorilated tau protein, resulting in neurofibrillary tangles, and extracellular clusters of insoluble β-amyloid peptide, named senile or amyloid plaques *(Kumar et al., 2015)*. Considerable pieces of evidence have linked impairments of the cholinergic system with loss of memory function. Consistent with this, reduced cholinergic activity has been reported, even at early AD stages, after post mortem analysis of AD patient brains *(Allen et al., 1997)*. Though the familial form of the disease is relatively well-defined, it only accounts for 5% of total AD cases *(Chin-Chan et al., 2015)*. By cons, late-onset AD is the most prevalent form, and both genetic and environmental factors are key mediators in its not-yet-fully understood aetiology *(Godfrey et al., 2003)*.

The *APOE4* genotype is the largest genetic risk for AD accounting for approximately 60% cases *(Higgins et al., 1997)*. Indeed, being carrier of one ε*4* allele increases the risk for AD in 2–3 folds, whereas the risk rises about 12-fold when carrying two ε*4* alleles *(Roses, 1996)*. Interestingly, several lines of evidence supported an *APOE4* – sex interaction in humans. Women carrying ε*4* have been shown to display more pronounced AD-like changes in neuroimaging, neuropathological, and neuropsychological measures than men *(Beydoun et al., 2013; Ungar et al., 2014)*. In contrast, the *APOE2* genotype has been traditionally related to confer neuroprotection and associated with increased longevity *(Reinvang et al., 2013)*.

Multiple mechanisms have been proposed to explain such increased vulnerability towards developing AD, but the exact sequence of events remains a major challenge. Various lines of evidence support the premise that apoE4 increases the rate and extent of amyloid plaques reflected by poor β-amyloid clearance ability, and contributes to tau pathologies odds *(Arold et al., 2011; Du et al., 2009; Huang et al., 2004)*. Furthermore, it has also been suggested that the apoE4 phenotype might be related to diminished levels of functional apoE required to maintain neuronal health *(Sullivan et al., 2011)*. Other potential mechanisms have been contemplated, including impairment of the antioxidative defence system, diminished protection against environmental insults, dysregulation of neuronal signalling pathways, disruption of cytoskeletal structure, and potentiation of neuronal apoptosis *(Huang et al., 2004)*.

1. 3. 4. 2. Cognitive performance and neurobehaviour

Epidemiological evidence

Following the discovery of its implication in AD outcome, a growing number of studies attempted to examine the role of the *APOE4* genotype in normal brain function and cognition. The first evidence came from the observation of faster and earlier cognitive decline in AD patients who had at least one ε*4* allele *(Reitz and Mayeux, 2009)*. Lately, epidemiological approaches involving mild AD patients concluded that ε*4* carriers had significantly greater memory retention impairments, and showed sharper medial temporal lobe (MTL) atrophy,

while non-carriers obtained worse scores in tests measuring executive control and lexical access *(Wolk et al., 2010)*. The *APOE4* genotype also affects negatively cognition in the preclinical stages of the disease *(Reitz and Mayeux, 2009)*. However, beyond a disease condition, it has been demonstrated that old, but non-demented apoE4 individuals had impaired attention and working memory relative to non-carriers *(Greenwood et al., 2005)*, and perform worse both object recognition and episodic memory tasks, as well as spatial navigation tests *(De Blasi et al., 2009; Kukolja et al., 2010)*. More recently, literature has emerged that offers contradictory findings about the role of apoE4 in cognitive function of young individuals. Thus, whilst some authors argued young ε*4* carriers were not able to retain a spatial test task, neither able to spatially learn a route *(Acevedo et al., 2010)*, others concluded there were no differences in cognitive skills between both carriers and non-carriers *(Dennis et al., 2010; Reiman et al., 2004)*.

In recent years, and despite the controversy, the hypothesis of a possible cholinergic dysfunction to explain some of the cognitive deficits related to the *APOE4* genotype has gained strength. Higher levels of AChE *(Eggers et al., 2006)*, greater number of mAChRs *(Cohen et al., 2003)* or reduced activity of cholinergic neurons *(Salehi et al., 1998)* have been proposed as potential factors contributing to their cognitive shortfalls.

Because of its strong association with AD, a substantial proportion of research has extensively focused on deciphering the features of the *APOE4* genotype; meanwhile the other two apoE isoforms have received scant attention. In this regard, some epidemiological studies highlighted not only the neuroprotective role of apoE2, but also the advantageous cognitive condition of its carriers *(Suri et al., 2013)*. In agreement, healthy older individuals carrying ε*2* had reduced cognitive decline, and faster processing of information *(Suri et al., 2013; Wilson, 2002)*. Furthermore, it has been suggested that the *APOE2* genotype might be protective against episodic memory impairments in normal ageing *(Wilson et al., 2002)*.

Evidence from experimental animals

Research assessing cognitive functioning in experimental animal models, although largely limited to explore learning and memory processes, has reported significant apoE isoform-dependent effects. Accordingly, both male and female *APOE4* transgenic mice showed altered acquisition and retention in a MWM, which progressively worsened with age *(Pfankuch et al., 2005)*. Moreover, old APOE4 transgenic mice were the worst in performing a radial arm maze task *(Hartman et al., 2001)*. Human apoE targeted replacement (TR) mice have also provided valuable information on *APOE* behavioural attributes. Indeed, both apoE4 TR male and female mice displayed memory impairments in a MWM *(Bour et al., 2008; Grootendorst et al., 2005)*. Similarly, several works conducted in our research group disclosed a negative influence of the *APOE4* genotype in acquiring and retaining a MWM *(Reverte et al., 2013, 2012)*. Focusing exclusively on dry mazes, Rodriguez and co-workers found that young apoE4 mice exhibited significantly impaired spatial learning and memory in a Barnes maze (BM) task compared to apoE3 mice. Such deficits were accompanied by a reduced dendritic spine density in the medial entorhinal cortex, an area of the brain which transmits spatial information to the hippocampus, and plays a critical role in spatial representation *(Rodriguez et al., 2013)*.

1. 3. 4. 3. Obesity

Epidemiological evidence

Although the three *APOE* polymorphisms seem to modulate differently the ability of the protein to predispose to obesity, human data are scarce, with the existing evidence being rather inconsistent. Data from the Atherosclerosis Risk in Communities study, including 15,000 individuals, showed that human apoE isoforms are associated with a low-to-high body mass index (BMI) following the apoE4 < apoE3 < apoE2 rank order *(Volcik, 2006)*. Accordingly, the presence of ε*2* allele is predictive for obesity status in a minority population of Croatia *(Zeljko et al., 2011)*. However, under specific health conditions, this order may be reversed *(Volcik, 2006)*. Besides, ε*3* compared to ε*4* has often been associated with higher BMI and body weight in both children and adults *(Ellis et al., 2011; Gottlieb et al., 2004)*.

Evidence from experimental animals

In line with these findings, some studies with human apoE TR mice have suggested that the *APOE3* genotype contributes to the development of diet-induced obesity *(Arbones-Mainar et al., 2008; Huebbe et al., 2015; Karagiannides et al., 2008)*. ApoE3 mice subjected to a western-type diet were phenotypically more obese than apoE4 mice, while their total and subcutaneous amounts of fat also increased *(Arbones-Mainar et al., 2008)*. In agreement with this, Huebbe and collaborators reported that apoE3 mice were heavier than apoE4 not only when they were on a high-fat diet, but also on a low-fat diet, arguing that they were more prone to accumulate fat in adipose tissue owing to its efficiency at harvesting dietary energy *(Huebbe et al., 2015)*.

1. 3. 5. *Apolipoprotein E* — toxic interaction

Several studies have focused on the contribution of *APOE* genotype to the severity of the toxicity caused by environmental insults. The apoE4 isoform has been conventionally reported to confer less protection against the effects caused by heavy metals, including mercury and lead, due to its reduced ability to bind them *(Godfrey et al., 2003; Mutter et al., 2004; Stewart et al., 2002)*. In contrast, a recent study from our research group showed that male apoE3 TR mice were the most vulnerable of the three *APOE* genotypes to the lipophilic compound decabromodiphenyl ether *(Reverte et al., 2013)*. Indeed, apoE3 mice postnatally treated with one of the congeners exhibited long-lasting spatial learning deficits *(Reverte et al., 2013)*. Nowadays, no single study exists which assess the potential behavioural and metabolic interaction between *APOE* genotype and the pesticide CPF.

1. 3. 6. Transgenic *apolipoprotein E* animal models

The apoE effects on disease have been widely investigated using several rodent models. Initially, studies with apoE-deficient mice corroborated the suspected implication of apoE in learning and memory processes *(Oitzl et al., 1997; Raber et al., 1998)*. Then, the first transgenic mouse lines expressing human *APOE* variants were designed in order to further explore the detrimental role of the *APOE4* genotype in cognition. However, these early transgenic models, generated from apoE$^{-/-}$, were under human promoters, and often displayed varying levels of transgene

expression, or no equitable distribution of the human isoform *(Grootendorst et al., 2005)*. Lately, the human apoE TR mouse model was created by replacing the murine *apoE* gene by one of the three most relevant human *APOE* variants, keeping intact the murine regulatory sequences *(Sullivan et al., 1997)*. As a result, apoE TR mice express functional human apoE isoforms at physiological levels, being their subsequent phenotype similar to that found in humans *(Hauser et al., 2011)*, fact that makes it a suitable model for studying apoE-related diseases.

1. 4. Neurobehavioural endpoints

Neurobehavioural research has been a very useful tool in neuroscience and toxicology, as it has allowed the *in vivo* evaluation of toxicants related-effects *(Weiss, 1994)*. Rodents share a number of neuroanatomical, neurochemical and behavioural commonalities with humans, and therefore are widely used for neurobehavioural assessment. Nowadays, a wide range of functional, cognitive and emotional abilities can be evaluated in rodent models using customized tests *(Sartori et al., 2011)*.

1. 4. 1. Learning and memory

Neural processes involving learning and memory, modulated by several endogenous systems, are crucial to ensure the adaptation of an individual to its environment.

Definitions

Learning is the process that leads to a permanent change in behaviour as a result of practice and subsequent acquisition of knowledge *(Kandel et al., 2013)*. Meanwhile, memory is the process by which this knowledge is encoded, stored and later retrieved *(Kandel et al., 2013)*.

There are two main types of memory that differ temporally: short and long-term memory. The latter can be divided into declarative (explicit or conscious) or non-declarative (implicit or unconscious). The declarative memory encompasses the knowledge of facts and their meaning, and can be sub-divided into episodic memory (i.e., association of an event to temporal and spatial contexts) and semantic memory (i.e., knowledge of the event regardless of the context).

The non-declarative memory refers to the acquisition of motor skills and habits *(Sharma et al., 2010)*. Episodic memory is especially vulnerable to normal ageing and is more likely to be impaired in neurodegenerative processes *(Graef et al., 2011)*.
Within the short-term memory, the working memory arises as the limited capacity allowing the temporary storage and manipulation of information necessary for a complex task *(Baddeley and Hitch, 2000)*.

Navigation is the intrinsic ability of organisms to learn to find their way through the environment without getting lost. It can be basically divided into spatial (allocentric) and egocentric navigation. The first one is characterized by the ability to navigate using distal cues, while the second wayfinding involves navigating using internal cues *(Vorhees and Williams, 2014)*. Typically, spatial memory stands as a subtype of episodic memory because it stores information within the spatio-temporal frame *(O'Keefe and Nadel, 1978)*.

Neurobiology of learning and memory

The major brain structure involved in declarative memory is the MTL, comprising the hippocampus together with surrounding areas (i.e., entorhinal, perirhinal and parahippocampal cortex) *(Corkin et al., 1997)*. However, interactions between the MTL and prefrontal cortex seem to be critical for successful memory storage *(Reber et al., 2002)*. At first, neocortical associative areas send sensorial information to the parahippocampal and perirhinal cortices that in turn send afferences to the entorhinal cortex. Lately, this structure gives afferents to the hippocampal formation (i.e., dentate gyrus; CA3, CA2 and CA1; and the subiculum). Once there, the information flows in reverse to get back to neocortical associative areas *(Rudy, 2009)*. On the other hand, non-declarative memory depends largely on the neostriatum, cerebellum and amygdala *(Sharma et al., 2010)*.

Spatial navigation involves several brain areas, such as striatum, basal forebrain, and neocortical areas. Nonetheless, numerous electrophysiological and lesion studies support that the primary region crucial for mediating this ability is the hippocampus *(Bird and Burgess, 2008;*

Buzsáki and Moser, 2013). Indeed, Nadel and O'Keefe observed that hippocampal place cells fired when the rat was in a specific location *(O'Keefe and Nadel, 1978)*. During the initial minutes of exposure to a novel space, these preferred locations undergo refinement *(Hill, 1978; Wilson and McNaughton, 1993)*, and are retrieved when the environment is subsequently revisited. Thereby they suggested that upon the use of external cues, the hippocampus creates a neural representation of the physical space, they called cognitive map *(Eichenbaum et al., 1999)*. Parietal cortex, whose cells provide information about head position and movement, seem to send additional information to place cells *(Save et al., 2005)*. It has been shown that basal forebrain lesions impair the performance of both the MWM *(D'Hooge and De Deyn, 2001)* and BM *(Greferath et al., 2000)*, likely because it provides innervation to the hippocampus and neocortex. The striatum is implicated in the response flexibility, motor control and procedural consolidation in the MWM *(D'Hooge and De Deyn, 2001)*, and lesions in this brain area impair spatial learning in the BM *(O'Leary and Brown, 2013)*. Furthermore, some authors suggest it is essential for spatial memory retrieval *(Iaria et al., 2003; Méndez-Couz et al., 2015)*.

Various neurotransmitter systems are involved in spatial learning and memory, being the cholinergic system critical for both processes. During spatial acquisition learning, ACh is released into the extracellular space in hippocampus and cortex, enabling its interaction with cholinergic receptors. However, during consolidation of spatial reference memory, ACh levels are low. These process-dependent requirements explain why the blockade of ACh receptors during acquisition impedes subsequent memory formation *(Deiana et al., 2011)*. Although controversially, the glutamatergic system has also been related to spatial learning and memory processes. Thus, transgenic mice lacking NMDA receptors in the CA1 region of the hippocampus show spatial memory impairments *(Tsien et al., 1996)*. The amine system does not play an imperative role in spatial navigation, but noradrenaline, dopamine (DA) and serotonin (5-HT) do it, as they have been shown to alter various aspects of the MWM performance *(Braun et al., 2015; Du et al., 2007; Warner and Drugan, 2012)*.

Assessment of learning and memory in rodents

The Barnes maze (BM) test was firstly developed in 1979 by Carol Barnes to assess visuo-spatial learning and memory in aged rats *(Barnes, 1979)*. Sixteen years later, Bach and co-workers adapted the task for its use in mice *(Bach et al., 1995)*. Thenceforth, the BM has been successfully used in different inbred mouse strains *(Holmes et al., 2002; Koopmans et al., 2003; O'Leary et al., 2011)*; in transgenic mouse models of AD *(O'Leary and Brown, 2009)*; and in mice carrying targeted mutations within genes implicated in learning and memory processes *(Seeger et al., 2004)*.

The dry-land maze consists of an elevated circular platform with many holes (i.e., 12, 20 or 40 depending on the diameter) evenly spaced around the perimeter. Beneath one of them, there is a removable, small, dark escape box designed to allow the animal to hide from adverse stimuli *(Sharma et al., 2010)*. This test has been considered appropriate for mice owing to their propensity to escape through small holes, and their intrinsic predilection for dark environments over open areas *(Bach et al. 1995)*. Furthermore, the BM has a number of advantages over the MWM, also aimed at assessing spatial learning and memory. Indeed, the BM avoids stress induced by swimming, prerequisite in the MWM, and seems to be less physically taxing *(Harrison et al., 2009)*.

In the standard reference memory task, animals should use distal visual cues in the extra-maze environment to locate the escape box that allows them to escape from aversive bright light and open space *(Harrison et al., 2009)*. Nonetheless, rodents can use different strategies to locate the target hole: a praxic strategy, in which the animal learns the sequence of movements required to reach the escape box; a taxic strategy, in which the animal uses proximal cues to the same end; or a spatial strategy, in which the animal reaches the escape box by using external cues. To prevent the mice using internal or proximal cues, each trial starts by putting them in the middle of the arena under a coloured box, thereby ensuring the animals to be in random orientation. However, non-controlled proximal cues, such as irregularities in the maze surface, can bring the mice additional wayfinding information. To avoid it, the maze can be rotated around its central axis in each trial, as long as the position of the escape box with respect to the external cues is maintained. Besides, to avoid odour cues, both the surface and the escape box must carefully be cleaned with ethanol *(Sharma et al., 2010)*.

1. 4. 2. Impulsivity, compulsivity and attention

Definitions

Impulsivity can be broadly defined as the tendency to act with inadequate degree of deliberation, forethought, or control *(Moeller et al., 2001)*. Indeed, for this action to occur, several factors must be met: stimulus, strong impulsion and dysfunctional inhibitory processes *(Bari and Robbins, 2013)*. By cons, compulsivity is acting persistently and improperly to the situation, without apparent relation to the overall objective of the action, often resulting in undesirable consequences *(Dalley et al., 2011)*. Both traits appear to be characterized by difficulties in self-control *(Dalley et al., 2011; Robbins et al., 2012)* (**Figure 4**).

The behavioural phenotypes covered by the term impulsivity include: acting without forethought, failure to inhibit inappropriate behaviour, altered perception of time, insensitivity to negative consequences, propensity to engage in risky actions, or inability to wait for a reward *(Bari and Robbins, 2013)*. Therefore, impulsivity cannot be conceived as a unitary construct, but rather as a variety of phenomena that could be attributed to different biological bases *(Winstanley et al., 2004)*. Notwithstanding, there is some consensus to classify impulsive behaviours into two major categories: a) impulsive decision making, or impulsive choice, and b) impulsive action, or behavioural disinhibition, or in other words “stopping” versus “waiting” *(Robinson et al., 2009)*. Waiting impulsivity is also known as premature or anticipatory responding, and can be assessed using the 5-CSRTT *(Voon, 2014)*.

Compulsivity is characterized by highly stereotypical or ritualized behaviours, including repetitive thoughts and actions, which generally lead to functional impairment *(Dalley et al, 2011)*. Therefore perseverative responses can be considered as an index of compulsiveness, and are defined as repeated responses that appear to be inappropriate by not being demanded by the situation *(Crider, 1997; Sandson and Albert, 1984)*.

Figure 4

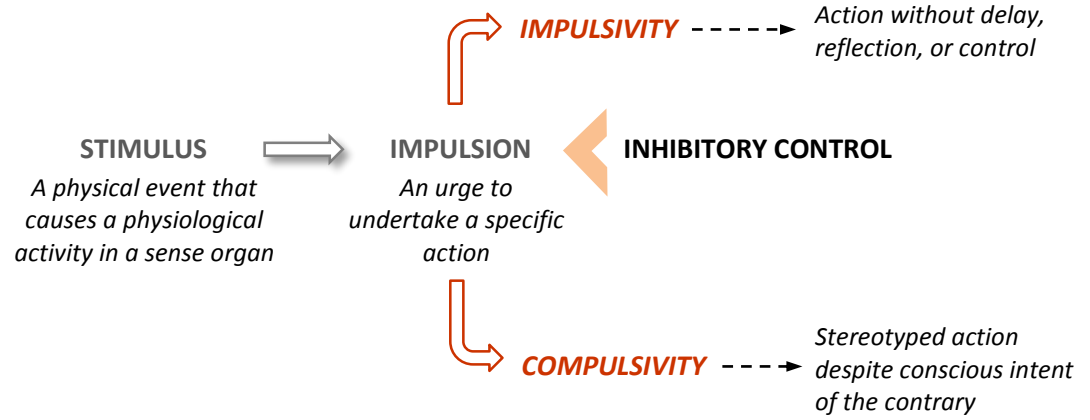


Figure 4 Impulsivity and compulsivity constructs. In susceptible individuals certain stimuli may activate strong urges that are not appropriate in a given environment. When inhibitory processes are functional, those impulsions are kept under control. However, strong impulsions and deficient inhibitory control will result in impulsive or compulsive acts or thoughts.

According to Sandson and Albert (1984), three major varieties of perseveration ensue: continuous, recurrent and stuck-in-set. The continuous form is the inability to terminate a discrete response, even if the task is already finished, and can be assessed in the 5-CSRTT. The recurrent perseveration is the inability to change to a different motor program, thus keeping in repeating the first even though the requirements of the task are different. Finally, the stuck-in-set form refers to a failure to respond differently to task demands, thus displaying the same pattern already learnt *(Sandson and Albert, 1984)*.

Although impulsive and compulsive traits may contribute to adaptive human behaviour, disordered regulation of both constructs may be detrimental in the development of several psychiatric and mental disorders *(Robbins et al., 2012)*. For instance, excessive impulsivity

characterizes attention deficit hyperactivity disorder (ADHD), mania, and personality disorders *(Clark and Robbins, 2002)*, while compulsivity is a major component of obsessive-compulsive disorder, schizophrenia and autism. Both traits are key mediators of substance abuse and eating disorders *(Robbins et al., 2012)*.

Attention is a vast complex psychological concept influencing almost all aspects of cognitive behaviour, as it is necessary in tasks requiring stimulus selection, response selection and performance monitoring *(Lustig et al., 2013)*. Generally, attention can be divided into two distinct constructs: input selection (i.e., the selection of task-relevant inputs for further processing) and rule selection (i.e., the process of choosing which rules to use in responding to selected inputs) *(Luck and Gold, 2008)*. The input selection covers several models of attention: selective attention, divided attention and sustained attention. Selective attention, which was firstly described by Donald Eric Broadbent in 1958 *(Broadbent, 1958)*, includes two competing processes: bottom-up and top-down. Bottom-up attention occurs when the brain automatically attends to sensory cues in the environment that stand out in some way. Conversely, top-down attention involves conscious control of attention toward some target. Bottom-up attention has long been considered to be dependent on the posterior parietal cortex, whereas top-down attention is considered to be dependent on the PFC and its connections. Emerged from the criticism of the Broadbent's theory, the model of divided attention assumes that simultaneous or parallel stimuli processing may occur, thus requiring additional demand for number of items attended to, and thereby implying additional top-down control *(Mahone and Schneider, 2012)*. Finally, sustained attention can be defined as the subject's readiness to detect unpredictably occurring signals over prolonged periods of time, and can be measured in the 5-CSRTT *(Lustig et al., 2013; Robbins, 2002)*. Human imaging studies have demonstrated that activation of frontal and parietal cortical areas are associated with sustained attention performance. Further, data from experimental animal studies have revealed that cholinergic inputs originating in the basal forebrain are pivotal components of the neuronal circuitry *(Sarter et al., 2001)*.

Neurobiology of impulsivity and compulsivity

Nowadays, it is well-documented that the prefrontal cortex (PFC) is crucial for the modulation of inhibitory control. Indeed, selective lesions of the medial PFC impair as simple measures of behavioural inhibition as novelty *(Dalley et al., 1999)*, or responding in extinction *(Quirk et al., 2006)*. Besides, the striatum, which is highly connected to the PFC, remarkably contributes to several forms of impulsive behaviour *(Dalley et al., 2008)*. It has been suggested that fronto-striatial circuits implicated in inhibitory control may somehow overlap in both impulsive and compulsive traits *(Robbins et al., 2012)*.

Impulsivity of decision making (stopping impulsivity) depends upon interactions between PFC areas (i.e., right inferior frontal gyrus, anterior cingulate cortex, orbitofrontal cortex, and cortical motor areas) and the basal ganglia, within which stand out the dorsal striatum, the globus pallidus and the subthalamic nucleus *(Aron, 2007; Dalley et al., 2011)*. Thus, selective lesions of the orbitofrontal cortex have been reported to disrupt stopping impulsivity, but not premature responding in the 5CSRT task, that is waiting impulsivity *(Dalley et al., 2008)*.

On the other hand, waiting impulsivity depends upon top-down PFC interactions with the hippocampus, amygdala, and structures in the ventral striatum, including the nucleus accumbens. Furthermore, several specific structures of the PFC (i.e., anterior cingulate cortex, dorsal and ventral prelimbic cortex, and infralimbic cortex) make distinct contributions to waiting impulsivity via independent inputs to the nucleus accumbens *(Dalley et al., 2011)*. Thus, impulsivity on the 5CSRTT is generally most significantly increased upon infralimbic cortex lesions *(Chudasama et al., 2003)*.

In addition to the aforementioned, both varieties of impulsivity are also modulated by serotonergic neurons in the raphe nuclei, midbrain dopaminergic neurons in the substantia nigra or ventral tegmental area, and noradrenergic neurons in the locus coeruleus *(Dalley et al., 2011)*. Indeed, the alteration of these neurotransmitter systems results in an impairment of inhibitory processes. It has been recurrently hypothesized that decreased 5-HT transmission correlates with increased premature responding in the 5-CSRTT *(Winstanley et al., 2004)*, even

though underlying mechanisms remain unclear. The role of DA in impulsivity, however, has been fairly well elucidated. Waiting impulsivity increases, while stopping impulsivity decreases upon enhancement of DA signalling *(Dalley and Roiser, 2012)*. In contrast, waiting impulsivity is reduced upon challenging noradrenaline transmission *(Pattij and Vanderschuren, 2008)*. Existing research also recognizes the, sometimes underestimated, critical role played by the GABAergic system in impulsivity *(Hayes et al., 2014; Montes de Oca et al., 2013)*. Besides, it has been demonstrated that alterations in central cholinergic function contribute to the aetiology of diseases in which decision making is perturbed, such as AD, ADHD, and schizophrenia *(Hosking et al., 2014)*. In this regard, while nicotine has been considered as a cognitive enhancer, recent experimental data indicate its cognitive benefits may be accompanied by impulsiveness *(Hosking et al., 2014)*.

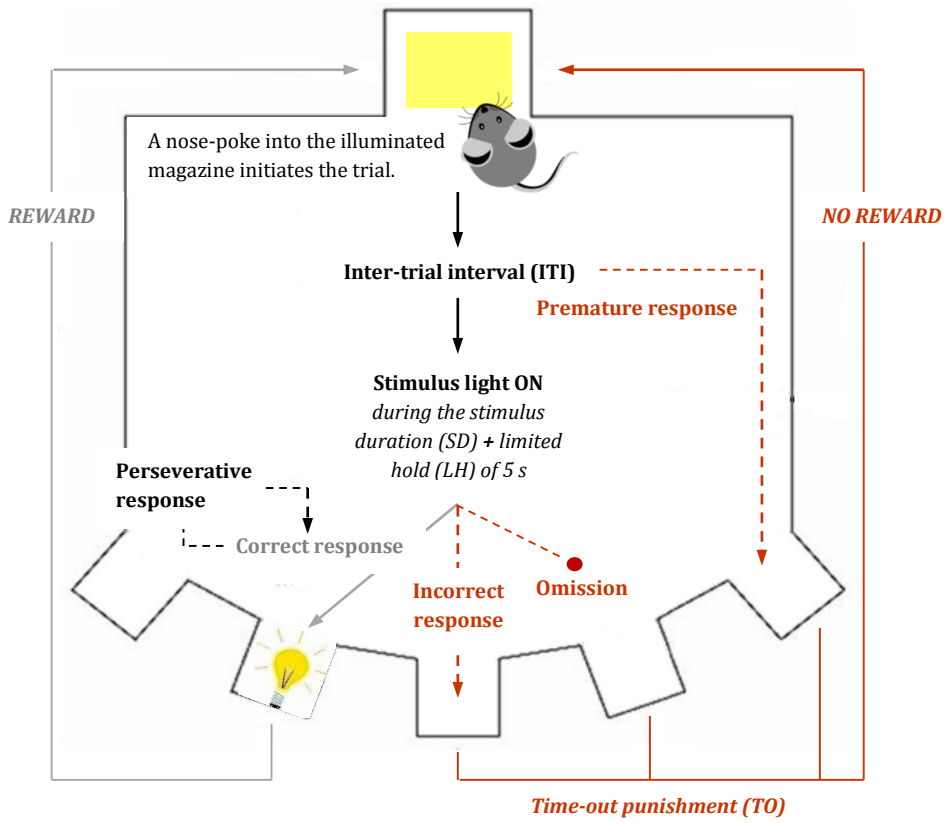
Assessment of impulsivity, compulsivity and sustained attention in rodents

To date, many behavioural tasks have emerged aiming to assess impulsivity in rodent models based on the two main categories of impulsive behaviour previously described. The impulsivity of decision making includes behavioural paradigms that assess impulsive choice, occurring when the animal tends to choose an immediate but smaller reward over a larger but delayed reward *(Dalley et al., 2011)*.

In 1983, Robbins and co-workers designed a test to assess attentional performance in rats, which was based on the continuous performance task used for the same purpose in humans *(Robbins, 2002)*. The result of an adaptation of the original version, the 5-choice serial reaction time task (5-CSRTT) enables various aspects of performance to be assessed simultaneously in both rats and mice *(Bari et al., 2008)*. So, the 5-CSRTT stands as a useful tool to assess not only sustained attention, but also inhibitory response control, and motivation. When used in mice, it may provide valuable information on both the genetic and neural bases of attention, waiting impulsivity and compulsivity, and may also help to understand to what extent affective states can modulate those processes *(Sanchez-Roige et al., 2012)*.

This task is conducted in operant chambers which consist of five operative evenly-spaced holes, distributed along a curved wall, equipped with infrared detectors and a bright light. In the opposite wall, there is a reward magazine also furnished with an infrared detector, automatically delivering a reward (**Figure 5**).

Figure 5



Adapted from Sanchez-Roige et al., 2012

Figure 5 Sequence of a session in a 5-choice serial reaction time task chamber. It should be noted that incorrect and premature responses can be performed at any non-illuminated hole.

Briefly, mice are progressively trained to detect a brief stimulus that is presented pseudorandomly in one of the five holes, in order to trigger a reward. Once reached a stable performance, the 5-CSRTT allows manipulating several baseline conditions in order to evaluate mice responses, so that mice attentional accuracy, inhibitory control and motivation. For instance, premature responding (i.e., responding before the onset of the visual stimulus), which reflects the inability to inhibit the response, provides a measure of waiting impulsivity *(Robbins, 2002)*. On the other hand, perseverative responding (i.e., responding persistently into the hole after the extinction of the stimulus) provides a measure of compulsivity *(Dalley et al., 2007)*.

1. 5. Energy homeostasis, feeding behaviour and obesity

1. 5. 1. Regulation of energy homeostasis

Despite the obvious disparities in body weight that occur within a population, it is noteworthy that intra-individual variations are remarkably stable over time. As expected, such weight stability is achieved by adjusting mechanisms of energy homeostasis, which are responsible for compensating the compendium of threats to which an individual is exposed from day to day. It is now well-recognized that food intake, energy expenditure and body adiposity are homeostatically regulated *(Keesey and Powley, 2008)*.

Energy homeostasis requires peripheral (i.e., gastrointestinal system and adipose tissue) and central signals to converge in certain brain regions to jointly coordinate the regulation of both short-term and long-term balances between energy intake and energy expenditure *(Korner et al., 2009)*. These brain areas include the hypothalamus, particularly the arcuate nucleus (ARC) that stands as the major site for sensing and integrating such signals. The latter houses at least two opposite neuronal circuitries: one appetite-stimulator and the other appetite-inhibitor. Both of them send signals primarily to the paraventricular nucleus (PVN) of the hypothalamus, which directly modulates feeding behaviour *(Gale et al., 2004)*. The appetite-stimulatory circuit consists basically of neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons that promote appetite and reduce energy expenditure. NPY directly signals to the PVN, whereas AgRP acts

indirectly by blocking the melanocortin type 4 receptor (MC4R), which is an appetite-inhibitory receptor in the PVN. The appetite-inhibitory circuit includes cocaine- and amphetamine-regulated transcript and proopiomelanocortin neurons, the latter producing α-melanocyte–stimulating hormone that operates mainly through the MC4R to inhibit appetite *(Luquet and Magnan, 2009; Schwartz et al., 2000)*.

The currently accepted model of energy intake proposes that intrinsic signals become integrated together with other extrinsic regulators of food intake, such as food availability and palatability, emotions, habits or social behaviour. Likewise, both meal size and cessation are likely to be governed by both intrinsic and extrinsic factors. In either case, such peripheral hormonal signals as leptin, insulin and ghrelin are crucial for maintaining energy homeostasis *(Donovan and Tecott, 2013; Korner et al., 2009; Morris and Rui, 2009)*.

Leptin

Leptin is an anorexigenic peptide mainly produced by adipocytes, of which circulating levels are positively correlated with the amount of body fat *(Harris, 2000)*. Leptin provides information on changes in both energy balance and the amount of fuel stored as fat to the CNS upon its interaction with specific leptin receptors widely distributed throughout the brain *(Morton, 2007)*. The most abundant leptin receptor, LepRb, is primarily expressed in the hypothalamus and is largely responsible for its anorexigenic effects *(Park and Ahima, 2015)*. Under normal conditions, leptin inhibits food intake and promotes energy expenditure, while decreased leptin signaling induces food intake and fat accumulation *(Morton, 2007)*. About two decades ago, early evidence of elevated leptin expression in obese individual aroused *(Lönnqvist et al., 1995)*. Nowadays it has been demonstrated that obese individuals not only exhibit high levels of leptin, but also that they fail to reduce excess adiposity, thereby indicating leptin resistance *(Park and Ahima, 2015)*. The underlying mechanisms are currently under intense investigation as they may serve as a potential therapeutic option for treating obesity. It has been suggested that leptin resistance may be due to defective transport of leptin into the brain, and/or reduced hypothalamic leptin signalling *(Gale et al., 2004 Morris and Rui, 2009)*.

Insulin

Insulin is a peptide secreted by the pancreatic β-cells in response to rising blood glucose levels after meals. Though it is primarily known for promoting glucose uptake and utilization by peripheral tissues, it also has a prominent role in mediating energy balance within the brain *(Donovan and Tecott, 2013)*. In a manner similar to leptin, insulin has been shown to act as an anorexigenic hormone. Indeed, its receptors are widely distributed throughout the brain, particularly in the ARC *(Luquet and Magnan, 2009; Schwartz et al., 2000)*, and when administered centrally it reduces both food intake and body weight *(Bruning, 2000; Gale et al., 2004)*. By cons, brain-specific knock-out of the insulin receptor causes an increase in food intake and makes individuals more susceptible to diet-induced obesity *(Obici et al., 2002)*. Furthermore, like leptin, insulin may be considered as an adiposity signal, as its circulating concentrations are also proportional to adiposity. In point of fact, obesity is generally associated with both hyperinsulinemia and hyperleptinemia, which are indicative of insulin and leptin resistance, respectively *(Gale et al., 2004)*.

Diabetes is a metabolic disease characterized by hyperglycaemia that results from defects in insulin secretion or action or a combination of both. Type 1 diabetes represents 3 to 5% of all diabetes cases and is caused by a lack of insulin secretion by autoimmune destruction of the β-cells of the pancreas. On the other hand, type 2 diabetes is the most common form of the disease, and gradually develops due to a defect in insulin secretion in the context of a gradual peripheral resistance to insulin action *(Jeon et al., 2015)*. It is a complex disease resulting from the combination of environmental and genetic factors. Indeed, it has been suggested that excessive caloric intake, decreased physical activity, smoking and alcohol consumption, and several hazardous agents may play a crucial role in the aetiology of the disease *(Bi et al., 2012)*. As is the case in obesity, current type 2 diabetes burden has no specific precedent. According to the International Diabetes Federation (IDF), the European prevalence of diabetes was estimated to be 7.9% in 2014 (i.e., 52 million people), whilst the predictions point to 68.9 million diabetic patients in 2035 *(IDF, 2014)*. Spain actually occupies the fourth place in the European ranking of countries with higher rates of the disease with a prevalence of 10.6% *(IDF, 2014)*.

Ghrelin

Ghrelin is an endogenous orexigenic peptide, primarily synthetized in the stomach that was first discovered as an endogenous ligand for the growth-hormone-secretagogue receptor (GHS-R). Two major forms of ghrelin coexist in the blood: acyl ghrelin and des-acyl ghrelin *(Hosoda et al., 2000)*, but it has been suggested that only the acylated form binds the GHS-R, and therefore is the only to be biologically active. However, increasing evidence points to a neglected metabolic role of des-acyl ghrelin *(Delhanty et al., 2013; Heppner et al., 2014)*. The inactivation of acyl ghrelin into the deacylated form largely depends on the BChE enzyme *(De Vriese et al., 2004)*. The pattern of ghrelin release suggests that it governs feelings of hunger: acyl ghrelin levels increase by fasting, while they decrease after the mealtime *(Kojima et al., 1999)*. Accordingly, central or peripheral administration of acyl ghrelin stimulates food intake, whereas chronic administration causes weight gain by not only stimulating food intake, but also decreasing both energy expenditure and the utilization of fat, and increasing utilization of carbohydrates *(De Vriese and Delporte, 2008)*.

1. 5. 2. Obesity

From a broad perspective, overweight and subsequent obesity are defined as abnormal or excessive fat accumulation that may endanger the healthy status of an individual *(Haslam and James, 2005)*. A crude population measure of obesity is the BMI, which is calculated by dividing the patient's weight in kilograms by the square of the individual's height in meters (kg/m^2). According to the WHO, adults with a BMI in the range of 25 to 29.9 are classified as overweight, while a BMI exceeding 30 is synonymous with obesity *(WHO, 2015)*. The leading cause of obesity and overweight is an energy imbalance between calories consumed and calories expended. Currently, high-energy and high-fat diets are within everyone's reach, and the increasingly sedentary nature of many forms of work, changing modes of transportation, and increasing urbanization has prompted the progressive decrease of physical activity *(Bray and Popkin, 2014; Mitchell et al., 2011)*.

Either directly or indirectly, the disruption of any of the mechanisms involved in energy homeostasis gives rise to life-threatening conditions, which include obesity and type 2 diabetes,

pathologies that are strongly linked. In fact, obesity clearly increases the likelihood of developing type 2 diabetes. A great deal of epidemiological studies has confirmed the links between excess weight and the development of insulin resistance and diabetes, suggesting that patients with excessive weight are at substantial risk for developing diabetes *(Brown et al., 2009; Haslam and James, 2005)*. Besides, raised BMI is a major risk factor for such other non-communicable diseases as cardiovascular disease, musculoskeletal disorders, and several types of cancer (e.g., breast, colon, etc.) *(WHO, 2014)*.

A number of authors have considered both impulsivity and compulsivity as potential factors contributing to the obesity epidemics *(Mole et al., 2015; Schag et al., 2013; Smith and Robbins, 2013)*, and incipient epidemiological data begin to suggest overlaps between disorders of pathological food and drug use *(Avena et al., 2011; Ziauddeen et al., 2012)*. A specific subgroup of obese individuals suffers from binge eating disorder, defined by the American Psychiatric Association as "recurring episodes of eating, in a discrete period of time, an amount of food that is definitely larger than most people would eat during a similar period of time [with a]…lack of control during the episodes". Like drug addicts, food-addicted individuals experience a lack of control in the face of food, have a continuation of overuse despite severe health consequences, and are unable to fight the urge to eat. In addition to similar behavioural traits, substance-dependent and obese individuals also show analogies in their brain structure and neurochemical profiles, including changes in fronto-striatal circuitry and abnormalities in the dopamine neurotransmitter system *(Smith and Robbins, 2013)*.

RATIONALE

2. RATIONALE

As it has been repeatedly mentioned throughout this dissertation, OPs in general and CPF in particular, are extensively used as pesticides. Although efforts have been taken to reduce CPF applicability, a great deal of evidence suggests it is still threatening human and natural ecosystems. With regard to this, recent data reveal CPF residues are widely distributed throughout both rural and urban areas *(Ccanccapa et al., 2015; Page et al., 2014; Weldon et al., 2011)*, implying that virtually everyone is exposed to its toxicity, to a greater or lesser extent. The most important risk group is made up of those individuals who daily and massively handle these substances, including general pesticide applicators, crop dusters, greenhouse workers, and farmers. In these cases, workers are steadily exposed to OP pesticides and are susceptible to acute poisoning, which still today constitutes a major public and occupational health concern *(Faiz et al., 2011; Roldán-Tapia et al., 2006)*. The inhibition of AChE induced by CPF, resulting in an accumulation of ACh at cholinergic synapses, is responsible for most of the symptoms observed in acute poisonings. Nevertheless, of particular interest are those exposure patterns that initially go unnoticed but may progressively contribute to the development of silent chronic exposures that may lately trigger undesirable consequences to health. Thus, for example, a considerable amount of epidemiological studies has revealed deficits in different domains of cognitive function after chronic exposure to OPs *(Farahat et al., 2010; Mackenzie Ross et al., 2010; Ross et al., 2013; Stephens and Sreenivasan, 2004)*. In addition, albeit less frequently, affective psychiatric disorders have also been described in OP pesticide applicators *(London et al., 2005; Mackenzie Ross et al., 2010; Roldán-Tapia et al., 2006, 2005; Steenland et al., 2000)*. An array of experimental investigations endorses human data. However, research on such cognitive functions as attention, as well as on complex behavioural processes including inhibitory control and motivation is rather lacking. In addition, in light of the current global prevalence of both obesity and diabetes, there has been renewed interest in ascertaining the contribution of OP pesticides to the aetiology of both health conditions *(Gonzalez et al., 2012; Jaacks and Staimez, 2015; Meggs and Brewer, 2010; Montgomery et al., 2008; Saldana et al., 2007; Slotkin, 2011; Starling et al., 2014)*. Nevertheless, evidence remains sparse, and underlying mechanisms are not yet fully elucidated.

Given the ubiquity of CPF exposures, one of the critical issues is to establish whether there are subpopulations that are particularly susceptible to its detrimental effects. In this context, the existing research focuses on exploring genetic polymorphisms in its biotransformation enzymes or its target molecules *(Costa et al., 2013; Crane et al., 2012; Furlong et al., 2005; Lockridge and Masson, 2000)*, whereas less is known about the interaction of CPF with other genetic risk factors.

Human *APOE* polymorphisms have been shown to confer different vulnerability to neurodegeneration: while the *APOE4* genotype is considered to be the largest genetic risk for AD, ε*2* carriers are suspected to be protected from neurodegeneration and have been associated to an increased longevity *(Higgins et al., 1997; Reinvang et al., 2013; Suri et al., 2013)*. Notwithstanding, apoE seems to further modulate cognitive function in the absence of a disease condition. Thus, poor cognitive outcome has also been attributed to the *APOE4* genotype, but studies addressing the behavioural features of the other two apoE isoforms are scarce. It has been controversially hypothesized that *APOE4*-related cognitive shortfalls may be partly due to a possible cholinergic dysfunction *(Cohen et al., 2003; Eggers et al., 2006; Salehi et al., 1998)*. Indeed, it is well-known that the cholinergic system is involved in such higher functions as sustained attention and impulse action control *(Cardona et al., 2006; Middlemore-Risher et al., 2010; Montes de Oca et al., 2013; Paolone et al., 2013)*. In the meantime, the most common apoE isoform has drawn less attention. In fact, the *APOE3* genotype has been traditionally considered as the healthy one due to the ability of its carriers in optimally accomplishing the major biological function of the protein (i.e., maintaining lipid homeostasis). However, it has been recently suggested that the *APOE3* genotype is the most efficient among the three *APOE* genetic variants at harvesting dietary energy, which could explain its vulnerability towards developing diet-induced obesity *(Arbones-Mainar et al., 2008; Huebbe et al., 2015; Karagiannides et al., 2008)*.

Based on the above, this dissertation seeks to determine whether *APOE* genotypic variability could differently modulate the toxicity of CPF. Furthermore, it also attempts to further characterize the behavioural traits of the three *APOE* genotypes, mainly in terms of executive functioning, about which there are little available data.

OBJECTIVES

3. OBJECTIVES

The main objective of this investigation was to assess the behavioural and metabolic effects of both *APOE* genotype and the pesticide CPF, as well as to determine whether the interaction between both factors contributed to the expression of these effects. Experiments were performed using human apoE TR mice for the three human *APOE* gene polymorphisms.

The following specific objectives were considered:

1. To assess the effects of: a) *APOE* genotype, b) chronic dietary exposure to CPF and c) the interaction between both factors on body weight status, and spatial learning and memory in a Barnes maze task in apoE TR adult male mice.

2. To determine the effects of subchronic dietary CPF exposure on metabolism and hormonal balance in apoE3 TR male mice, and to compare them with those from C57BL/6N male mice.

3. To characterize the effects of *APOE* genotype on attentional performance and inhibitory control in a 5-choice serial reaction time task in apoE TR female mice.

4. To explore the neurochemical and pharmacological bases of the potential *APOE*-related differences in attentional performance and inhibitory control in apoE TR female mice.

5. To evaluate the effects of subchronic dietary CPF exposure and its interaction with *APOE* genotype on attention, impulsivity, compulsivity, and motivation in a 5-choice serial reaction time task in apoE TR female mice.

RESULTS

4. RESULTS

Four original articles, either in the process of being published or already available in the scientific literature, are part of the results of this thesis. The specific objectives and corresponding publications are set out in **Table 3**.

Table 3 Specific objectives of the thesis and corresponding publications

Specific objectives	Publications
1	Peris-Sampedro F, Basaure P, Reverte I, Cabré M, Domingo JL, Colomina MT. ***Chronic exposure to chlorpyrifos triggered body weight increase and memory impairment depending on human apoE polymorphisms in a targeted replacement mouse model.*** Physiol Behav 2015; 144:37-45.
2	Peris-Sampedro F, Cabré M, Basaure P, Reverte I, Domingo JL, Colomina MT. ***Adulthood exposure to a common pesticide leads to an obese-like phenotype and a diabetic profile in apoE3 mice.*** Environ Res 2015; 142:169-76.
3 4	Reverte I, Peris-Sampedro F, Basaure P, Campa L, Suñol C, Moreno M, Domingo JL, Colomina MT. ***Attentional performance, impulsivity and related neurotransmitter systems in apoE2, apoE3 and apoE4 female transgenic mice.*** Psychopharmacology (Berl) 2015. DOI: 10.1007/s00213-015-4113-9.
5	Peris-Sampedro F, Reverte I, Basaure P, Cabré M, Domingo JL, Colomina MT. ***Apolipoprotein E genotype and the pesticide chlorpyrifos modulate attention, motivation and impulsivity in female mice in the 5-choice serial reaction time task.*** Neurotoxicology 2015. Currently under review.

Publication 1 **Chronic exposure to chlorpyrifos triggered body weight increase and memory impairment depending on human apoE polymorphisms in a targeted replacement mouse model.**

Peris-Sampedro F, Basaure P, Reverte I, Cabré M, Domingo JL, Colomina MT.
Physiol Behav 2015; 144:37-45. DOI: 10.1016/j.physbeh.2015.03.006. PMID: 25747767.

What is already known?

The onset of cognitive deficits and behavioural disorders after exposure to OP pesticides, and to CPF in particular, has been recurrently reported in the scientific literature. Consistently, data from animal models of repeated CPF exposure highlighted learning and memory impairments. The three apoE isoforms confer different cognitive skills on their carriers, but no single study exists which inquire about potential interactions between *APOE* genotype and CPF.

What this study adds?

The results of this study support the premise that *APOE* polymorphisms condition cognitive abilities in the absence of disease. Furthermore, this investigation was the first to demonstrate that *APOE* genotype modulates the toxicity of CPF. Strikingly, the *APOE3* genotype conferred on their carriers increased vulnerability to become overweighed upon chronic exposure to the pesticide.

Highlights

APOE genotype influenced spatial learning and memory processes in the BM task, and modulated the toxic effects of CPF. In particular, apoE3 mice were the only to fatten upon the exposure to the pesticide. Moreover, CPF increased search velocity in the BM task in apoE2 mice, and led to memory impairments in apoE3 mice.

Physiology & Behavior 144 (2015) 37–45

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Chronic exposure to chlorpyrifos triggered body weight increase and memory impairment depending on human apoE polymorphisms in a targeted replacement mouse model

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HIGHLIGHTS

- Repeated adulthood exposure to CPF increased body weight only in apoE3 mice.
- Learning and memory in the Barnes Maze task differed among the apoE genotypes.
- Search velocity in the Barnes Maze task was increased in CPF-exposed apoE2 mice.
- Repeated adulthood exposure to CPF led to a mild memory impairment in apoE3 mice.
- The apoE genotype modulated the toxic effects of the pesticide CPF.

ARTICLE INFO

Article history:
Received 19 January 2015
Received in revised form 25 February 2015
Accepted 3 March 2015
Available online 5 March 2015

Keywords:
Chlorpyrifos
Pesticides
Mice
Apolipoprotein E
Obesity
Learning

ABSTRACT

Despite restrictions on their use, humans are still constantly exposed to organophosphates (OPs). A huge number of studies have ratified the neurotoxic effects of chlorpyrifos (CPF) and suggested its association with neurodegenerative diseases, but data are still scarce. Human apolipoprotein E (apoE) plays an important role in lipid transport and distribution. In humans, the apoE4 isoform has been linked to an increased risk of Alzheimer's disease (AD). ApoE3 is the most prevalent isoform worldwide, and has been often established as the healthful one. The current study, performed in targeted replacement (TR) adult male mice, aimed to inquire whether genetic variations of the human *apoE* respond differently to a chronic dietary challenge with CPF.
At four/five months of age, mice carrying apoE2, apoE3 or apoE4 were pair-fed a diet supplemented with CPF at 0 or 2 mg/kg body weight/day for 13 weeks. Cholinergic signs were monitored daily and body weight changes weekly. In the last week of treatment, learning and memory were assessed in a Barnes maze task. Dietary CPF challenge increased body weight only in apoE3 mice. Differences in the acquisition and retention of the Barnes maze were attributed to apoE genetic differences. Our results showed that apoE4 mice performed worse than apoE2 and apoE3 carriers in the acquisition period of the spatial task, and that apoE2 mice had poorer retention than the other two genotypes. On the other hand, CPF increased the search velocity of apoE2 subjects during the acquisition period. Retention was impaired only in CPF-exposed apoE3 mice. These results underline that gene × environment interactions need to be taken into account in epidemiological studies. Given that apoE3, the most common polymorphism in humans, has proved to be the most sensitive to CPF, the potential implications for human health merit serious thought.

1. Introduction

Although pesticides have generally improved agricultural productivity, their widespread use has resulted in severe environmental pollution, and endangered human health. The highly-lipophilic organophosphorus (OPs) compound chlorpyrifos (CPF) is one of the most frequently used non-persistent pesticides worldwide, even though the US Environmental

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http://dx.doi.org/10.1016/j.physbeh.2015.03.006
0031-9384/

Protection Agency (EPA) banned it from residential use in 2001, and its decreasing use in Europe [65]. It is used extensively because of its effective and cost-competitive broad spectrum of activity, and has often been selected to replace persistent organochlorinated compounds [41]. Nearly everybody present organophosphate residues in their bodies but display no symptomatology [11], so most exposures are below the acute poisoning threshold and are therefore undetected, like a silent pandemic [28]. In the general population, the dietary intake of pesticides is the most important source of exposure [4,49,65].

To date numerous studies have endorsed the neurotoxic and behavioral effects of CPF in both human [6,20,21,55,62] and animal models [10,37,40,63,67]. CPF exerts its insecticidal activity by irreversibly inhibiting cholinesterases (ChE) and disrupting cholinergic function in the nervous system. While the acute neurotoxicity of CPF has been associated with systemic and brain ChE inhibition, an increasing body of experimental data suggests that CPF also targets non-cholinergic neurotransmitters such as serotonin, dopamine, glutamate and hormones [54,70,74]. Considering the number of potential targets, CPF exposure would be expected to have a wide range of effects. Specifically, CPF has been associated with learning and memory impairment, increased anxiety, and alterations in activity and impulsivity [9,13,17,51,60]. Heretofore, epidemiological studies have provided enough evidence supporting the existence of powerful links between OP exposure, long lasting cognitive impairments, and an increased risk of neurodegenerative diseases [3,23,31,46,78]. Besides, in recent years increasing references in the scientific literature point to lasting metabolic disturbances after perinatal exposure to CPF, and suggest that it is also an "endocrine disruptor" [43,68,69,76].

Apolipoprotein E (apoE) is a 34.2 kDa glycoprotein which is mainly involved in the metabolism of lipids, including cholesterol, and promotes the clearance of atherogenic lipoproteins such as very low density lipoprotein (VLDL) and chylomicron remnants from the circulation [33]. ApoE is mainly synthesized in the liver, but it is also produced by such other cell types as adipocytes, macrophages and astrocytes, which reveals its multiple functions [25,26,73]. Human *apoE* has three major allelic variants: *ε2*, *3* and *4*. These result in three main isoforms (apoE2, E3 and E4), of which apoE3 appears to be the most common in humans, followed by apoE4 and apoE2 [15]. Each form has a different influence on neuronal signal transduction, the properties of transporter proteins, receptors, and such enzymes as lipoprotein lipase [35]. In this sense, apoE4 has been related to disturbed lipid homeostasis [53,71] and decreased cerebral glucose metabolism [35], thus contributing to the pathophysiology of Alzheimer's disease (AD). Although the apoE3 isoform is commonly known as the "healthful" one, several studies in rodents have revealed that its carriers tend to be more prone to developing obesity [2,32,34], and more susceptible to the neurotoxic long-term effects of the decabromodiphenyl ether compound [56,58]. Human *apoE* targeted replacement (TR) mice are an appropriate model for characterizing neurobehavioral and metabolic differences attributable to the apoE genotype [7,38,66], and testing individual responses to toxic exposures.

The present study aimed to assess physical effects, spatial learning and memory in transgenic adult male mice carrying different polymorphisms of human *apoE* (*ε2*, *3* and *4*) after chronic moderate oral exposure to CPF. It also attempted to establish interactions between toxic exposure and genetic factors.

2. Materials and methods

2.1. Animals and care

Human apoE TR mice were used for this study. These animals have a C57BL/6NTac background and express functional human apoE isoforms at physiological levels, without altering any known endogenous regulatory sequences [72]. Adult male mice homozygous for each of the three *apoE* human alleles (*ε2*, *3* and *4*) were purchased from Taconic (Taconic Europe, Lille Skensved, Denmark). They were quarantined for 7 days, and then properly identified and housed in plastic cages containing 2–6 individuals of the same genotype. The animal room was maintained at a temperature of 22 ± 2 °C and a relative humidity of 50 ± 10%. The room was equipped with a 12-h light–dark automatic light cycle (light: 08:00–20:00 h). All the mice were allowed free access to food and water and given a normal chow diet (Panlab, Barcelona, Spain) until the beginning of the study. 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).

2.2. Treatment

Fifty-nine male mice between four and five months of age were weighed and randomly distributed in six experimental groups (n = 8–11): control apoE2, CPF-treated apoE2, control apoE3, CPF-treated apoE3, control apoE4, and CPF-treated apoE4. Mice were subjected to pair-feeding and were provided with 4 g of food/animal/day for 13 weeks as follows: the control groups received normal chow while CPF-treated groups were fed rodent chow supplemented with CPF at a dose of 2 mg/kg body weight/day. The dose was chosen to achieve a moderate inhibition of plasma cholinesterase with no clinical effects [14] in such a way that pesticide exposure could be compared with continuous non-occupational exposure in humans. Before the treatment period started, the baseline weight of the animals was recorded. During treatment, body weight and the appearance of possible cholinergic signs were monitored. Following the exposure period, mice were allowed access to food ad libitum and given normal chow diet. Food intake was then assessed for one week. The average daily food consumption obtained was divided by the number of animals in the cage.

2.3. Determination of cholinesterase activity

Twelve animals (n = 2/experimental group) were used to assess plasma cholinesterase activity 8 weeks after the start of the treatment. They were euthanized by cardiac puncture after anesthesia with carbon dioxide. Plasma was obtained by centrifugation at 3000 rpm for 20 min at 4 °C. Cholinesterase activity was determined spectrophotometrically using the Ellman method [19]. The result of the enzymatic activity was obtained from the activity value of the control subjects, and represented as a percentage.

2.4. Barnes maze

The effects of CPF on spatial learning and memory were assessed using a Barnes maze during the last week of treatment. The apparatus consists of a white methacrylate circular arena (92 cm diameter) elevated 1 m above the floor, with 20 circular holes (4.5 cm diameter) equally spaced around the perimeter. Each hole was located 2.5 cm from the edge of the maze, and assigned a number from 1 to 20. A dark escape box was placed beneath one of the holes. Bright and intense light was used to encourage them to escape into the dark box. During the acquisition period, mice were subjected to a daily session of two trials with an inter-trial interval of 30–60 min for five consecutive days. During each trial, animals were placed in the center of the maze. Mice were allowed to move freely, and the trial finished when they entered the escape box or after 180 s. If an animal failed to find the escape box within this time, it was guided and placed in it by the experimenter. After each trial, mice remained in the escape box for 30 s before being returned to their holding cages. To avoid proximal cues and ensure hippocampus-dependent learning, the maze was rotated 90° in each trial but the position of the escape box was maintained with respect to the external cues. The execution of the task by mice was recorded by a video camera (Sony CCD-IRIS). A video-tracking program (Etho-Vision©, Noldus Information Technologies, Wageningen, The Netherlands) was used to measure the latency of escape to the target hole, the total distance traveled in the

arena, and the average search velocity. The search strategies used by the mice to locate the target hole during the training sessions were also recorded and analyzed. The strategies were scored as "random" when the mouse displayed a nonsystematic search with multiple crossings through the center of the maze, as "serial" when the animal moved around the edge of the maze and past at least three adjacent holes before entering the target hole, and as "spatial" if the mouse moved directly towards the target hole from the center of the maze. The apparatus and escape box were cleaned between each trial to prevent the mice from using olfactory cues. Retention of the task was evaluated by a probe trial performed 24 h after the last training session, and consisting of a 120 s free exploration without the escape box. During the probe trial, the search time spent in the target quadrant was recorded, and compared to the time mice would be expected to spend by chance in each quadrant without any previous learning. Moreover, preferences for different holes were also analyzed by comparing the frequency of visits to the target hole and the other holes.

2.5. Statistical analysis

Data were analyzed using the SPSS Statistics 20.0 software. Body weight and food intake were analyzed by two-way analysis of variance (ANOVA) using the genotype and treatment as the main factors. A repeated measure multivariate analysis of variance (RMANOVA) with the period of time as the within-subject factor was also used when appropriate. Search strategies were studied by two-way ANOVA using the genotype and treatment as main factors. A paired *t*-test was used to analyze differences in the Barnes maze retention task. Post-hoc Tukey tests were used for multiple comparisons. The homogeneity of variance was determined using a Levene test. Statistical significance was defined by a probability value lower than 0.05 ($p < 0.05$).

3. Results

During the treatment period, no cholinergic signs were observed in any of the groups.

3.1. Body weight profile and food intake

Throughout the thirteen weeks of CPF exposure, body weight was analyzed by a RMANOVA using time as the within-subjects factor, and the genotype and treatment as the between-subjects factors. Both the genotype [$F(26,58) = 3.471$, $p < 0.001$] and the treatment [$F(13,58) = 9.226$, $p < 0.001$] were observed to influence the body weight profile over the experiment. The genotype was found to have an overall effect [$F(2,58) = 4.870$, $p = 0.011$]. Post-hoc analyses revealed that apoE2 mice had a higher body weight than apoE3 and apoE4 mice (Fig. 1). Also over the whole treatment period a trend was noted towards a genotype × treatment interaction [$F(26,58) = 1.466$, $p = 0.098$]. In order to define the effects of the treatment, each genotype was analyzed separately. The effect of CPF was only observed within the apoE3 group [$F(1,20) = 7.853$, $p = 0.011$) from the first week of treatment until the end. CPF-treated apoE3 mice increased their body weight more than their respective controls (Fig. 1). These differences in body weight were also clearly visible on the phenotypic level, where treated apoE3 mice appeared to be more obese than their control counterparts.

After the exposure period and under free access to food conditions, two-way ANOVA analysis revealed that apoE2 mice ate more than apoE3 or apoE4 mice [$F(1,21) = 3.748$, $p = 0.046$]. Interestingly, animals that had been exposed to CPF tended to increase their food ingestion [$F(1,21) = 3.885$, $p = 0.066$] (Fig. 2).

3.2. Plasma cholinesterase activity

Plasma ChE activity in CPF-exposed mice was 31.22% of that found in control groups 8 weeks after the start of CPF dietary exposure.

3.3. Spatial learning and memory in a Barnes maze

3.3.1. Acquisition

Learning performance during the five days of acquisition in the Barnes maze task was analyzed by a two-way RMANOVA (genotype x treatment). The session was used as the within-subject factor, while the dependent variables were the escape latency to the target hole,

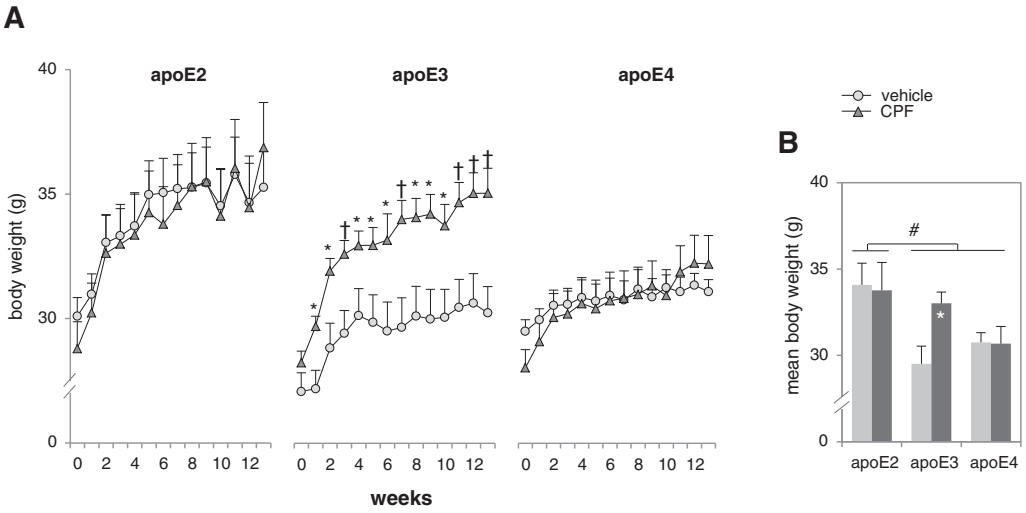

Fig. 1. Body weight profile. (A) Body weight progression of the three genotypes over the 13-week experiment. (B) Mean body weight of the experimental period for each group. Data are expressed as mean values ± SEM. The symbol # indicates significant differences ($p < 0.05$) between genotypes. The asterisk indicates significant differences between CPF-exposed apoE3 mice and their respective control group at $p < 0.05$, while the symbol † indicates significant differences at $p < 0.01$.

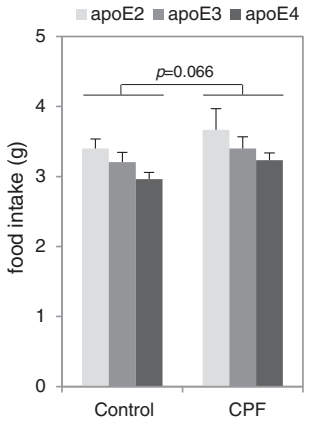

Fig. 2. Mean daily food intake assessed one week following CPF exposure in apoE2, apoE3 and apoE4 mice. Data are expressed as mean values ± SEM.

the total distance traveled in the arena, and the average search velocity. Performance during the acquisition period improved overall: escape latency to the target hole and total distance traveled in the arena decreased over sessions [$F(4,56) = 36.766$, $p < 0.001$; $F(4, 56) = 19.255$, $p < 0.001$, respectively] (Figs. 3 and 4). The average search velocity also changed during acquisition [$F(4,56) = 8.830$, $p < 0.001$]. The genotype was found to have an overall effect on the escape latency [$F(2,56) = 7.052$, $p = 0.002$] (Fig. 3), the total distance traveled in the arena [$F(2,56) = 6.198$, $p = 0.004$] (Fig. 4), and the average search velocity [$F(2,56) = 3.492$, $p = 0.038$] (Fig. 5). Post-hoc analyses showed that both the escape latency and the total distance traveled were higher for apoE4 mice than apoE2 and apoE3 mice, which suggests that this genotype has learning deficits in the spatial task (Figs. 3 and 4). Search velocity was higher in apoE2 than apoE4 mice (Fig. 5). An interaction between genotype and CPF treatment [$F(2,56) = 3.430$, $p = 0.040$] was also noted for the search velocity. Treatment also tended to decrease the escape latency [$F(1,56) = 3.212$, $p = 0.079$], indicating better acquisition in exposed animals.

To better analyze the effects of the treatment on latency, distance and velocity, each genotype was analyzed separately. CPF was observed to affect average search velocity in apoE2 mice [$F(1,18) = 5.533$, $p = 0.031$]. CPF-treated apoE2 subjects showed a higher velocity during the acquisition period than their respective controls (Fig. 5). Moreover, the escape latency in the arena tended to decrease in the CPF-treated apoE2 group [$F(1,18) = 3.481$, $p = 0.079$], suggesting higher levels of activity in these animals (Figs. 3 and 4).

To determine whether differences in search strategy accounted for discrepancies in performance among groups, the total frequency of each strategy used to locate the hidden escape box during the acquisition period was analyzed by a two-way ANOVA (genotype × treatment) (Fig. 6). An overall effect of genotype was found for both random [$F(2,58) = 7.319$, $p = 0.002$] and serial strategies [$F(2,58) = 4.787$, $p = 0.012$], and a tendency was also noted for spatial strategy [$F(2,58) = 3.089$, $p = 0.054$]. Post-hoc analyses showed that the random strategy was more frequent in apoE4 than apoE2 and apoE3 mice, and the serial strategy was less frequent in apoE4 than apoE2 mice. No effects of the treatment were noted.

3.3.2. Retention

Retention was evaluated by a single probe trial carried out 24 h after the last training session. A two-way ANOVA (genotype × treatment) was performed to analyze the total time spent in the target quadrant in which the escape box was previously located (Fig. 7). Genotype was observed to have a major effect [$F(2,58) = 2.282$, $p = 0.008$]: apoE4 mice spent more time in the target quadrant than apoE2. To better analyze retention, the time spent in the target quadrant was compared to the chance level (30 s) by means of a *t*-test (Fig. 7). Both control and treated apoE3 and apoE4 mice showed significant retention because they expressed a clear preference for the target quadrant. However, the apoE2 group did not show it, suggesting poorer retention among these subjects.

A paired *t*-test was carried out to compare how frequently each genotype entered the target hole with respect to the other holes in the maze (Fig. 8). CPF-exposed apoE3 mice were the group that entered the target hole least [$t = -2.360$, d.f.9, $p = 0.043$], indicating a slight memory impairment in this group.

In summary, genotype clearly affected the spatial task at seven/eight months of age. The three genotypes had different escape latencies and

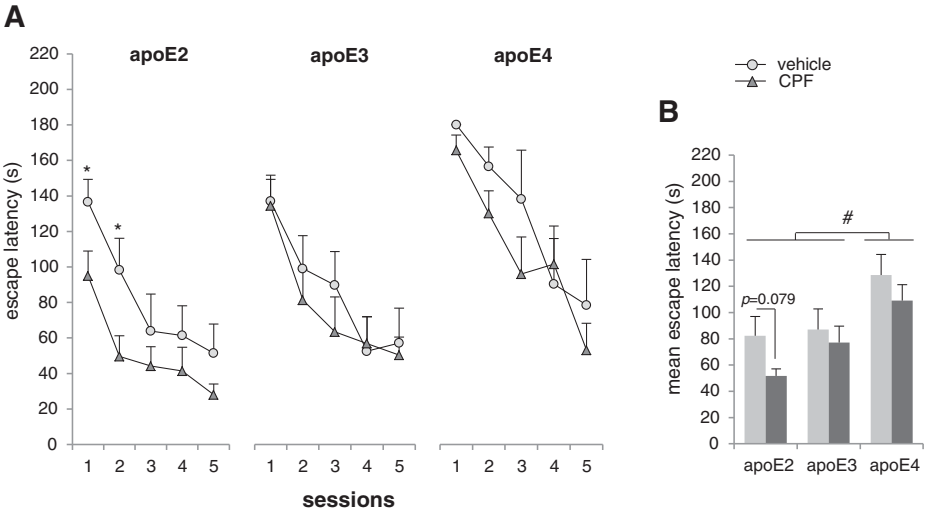

Fig. 3. Acquisition of a spatial learning task in the Barnes maze assessed at the end of the treatment period. (A) Escape latency to the target hole over the five training sessions. (B) Mean escape latency for each group. Data are expressed as mean values ± SEM. The symbol # indicates significant differences ($p < 0.05$) between genotypes. The asterisk indicates significant differences at $p < 0.05$ between CPF treatments within the apoE2 genotype on the first and second acquisition days.

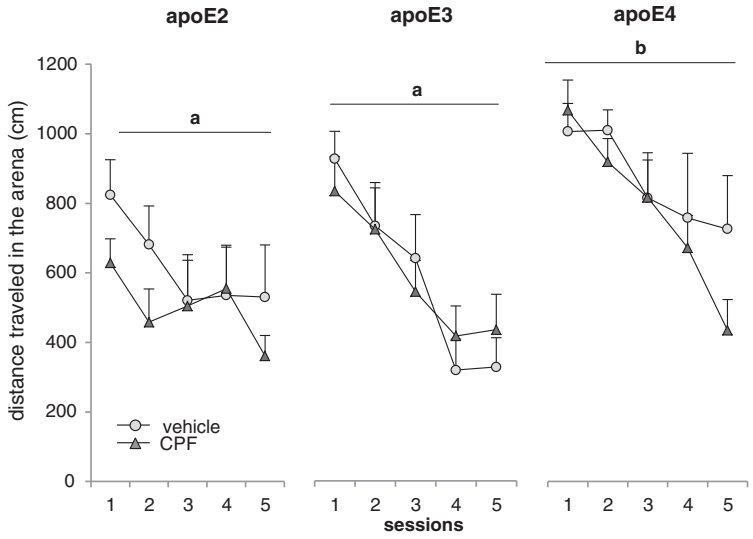

Fig. 4. Acquisition of a spatial learning task in the Barnes maze assessed at the end of the treatment period. Distance traveled over the five training sessions. Different letters (a, b) indicate significant differences ($p < 0.05$) between genotypes.

total distances traveled in the arena, being the apoE2 group which showed best results in the learning process. Treatment with CPF affected Barnes maze acquisition in apoE2 carriers by increasing their search velocity. As far as the probe trial is concerned, apoE2 mice showed poorer retention than apoE3 and apoE4 mice. The effect of CPF on the probe trial was observed among treated apoE3 mice, which made fewer entries into the target hole.

4. Discussion

This study was designed to investigate the effects of chronic oral exposure to low doses of the pesticide CPF on human apoE TR adult male mice. Despite a moderate inhibition of plasma cholinesterase, no clinical effect was observed probably because the inhibition came about gradually throughout the treatment period. The exposure to CPF induced alterations in body weight status, and in learning and retention in the Barnes maze task. These alterations depended on the apoE genotype. CPF increased body weight in apoE3 mice, but not in apoE2 or apoE4 carriers. After the exposure period, apoE2 mice ate more than apoE3 or apoE4 mice. Animals that had been exposed to CPF tended to increase their food ingestion. ApoE4 mice showed the worse learning performance in the Barnes maze task, and used the random strategy most frequently. CPF increased the search velocity of apoE2 mice, which may explain an apparent improvement in these subjects. Genotype was observed to have a clear influence in the retention assay: apoE2 mice showed poorer retention. Moreover, CPF-treated apoE3 animals exhibited slight memory impairment.

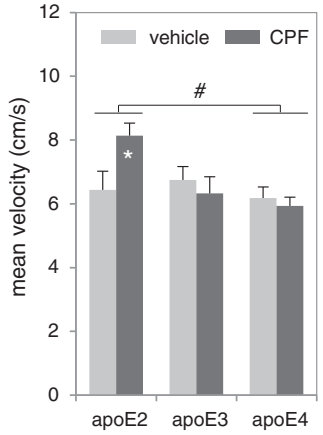

Fig. 5. Acquisition of a spatial learning task in the Barnes maze assessed at the end of the treatment period. Mean search velocity for each experimental group is depicted. The symbol # indicates significant differences ($p < 0.05$) between genotypes. The asterisk indicates significant differences between CPF-treated apoE2 mice and their respective controls at $p < 0.05$.

Differences in body weight were observed between apoE genotypes during the 13-week study. ApoE2 mice showed higher body weight than both apoE3 and apoE4 mice. Body weight differences could be linked to activity levels, food intake, or differences in metabolic efficiency [57]. Assuming that apoE2 subjects exhibited higher activity levels in the Barnes maze, the increased body weight found among this genotype is related to differences in food intake or in energy metabolism. In accordance with the present results, human apoE isoforms are associated with a low-to-high mass index following the apoE4 < apoE3 < apoE2 rank order [50,57]. A single amino acid substitution close to the LDL receptor binding site means that the apoE2 isoform is less able to bind to the receptor, which leads to a poor clearance of triglyceride-rich lipoproteins from plasma [52]. In this regard, ε2 carriers tend to have higher triglyceride plasma levels so they are more predisposed to Type III hyperlipidemia [22], and become a risk of metabolic-associated diseases. With reference to food intake, it was increased after the exposure to the pesticide raising the question as to CPF could be disturbing feeding behavior.

Interestingly, repeated exposure to low doses of CPF significantly increased body weight only in apoE3 males, which points out that the apoE3 genotype is more likely to promote obesity. This finding appears to have no specific precedent. Taking into account that, in the pair-feeding exposure design, CPF-treated apoE3 mice showed increases in their body weight, metabolic changes were expected upon CPF exposure. Previous studies have reported that apoE3 knock-in mice show higher vulnerability towards developing obesity and related metabolic disorders after 8 [2] and 24 weeks on a western-type diet [34]. Both studies reported that apoE3 mice gained more body weight than apoE4 mice. Arbones-Mainar and collaborators attributed this

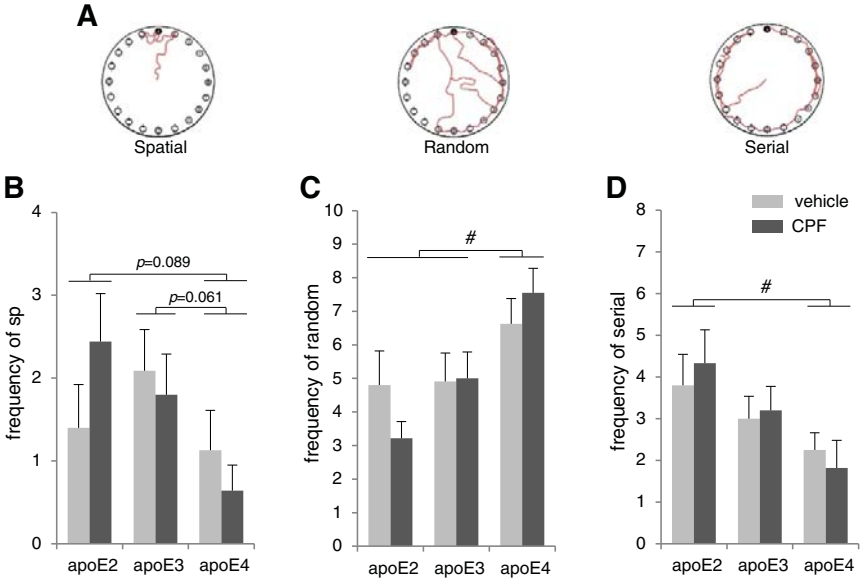

Fig. 6. Acquisition of a spatial learning task in the Barnes maze assessed at the end of the treatment period. (A) Representative images of search strategies used in the Barnes maze: spatial (direct path to the target hole), random (no distinguishable pattern) and serial (consecutive visits to the holes). Frequencies of spatial (B), random (C), and serial (C) search strategies are depicted for the whole acquisition period. Data are expressed as mean values ± SEM. The symbol # indicates significant differences ($p < 0.05$) between genotypes.

gain to higher amounts of total and subcutaneous fat in apoE3 mice than in apoE4 mice. They also suggested that apoE3 but not apoE4 isoform expression interfered with insulin sensing pathways, raising the interesting possibility that metabolic dysfunctions such as insulin sensitivity may be the result of the qualitative differences in fat depots observed in mice expressing different apoE isoforms. Along the same lines, Karagiannides and colleagues described hyperglycemia, hyperinsulinemia, hyperleptinemia, glucose intolerance, and insulin resistance in apoE3 knock-in mice fed a western-type diet for 24 weeks.

Several epidemiological studies have provided evidence to suggest that there is a link between exposures to CPF on the one hand, and higher rates of obesity [68] and diabetes [47,64] on the other. However, the investigations assessing the metabolic effects arising from exposure to CPF in animal models have been carried out during development. In this sense, an increase in body weight at 1 and 2 months after developmental exposure to CPF has been reported in rats [36]. By contrast, only two investigations have focused on the effects of CPF during adulthood, both of which found an increase in body weight in adult rats after repeated subcutaneous exposure to 60 mg/kg [18] and 5 mg/kg [44]. In the present study, we found an 11% increase in the weights of seven/eight-month-old apoE3 male mice given 2 mg/kg body weight/day CPF orally for 13 weeks. Similarly, Meggs and Brewer [44] reported an 8% increase in nine-month-old female rats after 3 months of subcutaneous exposure to 5 mg/kg/day CPF. Notwithstanding the interaction between CPF and the apoE3 genotype, this deserves further investigation.

The apoE genotype was observed to have a strong effect on the acquisition period in the Barnes maze: apoE4 mice learned worse than

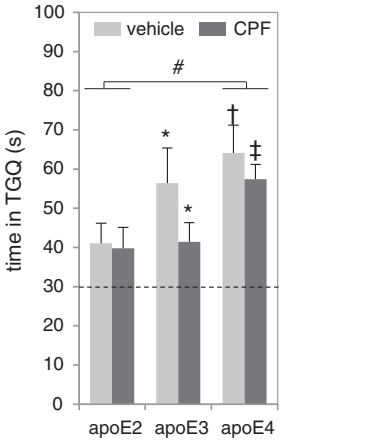

Fig. 7. Retention measured in the Barnes maze assessed at the end of the treatment period. Time spent in the target quadrant (TGQ) in probe trial performed 24 h after the last training session. The dashed line indicates the time mice would be expected to spend in each quadrant by chance without any previous learning (30 s). Data are expressed as mean values ± SEM. The symbol # indicates significant differences ($p < 0.05$) between genotypes. A significant change in performance over the chance level was found at $p < 0.05$ (*), $p < 0.01$ (†), and $p < 0.001$ (‡).

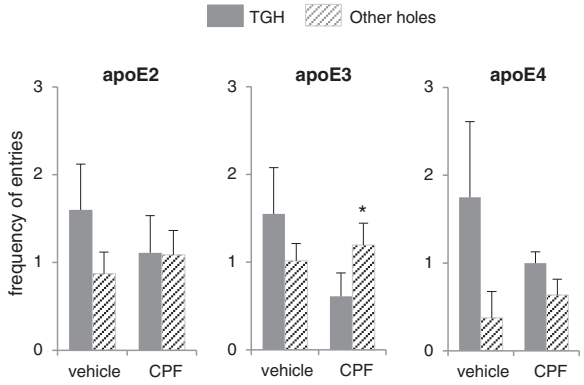

Fig. 8. Retention measured in the Barnes maze assessed at the end of the treatment period. The comparison between the frequency of visits to the target hole and the other holes is depicted for each genotype. Data are expressed as mean values ± SEM. The asterisk indicates significant differences at $p < 0.05$ between CPF treatments for the apoE3 genotype.

apoE2 or apoE3 mice at seven/eight months of age. In agreement with this, the apoE4 genotype has previously been reported to have a negative influence on learning and memory in young TR mice in both wet [56,57] and dry [61] mazes. The neural bases or systems involved in these deficits are still not well understood. For instance, the role of the cholinergic system in apoE4 learning and memory deficits is controversial. Although some authors have proposed that impairments in the cholinergic system could explain these shortfalls [42,77], other investigators have reported no differences [8]. Our results on search strategies during the acquisition period in the Barnes maze task indicated a worsening in the hippocampus-dependent learning of apoE4 individuals because of their greater use of the random strategy. In this regard, Rodriguez et al. [61] found a reduction in dendritic spine density in the medial entorhinal cortex of 3-month-old apoE4 mice, an area of the brain which transmits spatial information to the hippocampus, and plays a critical role in spatial representation. Interestingly, the entorhinal cortex is also a site of early dysfunction and neuronal loss in Alzheimer's disease [27]. On the contrary, apoE2 mice were the most active group with considerably lower latency to the target hole and less total distance traveled in the arena. In line with these results, a previous study carried out in our laboratory found an increased exploration of an open-field which disclosed a hyperactive behavior in both apoE2 males and females [57]. While some studies have assessed activity, learning and memory in apoE TR mice [7,30,56,57], none have focused on whether CPF may affect these processes differently in carriers of different apoE polymorphisms. Our present investigation reveals that chronic oral exposure to CPF increased the search velocity in these subjects, which may explain their apparent improvement. In animal models, activity after adult exposure to CPF has been reported to either increase [51] or decrease [63] in Tg2576 mice. In a recent study, increased vertical activity and a trend towards an anxiety trait were observed five months after repeated exposure to CPF in Tg2576 adult male mice [51]. The authors related this hyperactivity to an elevated plasma cholinesterase inhibition reached after the exposure. Otherwise, several other neurotransmitter systems and functions have also been associated with anxiety-related phenotypes, novelty exploration, and hyperactive behavior. Numerous epidemiological studies have linked prenatal exposure to organophosphates with an increased risk of attention-deficit hyperactive disorder (ADHD) [5,16,24,29,55]. The investigation carried out by de Cock et al. [16] suggested that both the disruption of thyroid hormone function and gamma-aminobutyric acid (GABA)-ergic mechanisms could be at the origin of this disorder. Nonetheless, because CPF effects have been related to several targets [1,12,45], the increased activity in the apoE2 genotype noted in this study cannot be attributed to one particular system.

In the probe trial — a single assay 24 h after the last training session — both control and treated apoE3 and apoE4 mice showed consolidation because of their clear preference for the target quadrant. ApoE2 mice, on the other hand, appeared to show poorer retention. Along the same lines, Reverte et al. [57] also found an impaired retention in apoE2 mice in a Morris water maze, pointing to an alteration in their memory consolidation, and they suggested that this could be related to their hyperactive behavior. In addition, our current results disclosed that the spatial strategy performed by this genotype over the acquisition period did not match with a good retention. This indicates that apoE2 mice probably used a contextual or memorized pathway rather than a spatial-guided learning, which could also reflect their inability to attend to spatial cues. In this sense, there is increasing evidence to suggest that the hippocampus not only contributes to spatial memory, but also plays a role in learning precise sequences and linking specific memories to context [39].

We also found that repeated exposure to CPF led to slight memory impairment in seven/eight-month-old apoE3 mice, suggesting that specific CPF effects on the metabolic system of these subjects could also be altering their memory performance. Although these effects did not imply a severe impairment, they depleted the advantage provided by their genetic background. Similarly, Ribes et al. [59] found that repeated exposure to very low doses of aluminum in the diet impaired the memory of wild-type mice and increased the total number of proliferating cells in the dentate gyrus of their hippocampus, thus indicating a reactive response of the brain to toxic injury.

Several studies have focused on the contribution of the apoE genotype to the severity of the toxicity caused by harmful elements. In this sense, the apoE4 isoform has been reported to be more sensitive to the effects of mercury, due to its reduced ability to bind metals [48]. Conversely, recent studies showed that apoE3 is the most vulnerable genotype to the lipophilic compound decabromodiphenyl ether [56,58]. In this sense, we also found more effects in apoE3 than in apoE4 genotype after CPF exposure, which is also highly-lipophilic. Taking into account that the apoE genotype can alter the lipid distribution in both plasma and brain [75], we hypothesized that the vulnerability associated with the apoE genotype may depend on the lipophilic characteristics of toxic agents.

In conclusion, the results of the present research show that genotypic variability may interfere in the detection of toxic effects and should be taken into account in epidemiological studies. In the other hand, the behavioral features described for apoE2 mice are of particular interest, because they open up the possibility that this genotype can be used as a model to study hyperactive behavior. Likewise, our results provide sufficient evidence to support the hypothesis that links exposure to CPF with an increased risk of the apoE3 genotype developing obesity and other related metabolic dysfunctions. The mechanisms underlying how CPF contributes to the prevalence of obesity within this genotype are still unknown, but it probably causes changes in the axis for weight control or feeding behavior and, among other things, may alter hormonal communications between the hypothalamus and adipose tissue. These findings should serve as a warning to those institutions responsible for regulating the use of pesticides with respect to human health. Notwithstanding these findings, further studies are needed to better characterize both low level toxicant exposures and the possible link between CPF exposure and metabolic and/or cognitive dysfunctions.

Disclosure statement

The authors declare that no conflict of interest has influenced the results presented in this article.

Acknowledgments

The authors would like to thank Amparo Aguilar for her skillful technical support. This research was supported by PSI2010-21743-C02-01, the Ministry of the Economy and Competitiveness (MINECO, Spain), the European Regional Development Fund (ERDF), the Commission for Universities and Research of the Department of Innovation, the Universities and Enterprise of the Generalitat de Catalunya (2013 FI_B 00170) and the European Social Fund.

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Publication 2 **Adulthood exposure to a common pesticide leads to an obese-like phenotype and a diabetic profile in apoE3 mice.**

Peris-Sampedro F, Cabré M, Basaure P, Reverte I, Domingo JL, Colomina MT.
Environ Res 2015; 142:169-76. DOI: 10.1016/j.envres.2015.06.036. PMID: 26162960.

What is already known?

Even though there has been renewed interest in ascertaining the contribution of environmental hazards to the global epidemics of obesity and type 2 diabetes; to date, very little research has focused on OPs. On the basis of our previous study, apoE3 mice are more vulnerable than apoE2 and apoE4 to the obesogenic effect of CPF.

What this study adds?

Overall, the results of this study expand the scanty existing literature on the obesogenic effect of OP exposures, and further demonstrate their contribution to the development of metabolic diseases. Consistently with the results provided by the first study, current data highlight a markedly vulnerability of the *APOE3* genotype towards the metabolic-disruptor role of CPF.

Highlights

In general, the exposure to CPF enhanced food intake, induced hyperglycemia and hypercholesterolemia, tended to elevate acyl ghrelin levels, increased insulin and leptin levels, and impaired HOMA-IR index scores. Nonetheless, apoE3 mice were more vulnerable than C57BL/6N to the metabolic-disruptor role of CPF. In particular, apoE3 mice exhibited higher insulin and leptin levels, as well as higher HOMA-IR index scores.

Environmental Research 142 (2015) 169–176

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Adulthood dietary exposure to a common pesticide leads to an obese-like phenotype and a diabetic profile in apoE3 mice

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ARTICLE INFO

Article history:
Received 11 June 2015
Received in revised form
25 June 2015
Accepted 26 June 2015
Available online 7 July 2015

Keywords:
Pesticide
Apolipoprotein E
Obesity
Diabetes
Leptin
Ghrelin

ABSTRACT

Increasing evidence links the widespread exposure to organophosphate (OP) pesticides to the global epidemics of type 2 diabetes and obesity. Our recent data highlighted gene × environment interactions: mice expressing the human apolipoprotein E3 (apoE3) isoform were more prone to develop obesity than those expressing apoE2 or apoE4 upon dietary challenge with chlorpyrifos (CPF), the most used OP worldwide. Thus, we aimed to further explore the contribution of the *APOE3* genotype on the emergence of obesity and related metabolic dysfunctions upon subchronic exposure to CPF. Seven-month-old targeted replacement apoE3 and C57BL/6N male mice were orally exposed to CPF at 0 or 2 mg/kg body weight/day for 8 consecutive weeks. We examined body weight status, food and water intake, lipid and glucose homeostasis, metabolic biomarkers concentrations, insulin levels and insulin resistance, and leptin and ghrelin profiles. CPF exposure generally increased food ingestion, glucose and total cholesterol concentrations, and tended to elevate acyl ghrelin levels. Nonetheless, excess weight gain and increased leptin levels were inherent to apoE3 mice. Moreover, the propensity towards a diabetic profile was markedly higher in these animals than in C57BL/6N, as they showed a higher homeostatic model assessment for insulin resistance index and higher insulin levels. Although both genotypes were metabolically affected by CPF, the results of the present investigation revealed that apoE3 mice were the most vulnerable to developing obesity and related disturbances following CPF administration through the diet. Since the *APOE3* genotype is the most prevalent worldwide, current findings have particular implications for human health.

1. Introduction

Over the last two centuries, the human lifespan has increased markedly because the development of industrialized societies has led to an improved quality of life. In this context, individuals are constantly and unconsciously exposed to a wide range of xenobiotics, the long-term effects of which are often unknown. Despite its obvious neurotoxic effect (Eaton et al., 2008), chlorpyrifos (CPF) is still the most widely used organophosphate (OP) pesticide in Europe, for both agricultural and urban purposes. It has been classified as a potent inhibitor of both systemic and brain cholinesterases (ChE), leading to the onset of acute neurotoxic symptomatology. However, an increasing body of reports have suggested that CPF also disrupts the serotonergic neurotransmitter system (Slotkin et al., 2015), targets serine hydrolase enzymes (Quistad et al., 2006b) and interferes with the signaling of hormones, some of which – for example, insulin and leptin-are related to energy homeostasis (Lassiter and Brimijoin, 2008; Slotkin et al., 2005). In accordance, sundry investigations have shown that CPF exposure induce a broad spectrum of effects, including metabolic disturbances (Lasram et al., 2014; Peris-Sampedro et al., 2014).

Abbreviations: OP, organophosphate; apoE, apolipoprotein E; CPF, chlorpyrifos; DTNB, 5, 5'-dithiobis-(2-nitrobenzoic acid); HOMA-IR, homeostatic model assessment for insulin resistance; ChE, cholinesterase; WHO, World Health Organization; apoE TR mouse model, apoE targeted replacement mouse model; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; AST, aspartate transaminase; ALT, alanine transaminase; ACh, acetylcholine; BChE, butyrylcholinesterase

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http://dx.doi.org/10.1016/j.envres.2015.06.036
0013-9351/

Type 2 diabetes accounts for over 90% of all cases of diabetes. Sedentary lifestyle, obesity, careless dietary habits, low socio-economic status and genetic vulnerability are well-known risk factors that contribute to its emergence (Zimmet et al., 2001). Nowadays, the prevalence of obesity and type 2 diabetes worldwide is increasing at epidemic rates. According to the World Health Organization (WHO), 13% of the adult population was obese (body mass index ≥ 30 kg/m^2) in 2014, while the predictions of the incidence of type 2 diabetes are not very encouraging, pointing to 366 million type 2 diabetes patients in 2030 (Wild et al., 2004). In the light of this trend, the risk factors commonly studied fail to explain by themselves the global boom of both diseases. Hence, "non-traditional" risk factors have been reconsidered (Arrebola et al., 2015; Howell et al., 2015). Some epidemiological evidence links general pesticide exposure (Arrebola et al., 2013, 2015; Suarez-Lopez et al., 2015) and more specifically OP exposure (Montgomery et al., 2008; Saldana et al., 2007) to a higher incidence of type 2 diabetes and related metabolic dysfunctions. Nevertheless, experimental studies are scarce. Very little research has investigated the metabolic and endocrine effects that emerge following adulthood exposure to CPF in rodents, being most studies focused on early-life exposure (Lassiter and Brimijoin, 2008; Slotkin et al., 2005). Current knowledge of adulthood exposure to CPF is limited to four studies carried out in rats. From these, two revealed a weight gain in treated subjects (Ehrich et al., 2004; Meggs and Brewer, 2007) and the other two pointed to disturbances of both glucose and lipid metabolisms in exposed animals (Acker and Nogueira, 2012; Elsharkawy et al., 2013). In general, these protocols were based on high CPF doses.

Apolipoprotein E (apoE) is a glycoprotein mainly involved in the maintenance of plasma lipid homeostasis, and is basically synthesized in the liver, but also in the brain and adipose tissue (Frühbeck, 2004; Gee and Keller, 2005). The human *APOE* gene is polymorphic and presents three major allelic variants ($\varepsilon 2$, $\varepsilon 3$, $\varepsilon 4$), coding for three main isoforms associated with a low-to-high prevalence following the apoE2 < apoE4 < apoE3 rank order (Corbo and Scacchi, 1999). While apoE3 is accepted as the healthy phenotype, recent experimental data have shown that it tends to be more prone to developing diet-induced obesity (Arbones-Mainar et al., 2008; Huebbe et al., 2015; Karagiannides et al., 2008), and more vulnerable to decabromodiphenyl ether (Reverte et al., 2013). In a recent study, we found that apoE3 mice were more vulnerable to gain excess weight upon CPF exposure than apoE2 and apoE4 mice (Peris-Sampedro et al., 2015).

The apoE targeted replacement (TR) mouse model was originally created by Sullivan et al. (1997). These animals have a C57BL/6N background but their murine *apoE* gene has been replaced by one of the three most prevalent human *APOE* alleles. Thus, apoE TR mice differ from C57BL/6N in that they carry and express functional human apoE isoforms at physiological levels. It has been established that this expression does not alter any known endogenous regulatory sequence (Sullivan et al., 1997), being the subsequent phenotype in mice similar to that found in humans (Hauser et al., 2011).

Based on our previous results and from evidence gathered in the literature, the main objectives of the current investigation were: (a) to provide greater insight into the metabolic disturbances, ranging from hormonal imbalance to disturbed eating behavior, as a result of CPF exposure, and (b) to investigate how the human $\varepsilon 3$ allele might favor their emergence. For these purposes, the metabolic profile of both apoE3 and C57BL/6N male mice were assessed and compared after an 8-week period of oral exposure to CPF.

2. Material and methods

2.1. Chemicals

CPF (*O*,*O*-diethyl *O*-3,5,6-trichloropyridin-2-yl phosphorothioate, purity 99.5%) was supplied by Sigma-Aldrich (Seelze, Germany). Standard rodent chow (Panlab, Barcelona, Spain) was supplemented with CPF at a concentration intended to deliver a dose of 2 mg/kg body weight/day, based on the results of our recent study (Peris-Sampedro et al., 2015). The protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) was also purchased from Sigma-Aldrich.

2.2. Animal care

Seven-month-old apoE TR male mice and C57BL/6N male mice were used. Mice homozygous for the human $\varepsilon 3$ allele were obtained from Taconic (Taconic Europe, Lille Skensved, Denmark), and C57BL/6N mice were purchased from Charles River (Charles River France, L'Arbresle, France). After a quarantine period, the animals were properly housed in plastic cages containing 2–3 individuals in an environmentally controlled room equipped with a 12-h light–dark automatic light cycle (light: 08:00–20:00 h), a temperature of 22 ± 2 °C, and a relative humidity of 50 ± 10%. Mice were allowed access to food and fresh water *ad libitum* and given a standard chow diet (Panlab, Barcelona, Spain) before the experiment started. The use of animals and the experimental protocol design were supervised and approved by the Animal Care and Use Committee of the Rovira i Virgili University (Tarragona, Spain). Likewise, efforts were made to alleviate animal suffering as established by the Spanish Royal Decree 53/2013 and and the European Communities Council Directive (86/609/EEC).

2.3. Treatment protocol

The animals were weighed and then distributed into four experimental groups ($n=10$/group): control apoE3, control C57BL/6N, CPF-exposed apoE3, and CPF-exposed C57BL/6N. Mice were fed either a standard or a CPF-supplemented rodent chow (2 mg/kg body weight/day) for 8 consecutive weeks, and were checked for cholinergic signs twice a week. After the treatment period, animals were subjected to a 3-h fast before being anesthetized with carbon dioxide and euthanized by cardiac puncture. Blood was immediately collected into 500 μL tubes containing EDTA (BD Microtainer®, Plymouth, United Kingdom), and centrifuged at 3000 rpm for 20 min at 4 °C to obtain plasma, which was aliquoted and stored at −80 °C.

2.4. Plasma cholinesterase activity

Plasma ChE activity was evaluated as an indicator of the systemic CPF effect (Eaton et al., 2008). It was determined spectrophotometrically using a commercial available kit, as recommended by the supplier. Briefly, the cholinesterase enzyme hydrolyzes butyrylthiocholine to give thiocholine and butyrate. The reaction between thiocholine and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) produces 2-nitro-5-mercaptobenzoate, a yellow compound which can be measured at 405 nm. The enzymatic activity of exposed animals was calculated on the basis of the activity value of the control mice, and represented as a percentage.

2.5. Body weight status and food and water consumption

The body weight status of the mice was recorded weekly over the treatment period. Food intake was estimated on a daily basis for a 7-day period by subtracting the uneaten pellets at the end of

the week from the total amount of food given at the beginning. To obtain a more accurate value, we made sure there was no leftover food scattered around the cage. The average daily food consumption obtained was divided by the number of animals in the cage. Water intake was estimated in the same way.

2.6. Analysis of metabolic biomarkers

Plasma concentrations of total cholesterol, triglycerides, albumin and creatinine, as well as total activity levels of aspartate (AST) and alanine (ALT) transaminases were determined after 3 h of food withdrawal as biomarkers of metabolic state in both control and CPF-fed mice. They were determined with commercially available kits supplied by QCA (Química Analítica Clínica S.A., QCA, Amposta, Spain). Briefly, every absorbance measurement was carried out in duplicate according to the manufacturer's instructions at a constant temperature of 37 °C with a semiautomatic COBAS MIRA analyzer (Hoffman-La Roche & Co., Basel, Switzerland). Before sacrifice, fasting glucose was measured by tail bleeding using a handheld glucometer (Accu-check Performa, Roche Diagnostics, Sant Cugat del Vallès, Spain). Each parameter was expressed in the international system of units (SI).

2.7. Measurement of insulin sensitivity

Insulin sensitivity was estimated by determining fasting plasma insulin levels and by computing an insulin resistance score: the homeostatic model assessment for insulin resistance (HOMA-IR). Plasma insulin levels were assessed in duplicate with a commercially available ELISA mouse kit supplied by Merck Millipore (Darmstadt, Germany), following the manufacturer's instructions, and were expressed in SI. Insulin resistance was estimated on the basis of both fasting glucose and fasting insulin values, using the HOMA-IR index first described by Matthews et al. (1985), as follows: HOMA-IR=(fasting insulin × fasting glucose)/22.5, where insulin and glucose concentrations were expressed in mU/L and SI, respectively. The conversion factor used for insulin was 1 mU/L=6 pmol/L, which was based on the first international standard for insulin issued by the WHO in 1987 (Vølund, 1993).

2.8. Quantification of plasma leptin, total ghrelin and acyl ghrelin levels

Plasma leptin levels were determined in order to provide further insight into the body weight status, while acyl and total ghrelin levels were assessed to evaluate more in depth feeding behavior. The concentration of plasma leptin was measured in duplicate with a mouse ELISA kit provided by Merck Millipore (Darmstadt, Germany), as recommended by the supplier, and was expressed in SI. Prior to storage, plasma aliquots intended for the determination of total ghrelin and acyl ghrelin levels were supplemented with AEBSF in order to prevent hormone degradation by proteases. At this point, both ghrelin statuses were also evaluated in duplicated with commercially available mouse ELISA kit from Merck Millipore. Acyl ghrelin and total ghrelin levels were expressed in pg/mL and ng/mL, respectively.

2.9. Statistical analysis

Data were analyzed with the SPSS statistical package (version 20.0), and reported as mean values ± SE. Two-way analysis of variance (ANOVA) were performed to establish the contribution of both the CPF and *APOE* genetic background to the inhibition of plasma ChE, food and water consumption, metabolic biomarkers, hormones profiles and HOMA-IR index values. Throughout the 8-week experiment the body weight profile was studied by two-way repeated-measures ANOVA with the period of time as the within-subject factor. Tukey's *post hoc* test was used for multiple comparisons. A correlation analysis, determined by linear regression, was performed to assess the relationship between body weight and circulating levels of leptin. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Plasma cholinesterase inhibition and signs of toxicity

During the experimental period, we noticed no apparent signs of cholinergic toxicity in any group. Assessed under fasting conditions at the end of the treatment, plasma ChE activity dropped to 32.12% in CPF-exposed mice.

3.2. CPF triggers body weight gain in apoE3 mice and increases food intake

Prior to the exposure period, *post hoc* analyses showed no significant differences in initial body weight between groups. The exposure led to gradual weight gain throughout the experiment [$F_{7,39}=13.662$, $p < 0.001$]. Thus, CPF-exposed mice showed higher body weights than their respective controls. A triple interaction (time × treatment × genotype) was noted during the experiment [$F_{7,39}=2.355$, $p=0.048$], and the genotype tended to have an overall effect [$F_{1,39}=3.128$, $p=0.085$]. Highest propensity to the CPF obesogenic effect was observed in apoE3 mice [$F_{1,19}=5.077$, $p=0.037$], which weighed more than their control counterparts from the fourth week until the end of the treatment (Fig. 1A). In contrast, we found only an upward trend in exposed C57BL/6N animals [$F_{1,19}=3.443$, $p=0.080$] (Fig. 1B). While water consumption was not altered over the experiment, CPF exposure increased food intake [$F_{1,39}=124.361$, $p < 0.001$] (Table 1).

3.3. CPF increases total cholesterol and fasting glucose levels

The levels of metabolic biomarkers are set out in Table 2. With regards to plasma lipids, CPF exposure generally increased total cholesterol levels [$F_{1,39}=4.736$, $p=0.036$]. There were no significant differences in plasma triglycerides between groups. The genotype affected plasma creatinine levels differently [$F_{1,26}=10.989$, $p=0.003$], having apoE3 mice higher concentrations than C57BL/6N animals. As expected, plasma albumin levels and both ALT and AST activities were unaltered between genotypes and were statistically indistinguishable among groups, indicating that there was no deterioration of renal and hepatic functions upon dietary CPF exposure. Both the genotype [$F_{1,39}=6.214$, $p=0.017$] and the treatment [$F_{1,39}=4.893$, $p=0.033$] altered fasting glucose concentration. The highest levels of glucose were inherent to the $\varepsilon 3$ allele carriers and the CPF-treated mice on the other hand.

3.4. Insulin levels and insulin resistance are higher in CPF-exposed apoE3 mice

Hyperglycemia emerged after prolonged exposure to CPF. To determine if these high levels of fasting glucose were related to higher rates of insulin resistance, both fasting insulin levels (Fig. 2A) and HOMA-IR (Fig. 2B) were assessed. Both the genotype [$F_{1,34}=17.010$, $p < 0.001$] and the treatment [$F_{1,34}=11.112$, $p=0.002$] influenced insulin levels, and a genotype x treatment interaction was found [$F_{1,34}=4.337$, $p=0.046$]. Data from both genotypes were then studied separately. Reanalyses showed that CPF exposure increased fasting plasma insulin levels in both apoE3

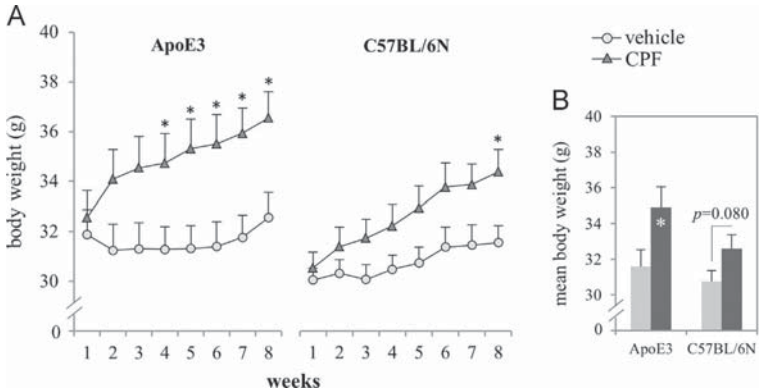

Fig. 1. The body weight progression was recorded weekly over the 8-week treatment period to evaluate the obesogenic effect of subchronic oral adulthood exposure to chlorpyrifos in both apoE3 and C57BL/6N male mice (A). The mean body weight of the experimental period was also depicted for each group (B). *Asterisks* indicate significant differences between CPF-exposed mice and their corresponding control group ($p < 0.05$).

Table 1
Mean daily intake of food and water in both apoE3 and C57BL/6N mice[a]

	Food intake (g)		Water intake (g)	
	Control	CPF	Control	CPF
ApoE3	2.65 ± 0.06	3.13 ± 0.07*	2.08 ± 0.04	2.06 ± 0.07
C57BL/6N	2.62 ± 0.03	3.20 ± 0.01*	2.12 ± 0.04	2.19 ± 0.05

[a] Statistically different changes versus the corresponding control group are indicated as *$p < 0.05$

[$F_{1,17}=8.143$, $p=0.011$] and C57BL/6N mice [$F_{1,16}=8.892$, $p=0.009$]. Strikingly, however, *post hoc* testing revealed that exposed apoE3 mice were more sensitive to CPF as their insulin levels were 54.62% higher than those found in their treated counterparts (Fig. 2A).

Both the genotype [$F_{1,34}=11.808$, $p=0.002$] and the treatment [$F_{1,34}=10.477$, $p=0.003$] were found to have an overall effect on HOMA-IR. A significant interaction between genotype and treatment [$F_{1,34}=4.245$, $p=0.048$] was also found. Further analysis confirmed an overall effect of the treatment on HOMA-IR in both apoE3 [$F_{1,17}=7.868$, $p=0.013$] and C57BL/6N mice [$F_{1,16}=6.382$, $p=0.023$], which indicates that CPF exposure leads to higher rates of insulin resistance. Likewise, *post hoc* analyses highlighted the propensity of *ε3* allele carriers to develop insulin resistance as their HOMA-IR values were 59.79% higher than those of C57BL/6N mice exposed to CPF (Fig. 2B).

3.5. Leptin levels of apoE3 mice are increased by CPF

Both the genotype [$F_{1,33}=11.655$, $p=0.002$] and the treatment [$F_{1,33}=11.037$, $p=0.002$] showed overall effects on leptin levels (Fig. 3A). In fact, only apoE3 mice were found to have significantly elevated leptin concentration after CPF exposure [$F_{1,16}=9.356$, $p=0.008$], suggesting greater amounts of fat depots in these subjects. In addition, we studied the relationship between body weight status at the end of the treatment and circulating levels of leptin, determined in plasma after a 3-h fast period (Fig. 3B). Body weight and leptin were strongly correlated ($r^2=0.729$, $p < 0.001$). The linear regression indicated that for each gram of weight gain, leptin levels increased in 1.827 μg/L.

3.6. CPF tends to increase acyl ghrelin levels

Repeated exposure to CPF tended to increase acyl ghrelin levels [$F_{1,26}=3.775$, $p=0.064$], which could explain the elevated rates of food intake found in the treated animals (Fig. 4A). Furthermore, total ghrelin levels were dependent upon genotype [$F_{1,28}=4.328$, $p=0.048$]: C57BL/6N mice appeared to have higher concentrations than apoE3 (Fig. 4B).

4. Discussion

The present study aimed to fully explore the metabolic effects of a subchronic dietary exposure to CPF, as well as to assess whether the human *APOE3* genotype could exacerbate their emergence. The metabolic disturbances arising out of the 8-week

Table 2
Plasma concentration of metabolic biomarkers in both apoE3 and C57BL/6N mice[a]

	ApoE3		C57BL/6N		Overall effects	
	Control	CPF	Control	CPF	Treatment	Genotype
FG (mmol/L)	9.07 ± 0.46	10.42 ± 0.63	8.10 ± 0.41	8.94 ± 0.45	**$p=0.033$**	**$p=0.017$**
Cholesterol (mmol/L)	4.14 ± 0.12	5.65 ± 0.86[†]	4.07 ± 0.13	4.48 ± 0.13*	**$p=0.036$**	$p=0.167$
Triglycerides (mmol/L)	1.41 ± 0.05	1.91 ± 0.35	1.59 ± 0.06	1.63 ± 0.11	$p=0.155$	$p=0.782$
Albumin (g/L)	41.96 ± 0.93	40.68 ± 0.71	41.46 ± 0.79	40.89 ± 0.58	$p=0.233$	$p=0.850$
Creatinine (μmol/L)	35.36 ± 0.00	35.36 ± 4.56	24.31 ± 3.24	25.05 ± 3.55	$p=0.910$	**$p=0.003$**
ALT (U/L)	10.52 ± 8.35	23.75 ± 12.12	16.77 ± 8.17	27.40 ± 9.20	$p=0.294$	$p=0.658$
AST (U/L)	109.94 ± 20.54	74.70 ± 17.84	91.05 ± 15.06	102.12 ± 22.97	$p=0.533$	$p=0.825$

[a] Statistically different changes versus the corresponding control group are indicated as *$p < 0.05$
[†] The symbol indicates a tendency ($p=0.097$) versus the corresponding control group. FG, fasting glucose.

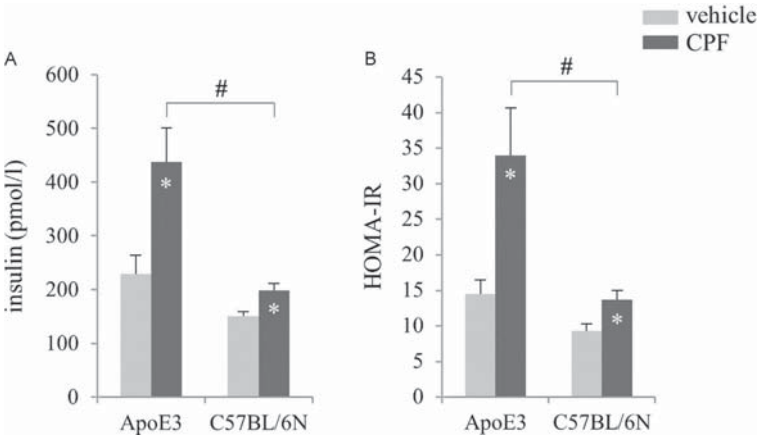

Fig. 2. The fasting plasma insulin levels (A) and the estimation of insulin resistance, which was based on the HOMA-IR index (B), were evaluated to estimate insulin sensitivity after subchronic oral adulthood exposure to chlorpyrifos in both apoE3 and C57BL/6N male mice. *Asterisks* indicate significant differences between CPF-exposed mice and their corresponding control group ($p < 0.05$), while the symbol # indicates significant differences between genotypes on the same treatment ($p < 0.05$).

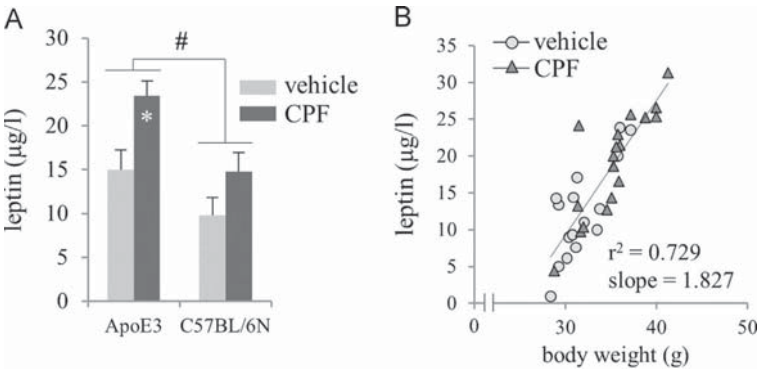

Fig. 3. Effect of subchronic oral adulthood exposure to chlorpyrifos on plasma levels of leptin (A). The correlation of body weight status at the end of the 8-week treatment period and circulating plasma levels of leptin was also depicted (B). *Asterisks* indicate significant differences between CPF-exposed mice and their corresponding control group ($p < 0.05$), while the symbol # indicates significant differences between genotypes ($p < 0.05$).

treatment period were evaluated in both apoE3 and C57BL/6N adult male mice and then compared. Specifically, body weight status, food intake, lipid and glucose homeostasis, metabolic biomarker concentrations, insulin levels and insulin resistance, and leptin and ghrelin profiles were investigated. Our results indicated that repeated dietary doses of CPF, devoid of signs of cholinergic toxicity, induced metabolic alterations in both genotypes. Nevertheless, this study shed novel and significant evidence supporting the vulnerability of human *ε3* carriers to the development of obesity and related metabolic disturbances in response to CPF. Indeed, although CPF broadly increased food intake, weight gain and higher plasma leptin levels were inherent to CPF-fed apoE3 mice. Furthermore, both exposed groups exhibited hyperinsulinemia and displayed insulin resistance, but these effects

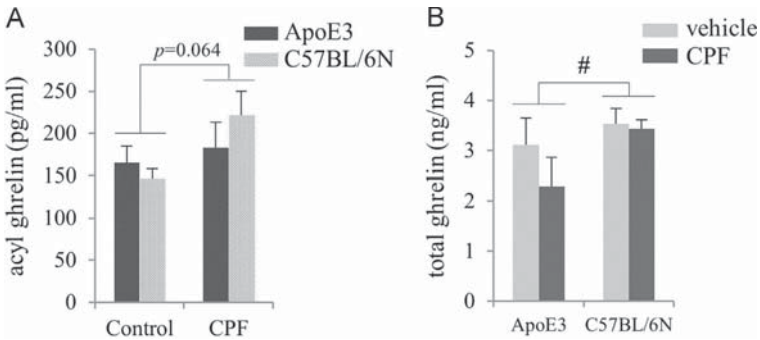

Fig. 4. Effect of subchronic oral adulthood exposure to chlorpyrifos on plasma levels of both acyl ghrelin (A) and total ghrelin (B). The symbol # indicates significant differences between genotypes ($p < 0.05$).

were more prominent in the *ε3* carriers. Total cholesterol and fasting plasma glucose levels increased overall in treated animals. CPF exposure also tended to increase plasma acyl ghrelin levels.

Despite the growing body of epidemiological data linking pesticide exposure with increased incidence of obesity (Kim et al., 2015), little attention has been paid to the contribution of CPF. To the best of our knowledge, only three experimental studies have revealed weight gain after early-life (Lassiter and Brimijoin, 2008) or adulthood exposures to CPF (Ehrich et al., 2004; Meggs and Brewer, 2007). The present investigation indicated that repeated exposure to CPF induced a weight gain in both apoE3 and C57BL/6N mice. Nevertheless, this increase was faster and steeper in *ε3* carriers, in agreement with our previous study (Peris-Sampedro et al., 2015). The present results also revealed an increase in leptin levels in exposed apoE3 animals. Moreover, body weight status at the end of the treatment period and circulating leptin levels were strongly correlated. The concentration of leptin has been positively correlated with the percentage of body fat in humans (Harris, 2000). This hormone operates as a satiety signal, inhibiting food intake and promoting energy expenditure (Pan et al., 2014). Nonetheless, its levels have been found to be elevated in obese individuals (Harris, 2000). The increase in leptin levels described here suggests that CPF-treated apoE3 mice had higher amounts of body fat, thus indicating that CPF exposure could be increasing their adiposity. Despite no significant changes in leptin levels, Lassiter and Brimijoin (2008) argued that the excess weight gain observed after developmental exposure to CPF could be due to an increased adiposity in rats. Accordingly, Meggs and Brewer (2007) found that subcutaneous exposure to 5 mg/kg/day CPF for 3 months increased adipose tissue in rats. Therefore, adipose tissue is expected to be a potential target for CPF, which is highly lipophilic in nature.

In recent years several studies have suggested that the *APOE3* genotype contributes to the development of diet-induced obesity (Arbones-Mainar et al., 2008; Karagiannides et al., 2008). ApoE3 mice subjected to a western-type diet were phenotypically more obese than apoE4 mice, while their total and subcutaneous amounts of fat also increased (Arbones-Mainar et al., 2008). In agreement with this, Huebbe et al. (2015) reported that apoE3 mice were heavier than apoE4 not only when they were on a high-fat diet, but also on a low-fat diet. When these authors explored the mechanisms by which the *APOE3* genotype could be contributing to increased fat depots, they suggested that the *ε3* carriers were more efficient at harvesting dietary energy (Huebbe et al., 2015). In the light of the above, the combination of apoE3 isoform expression with CPF exposure would provoke an additive effect, and greater body weight would be expected in exposed apoE3 mice. The present results are consistent with the genotype-dependent weight gain observed in our previous study (Peris-Sampedro et al., 2015), raising the issue of whether the human *ε3* allele could be promoting fat accumulation and, subsequently, favouring an obese-like phenotype after CPF exposure.

Increasing epidemiological and experimental evidence suggests that OPs disrupt glucose metabolism and cause insulin resistance, leading to type 2 diabetes (Lasram et al., 2014). Nevertheless, the data are sometimes contradictory and fail to define how they trigger them. Furthermore, only few studies have investigated whether adulthood exposure to CPF can contribute to the onset of insulin resistance or type 2 diabetes, the most frequently studied OPs being malathion and diazinon (Lasram et al., 2014). In the current investigation, repeated exposure to CPF induced moderate fasting hyperglycemia 8 weeks after the treatment started. In this context, only two studies have explored the role of CPF in disturbing glucose homeostasis throughout adulthood (Acker and Nogueira, 2012; Elsharkawy et al., 2013). Despite differences in experimental protocols, our data are in agreement with those reported by these studies, which found an increase in glucose levels in both Wistar and Sprague-Dawley adult male rats after a single acute dose of 50 mg/kg CPF (Acker and Nogueira, 2012) and following a 3 month-period of oral exposure to CPF at 30 mg/kg body weight (Elsharkawy et al., 2013). The mechanisms by which OPs exert their hyperglycemic function are the subject of intense debate. One of the most widely accepted is that they disrupt the gluconeogenesis and glycogenolysis pathways in the liver, but the findings are rather varied. The work conducted by Acker and Nogueira (2012) revealed increased activities of both tyrosine aminotransferase and glucose-6-phosphatase enzymes, pointing to enhanced CPF-related liver glucose production. However, they found an increase in hepatic glycogen levels, but not a decrease, which indicates that this route is not associated with hyperglycemia upon CPF exposure. A possible explanation for the elevated glucose levels observed in response to CPF is its cholinergic disrupting effect. It is well-known that acetylcholine (ACh) elicits the release of adrenaline and noradrenaline in the adrenal medulla (Butterworth and Mann, 1957). Indeed, it has been suggested that ChE inhibitors exacerbate this ACh-induced catecholamine release (Akiyama et al., 2003), which could trigger transient hyperglycemia by decreasing insulin-stimulated translocation of glucose transporters to the plasma membrane (Mulder et al., 2005). Ultimately, the excessive release of catecholamines could lead to insulin resistance (Ziegler et al., 2012).

OPs are known to generally alter lipid metabolism (Lasram et al., 2014). Just as found for glucose, repeated exposure to CPF increased total cholesterol levels in mice 8 weeks after the treatment started. Likewise, Elsharkawy et al. (2013) reported elevated plasma cholesterol levels following subchronic oral exposure to CPF in rats. They related this increase to liver cell damage, which was verified by light microscopic examination. While our exposure paradigm did not affect triglyceride concentrations, Elsharkawy et al. (2013) found reduced triglyceride levels in CPF-treated rats, which were also explained in terms of liver damage. Intrinsic mechanisms of CPF to promote hypercholesterolemia have not been yet disclosed. However, it is worth pointing out that CPF has been shown to target such key enzymes related to lipid metabolism as monoacylglycerol lipase and fatty acid amide hydrolase, among others (Quistad et al., 2006a).

Without underestimating the importance of exposure to other OPs for insulin resistance and type 2 diabetes outcomes (Lasram et al., 2014), the present study combining adulthood CPF exposure and human apoE3 isoform expression in mice appears to have no specific precedent. Repeated exposure to CPF led to increased insulin levels and the higher HOMA-IR values pointed to the development of insulin resistance, in both apoE3 and C57BL/6N mice. Hyperinsulinemia is considered to be indicative of insulin resistance, as well as a predictor of developing type 2 diabetes. Interestingly, although the insulin pathway was notably disturbed in both genotypes following CPF exposure, the effect was greater in apoE3 mice. Visceral obesity has been associated with insulin resistance (Yamashita et al., 1996). Indeed, it has been shown that leptin plays a role in modulating insulin action and sensitivity, and has been related to the emergence of insulin resistance and subsequent type 2 diabetes (Söderberg et al., 2007). Taking into account that the *APOE3* genotype appears to more efficiently harvest dietary energy through fat deposition, we hypothesize that apoE3 isoform expression aggravates insulin resistance and subsequent type 2 diabetes following CPF exposure.

Contrary to what might be expected, high levels of leptin in obesity fail to inhibit food intake (Harris, 2000). Our results indicated that repeated exposure to CPF generally increased food ingestion. In line with these findings, we recently found that CPF exposure tended to alter feeding behavior (Peris-Sampedro et al., 2015). In relation to this, the role of ghrelin deserves special

mention. It is an orexigenic hormone, secreted by the stomach, which stimulates food intake. Two forms of ghrelin coexist in the blood: acyl ghrelin and des-acyl ghrelin. However, only the acylated form has been shown to bind the growth hormone secretagogue receptor. The inactivation of acyl ghrelin into the deacylated form depends on hydrolization by the butyrylcholinesterase (BChE) enzyme (De Vriese et al., 2004). It is well-established that CPF inhibits both systemic and brain ChE enzymes. Accordingly, inhibition of BChE by CPF would be expected to increase acyl ghrelin levels, thereby leading to increased food intake. In support of this, we found that acyl ghrelin levels increased, although not significantly, after repeated exposure to CPF. Likewise, there is increasing evidence to suggest that acyl ghrelin could be a modulator of glucose homeostasis, and its elevated circulating levels could also be a triggering factor for type 2 diabetes (Huang et al., 2014). This finding shed novel information about how CPF exposure, in terms of BChE inhibition, would elicit type 2 diabetes outcome.

In conclusion, the results of the present study show that repeated exposure to the pesticide CPF can considerably disrupt not only glucose and lipid homeostasis but also feeding behavior in adult male mice. Together with recent results (Peris-Sampedro et al., 2015), the current data provide enough evidence to suggest that human apoE3 isoform expression increases vulnerability to developing obesity and related metabolic dysfunctions after CPF exposure. Although not conclusive, the results of this study suggest that CPF has hormonal targets, such as leptin or ghrelin, on which, to date, little has been reported in the scientific literature. The CPF dose used in our experiment, although free from cholinergic symptoms, is relatively high when compared with the dose that would be expected for typical non-occupational exposures. Therefore, further research is required to provide new insights into what doses would be exempt from metabolic effects. Given the wide distribution of the apoE3 phenotype worldwide, as well as the ubiquitous use of CPF, it is worth asking whether the combination of the two factors is contributing to the global incidence of obesity and type 2 diabetes.

Conflict of interest

The authors declare that there is no conflict of interest associated with their contribution to this manuscript.

Acknowledgments

This work was supported by the Ministry of the Economy and Competitiveness (MINECO, Spain) (Grant no. PSI2010-21743-C02-01) and the Commission for Universities and Research of the Department of Innovation, Universities and Enterprise of the *Generalitat de Catalunya* (Grant no. 2013 FI_B00170).

The authors would like to thank Amparo Aguilar, Esperanza Chernichero and Juan València (Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili), for their skillful technical support with animal care.

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Publication 3 **Attentional performance, impulsivity and related neurotransmitter systems in apoE2, apoE3 and apoE4 female transgenic mice.**

Reverte I, Peris-Sampedro F, Basaure P, Campa L, Suñol C, Moreno M, Domingo JL, Colomina MT. Psychopharmacology 2015. DOI: 10.1007/s00213-015-4113-9.

What is already known?

Learning and memory processes have been extensively studied in apoE TR mice. However, there is still considerable uncertainty about the extent to which *APOE* genotype contributes to other cognitive and behavioural processes, including sustained attention and inhibitory control.

What this study adds?

The results of this study further confirm that *APOE* polymorphisms strongly modulate attention and inhibitory control, being the individuals carrying the *ε4* allele the most behaviourally affected. The influence of apoE isoforms in the brain neuromodulatory system may explain the cognitive and behavioural differences attributable to the *APOE4* genotype.

Highlights

APOE genotype influenced attention and inhibitory control in the 5-CSRTT. In particular, apoE4 mice displayed increased premature and perseverative responding, and exhibited a steeper drop in accuracy when attention was challenged. Moreover, apoE4 mice showed less DA in the frontal cortex than apoE2 mice. Finally, the adverse effects of scopolamine on the 5-CSRTT performance were sharper in apoE3 mice relative to the other two groups.

Psychopharmacology
DOI 10.1007/s00213-015-4113-9

ORIGINAL INVESTIGATION

Attentional performance, impulsivity, and related neurotransmitter systems in apoE2, apoE3, and apoE4 female transgenic mice

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Received: 2 June 2015 / Accepted: 6 October 2015

Abstract
Rationale The apolipoprotein E (apoE) genotype influences cognitive performance in humans depending on age and sex. While the detrimental role of the apoE4 isoform on spatial learning and memory has been well-established in humans and rodents, less is known on its impact on the executive functions.
Objectives We aimed to evaluate the effect of apoE isoforms (apoE2, apoE3, apoE4) on visuospatial attention and inhibitory control performance in female transgenic mice, and to determine the neurochemical and neuropharmacological basis of this potential relationship.
Methods Female mice carrying apoE2, apoE3, and apoE4 were trained in the five-choice serial reaction time task (5-CSRTT). Upon a stable performance, we manipulated the inter-trial interval and the stimulus duration to elicit impulsive responding and engage attention respectively. We further performed a pharmacological challenge by administering cholinergic and GABAergic agents. Finally, we analyzed the levels of brain amino acids and monoamines by using reversed phase high-performance liquid chromatography (HPLC).
Results ApoE4 mice showed a deficient inhibitory control as revealed by increased perseveration and premature responding. When attention was challenged, apoE4 mice also showed a higher drop in accuracy. The adverse effect of scopolamine on the task was attenuated in apoE4 mice compared to apoE2 and apoE3. Furthermore, apoE4 mice showed less dopamine in the frontal cortex than apoE2 mice.
Conclusions We confirmed that the apoE genotype influences attention and inhibitory control in female transgenic mice. The influence of apoE isoforms in the brain neuromodulatory system may explain the cognitive and behavioral differences attributable to the genotype.

Keywords Apolipoprotein E · ApoE · Visuospatial attention · Impulsivity · 5-CSRTT · Acetylcholine · Dopamine · Glutamate · Striatum · Frontal cortex

Abbreviations

5-CSRTT	five-choice serial reaction time task
5-HIAA	5-Hydroxy-3-indolacetic acid
AD	Alzheimer's disease
ANOVA	Analysis of variance
apoE	Apolipoprotein E
CNS	Central nervous system
DOPAC	Dihydroxyphenylacetic acid
DA	Dopamine
Glu	Glutamate

Electronic supplementary material The online version of this article (doi:10.1007/s00213-015-4113-9) contains supplementary material, which is available to authorized users.

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Published online: 24 October 2015

GABA	Gamma-aminobutyric acid
HPLC	High-performance liquid chromatography
HVA	Homovanillic acid
ITI	Inter-trial interval
LH	Limited hold
NE	Norepinephrine
5-HT	Serotonin
SD	Stimulus duration
TR	Targeted replacement mice
TO	Time-out

Introduction

Apolipoprotein E (apoE), the main apolipoprotein in the brain, contributes to the synaptic development, integrity, and neural plasticity in the central nervous system (CNS), where it is locally synthesized primarily by astrocytes (Hauser et al. 2011).

ApoE in humans is present in three allelic variants (ε2, ε3, ε4) which modulate cognitive functions throughout the life span (Davies et al. 2015). Among them, the ε4 allele is a well-established risk factor for Alzheimer's disease (AD) while apoE3 (the most frequent isoform) is regarded as the "neutral form" and apoE2 as neuroprotective against AD (Arendt 2001; Raber et al. 2004; Reitz and Mayeux 2009). However, apoE seems to modulate the cognitive function in the absence of the disease. Particularly, apoE4 has been associated with impaired attention, as well as deficits in verbal and spatial memory in healthy subjects (Berteau-Pavy et al. 2007; De Blasi et al. 2009; Greenwood et al. 2005; Kukolja et al. 2010; Marchant et al. 2010). Interestingly, several lines of evidence supported an apoE4–sex interaction in humans. In fact, apoE4 women carriers have shown more pronounced AD-like changes in neuroimaging, neuropathological, and neuropsychological measures than men (Beydoun et al. 2013; Ungar et al. 2014).

At the preclinical level, initial studies on apoE knockout mice readily suggested an implication of apoE in learning and memory (Champagne et al. 2002; Raber et al. 1998). Subsequently, transgenic lines expressing human apoE isoforms under the control of neuron-specific enolase (NSE) or the glial fibrillary acidic protein (GFAP) promoter revealed impaired spatial learning and increased anxiety in apoE4 mice relative to apoE3 and wild-type controls (Hartman et al. 2001; van Meer et al. 2007). Then, the human apoE targeted replacement (TR) mouse model was created to emulate the human condition since it allows the expression of the apoE protein in the same pattern and level as non-demented humans (Sullivan et al. 1997). Consistently with earlier studies, apoE4-TR mice showed alterations in spatial learning tasks as well as decreased locomotor activity and increased anxiety relative to apoE3 (Reverte et al. 2012; Reverte et al. 2014; Siegel et al. 2012). Notably, preclinical studies reported a decreased learning performance in female apoE4 mice relative to the male counterparts, similarly to that reported in humans (Grootendorst et al. 2005; Reverte et al. 2012; van Meer et al. 2007).

While spatial learning and memory have been extensively studied in apoE transgenic mice, other executive functions such as visuospatial attention and inhibitory control have not been systematically investigated. The aim of the present study was to characterize the differences in attention and inhibitory control between the three major isoforms for the apoE found in humans (apoE2, apoE3, apoE4). We first assessed impulsivity in the context of general attentional abilities by using the five-choice serial reaction time task (5-CSRTT) (Robbins 2002) in female apoE transgenic mice (apoE2, apoE3, and apoE4). Subsequently, we investigated the neuropharmacological basis of these effects. Based on recent evidence supporting abnormal neuronal maturation caused by the dysfunction of GABAergic interneurons in the hippocampus (Li et al. 2009) and a deficient cholinergic system (Yun et al. 2005) in apoE4 mice, we assessed the effects of a GABAergic agonist (alprazolam), a GABAergic antagonist (picrotoxin), and a cholinergic antagonist (scopolamine) in female apoE-TR mice pretrained in the 5-CSRTT. Finally, on a separate cohort of female apoE-TR mice, we further determined the levels of brain amino acids, monoamines, and their metabolites in the frontal cortex, striatum, hippocampus, and thalamus.

Material and methods

Subjects

The human apoE targeted replacement (TR) mice are generated by replacing the murine apoE gene with one of the three apoE human alleles in the C57BL/6 N mice (Sullivan et al. 1997). Adult homozygous (ε2, ε3, and ε4) apoE-TR female mice were obtained from Taconic (N=35, Taconic Europe, Lille Skensved, Denmark). A *wild-type* group was not included because our goal was to determine differences in impulsivity and attentional control between the three apoE genotypes so to recapitulate the human spectrum. It is also worth noticing that several studies confirmed a very similar phenotype between apoE3 and the wild-type (WT) or an intermediate phenotype between apoE3 and apoE4 in the WT; please refer to (Bour et al. 2008; Grootendorst et al. 2005; Li et al. 2009; Levi et al. 2003). Subjects were housed in pairs in a room at controlled temperature (22±2 °C) and humidity (50±10 %) and under a 12-h light/dark automatic cycle (light ON at 08:00–20:00). Mice were fed with standard rodent chow (Panlab, Barcelona, Spain). During the behavioral training, mice were food-restricted to achieve the 80–85 % of their free feeding

weight, while water was available ad libitum. Nine animals were removed from the experiments because of poor health or poor performance (apoE2=2, apoE3=4, apoE4=2). Another group of adult female apoE transgenic mice (N=21, Taconic) was housed in groups of two to four per cage with food and water available ad libitum until killing for neurochemical analyses. Experimental procedures complied with the Animal Care and Use Committee of the Universitat Rovira i Virgili (Tarragona, Spain), the Spanish Royal Decree 53/2013 on the protection of experimental animals, and the European Communities Council Directive (86/609/EEC).

5-CSRTT

Apparatus

Mice were trained in operant chambers (24×20×15 cm) placed inside ventilated sound-attenuating cubicles (Med Associates Inc., St. Albans, VT, USA). Each chamber consisted of a curved wall containing nine round apertures equipped with infrared detectors and bright yellow led (1.7 W) at the rear. Four of the nine apertures were blocked with a metal plate, thus allowing five functioning apertures equally spaced 2.5 cm apart. A magazine was located centrally in the opposite wall, equipped with an infrared detector and connected to a liquid dipper delivering 0.01 ml of grape juice (grape juice and 15.13 % sugar, López Morenas, SL, Spain). The chambers were controlled by a PC using a Fader Control interface and Med Pc software (Med Associates Inc., St. Albans, VT, USA).

Habituation to the reinforcer (grape juice) and to the 5-CSRTT apparatus

Prior to training, the preference for the grape juice was tested in a two-bottle choice procedure (Bachmanov et al. 2001). One bottle containing water and one bottle containing grape juice were placed in the home cage. The position of the bottles was counterbalanced across mice. The water and grape juice intakes were recorded after 24 h.

Mice were also habituated to the 5-CSRTT chambers with a 20-min session in which the magazine light remained illuminated and each nose-poke in the magazine triggered the liquid dipper (available for 3 s).

5-CSRTT training

The behavioral training was carried out during the light phase. The training consisted of a 20-min daily session for 5 days a week over a period of 20 weeks. All sessions in the 5-CSRTT were conducted with the houselight of the apparatus extinguished (Humby et al. 2005).

Pretraining and training procedures were adapted from previous studies (Moreno et al. 2010; Oliver et al. 2009; Robbins 2002) (Supplementary Table S1). During the *pretraining 0 stage*, the five apertures remained illuminated throughout the session and a drop of grape juice was placed in each aperture to elicit exploration. A nose-poke in one of the apertures triggered the liquid dipper delivering the grape juice in the magazine, which was available until collection. Mice were trained at this stage until they performed five nose-pokes in 20 min. In *pretraining 1 stage*, three random apertures remained illuminated throughout the session. A response into an illuminated aperture triggered the liquid dipper delivering the grape juice in the magazine, which was available until collection. Mice were trained at this stage until they performed 20 correct responses in 20 min.

During *training* stages, mice learned to detect the location of a brief visual stimulus (cue light) presented in one of the five apertures in a pseudo-random order. During the acquisition of the task, the stimulus duration (SD) was progressively reduced from 30 to 1 s in ten stages. Each session consisted of 20-min or 70 discrete trials. Each trial started with the mouse nose-poking into the illuminated magazine. After an inter-trial interval (ITI) of 5 s, the stimulus was presented.

A *correct* response was recorded upon successful detection of the spatial location of the visual stimulus, and it was rewarded with 0.01 ml of grape juice. A failure to respond within a limited hold period of 5 s was recorded as an *omission* and was signaled by a 5-s time-out period during which the houselight was illuminated. Similar feedback was given on trials when mice responded in an adjacent aperture (an *incorrect* response), or prior to the onset of the light stimulus (a *premature* response). Furthermore, an additional response to an aperture occurring after a correct response but before the reward collection was recorded as a *perseverative response*.

Mice were trained until they showed for 5 consecutive days a stable performance: correct trials >50 %, accuracy>80 %, and omissions<25 %.

Behavioral challenge

The behavioral testing spanned over a period of 8 consecutive weeks and started upon stable baseline response (Robbins 2002; Sanchez-Roige et al. 2012). A total of 27 female mice were tested (apoE2=9, apoE3=9, apoE4=9). The mean age at the beginning of the challenge was 7.9±1.6 months.

Impulsivity and attentional performance were assessed once a week, typically on Wednesday. Monday, Tuesday, Thursday, and Friday mice were trained with standard baseline parameters. The challenge to elicit impulsive responding consisted in increasing the ITI from 5 s (baseline) to 7 s (weeks 1 and 2) and 10 s (weeks 3 and 4), respectively. The attentional performance was assessed by reducing the

stimulus duration from 1 s (baseline) to 0.8 s (weeks 5 and 6) and 0.5 s (weeks 7 and 8), respectively (Fig. 1).

Pharmacological challenge

All drugs were injected intraperitoneally (i.p.) according to a Latin square design. During the testing weeks, 0.9 % saline was injected i.p. on Tuesdays and Thursdays (baseline condition), while on Wednesdays and Fridays, a given drug/dose was administered 30 min (alprazolam, scopolamine) or 10 min (picrotoxin) before the session (Fig. 1). Mice were subjected to standard sessions of the 5-CSRTT with the same parameters used for the assessment of baseline responding. Mice received infusions of 0.9 % saline, the GABAergic agonist alprazolam (0.06 and 0.12 mg/kg), the GABAergic antagonist picrotoxin (0.25 and 0.5 mg/kg), and the cholinergic antagonist scopolamine (0.8 and 1.6 mg/kg). The dose selection was based on previous studies (Kulkarni and Sharma 1993; Sanchez-Roige et al. 2012; Siegel et al. 2010). Mice were habituated to the i.p. injection (0.9 % saline) daily 20 min before the training session over a period of 1 week. We further performed a pilot study to ensure that the selected doses of picrotoxin did not induce convulsion and the doses of alprazolam did not induce high sedation in mice of any genotype (data not shown).

Neurochemical analyses

A group of naïve female mice (apoE2=5, apoE3=7, apoE4=9; age 7±2 months) were used for this study. Mice were killed by rapid decapitation and the brains were quickly removed and dissected. The frontal cortex, striatum, thalamus, and hippocampus were frozen in liquid nitrogen and stored at −80 °C before processing. Brain region samples were weighed and homogenized in 0.4 N perchloric acid with 0.1 % metabisulfite, 0.01 % EDTA, and 1 mg/ml cysteine. The homogenates were centrifuged at 15,000 rpm for 20 min at 4 °C, and supernatants were collected, filtered (Millipore filters 0.45 micron), and stored at −80 °C until biochemical analyses. The levels of glutamate (Glu), gamma-aminobutyric acid (GABA), norepinephrine (NE), dopamine (DA), serotonin (5-HT), and the metabolites dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxy-3-indolacetic acid (5-HIAA) were measured using reversed phase high-performance liquid chromatography (HPLC).

Monoamine measurements

Levels of norepinephrine (NE), dopamine (DA), serotonin (5-HT), and their metabolites dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxy-3-indolacetic acid (5-HIAA) were measured by reversed phase HPLC with amperometric detection (+0.7 V). The mobile phase, containing 0.1 M KH_2PO_4, 0.1 mM Na_2-EDTA, and 2.1 mM 1-octane sulfonic acid, plus 15 % methanol, adjusted to pH 2.65 with 85 % H_3PO_4, was delivered at 1 ml/min flow rate. Monoamines were separated on a 3-μm particle size column C18 (10 cm×0.46 cm).Tissue contents of the monoamines are given as picomoles per milligram of tissue. As indices of DA and 5-HT turnover, DOPAC/DA, HVA/DA, and 5-HIAA/5-HT ratios were calculated.

Amino acid measurements

Levels of glutamate and GABA were measured by reversed phase HPLC with fluorescence detection using excitation and

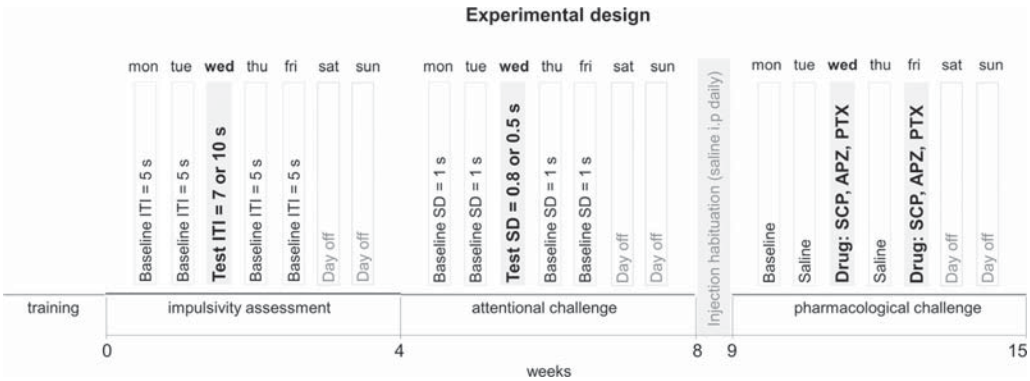

Fig. 1 Experimental design of the behavioral and pharmacological challenges in the 5-CSRTT. Upon training completion, once the animals showed a stable performance in the task, the inter-trial interval (ITI) was increased (7–10 s) and the stimulus duration (SD) was decreased (0.8–0.5 s) to challenge impulsivity and attention, respectively. Each parameter was manipulated once a week during 8 weeks: first and second weeks, ITI=7 s; third and fourth weeks, ITI=10 s; fifth and sixth weeks, SD=0.8 s; and seventh and eighth weeks, SD=0.5 s. After the behavioral challenge, mice were habituated to saline injections for 1 week. During the pharmacological challenge, alprazolam (*APZ*, 0.06 and 0.12 mg/kg), picrotoxin (*PTX*, 0.25 and 0.5 mg/kg), and scopolamine (*SCP*, 0.8 and 1.6 mg/kg) were injected twice a week before the testing session. The order of drug administration was assigned to each mouse using a Latin square design

emission wavelengths of 360 and 450 nm, respectively. The mobile phase consisted of two components (solution A, containing 0.05 M Na_2HPO_4, 28 % MeOH, adjusted to pH 5.65 with 85 % H_3PO_4; and solution B, MeOH/H_2O 8:2 ratio) and was delivered at 0.8 ml/min. Glutamate and GABA were separated in a 5-μm particle size C18 column (10 cm×0.4 cm). The samples were precolumn derivatized with OPA reagent and injected after a 2.5-min reaction time. A gradient was established from 100 % solution A to 100 % solution B. After washing out late eluting peaks, the mobile phase returned to initial conditions. The total gradient programmed time was 20 min.

Statistical analyses

Data were analyzed with the SPSS Statistics 17.0 software. One-way ANOVA (genotype) was used to analyze the number of sessions required at each stage of the training. Repeated-measure ANOVA (genotype) was used to analyze the performance in the 5-CSRTT during baseline, ITI, SD, and pharmacological manipulations. For the behavioral and pharmacological challenges, two measures of each variable taken in two different sessions (5-, 7-, and 10-s ITI; 1-, 0.8-, and 0.5-s SD; vehicle and each drug dose) were used as within-subjects factor and the genotype as the between-subjects factor. A post hoc Tukey test was used to follow-up significant main effects and interactions. Amino acid and monoamine levels in each brain region were analyzed by one-way ANOVA (genotype). The homogeneity of the variance was determined by the Levene's test. Statistical significance was set at $p<0.05$.

The variables considered in the analysis of the performance in the 5-CSRTT were as follows: trials completed (correct responses+incorrect responses+omissions), *% accuracy* (correct responses/(correct+incorrect responses)×100), *% of omissions* (omissions/trials completed×100), *% of premature responses* (premature responses/trials completed x 100), *perseverative responses* (number of responses made after a correct response and before the collection of the reward), *correct latency* (latency to made a correct response after the onset of the stimulus), and *reward latency* (latency to collect the reward after a correct response).

Results

Habituation to the reinforcer and 5-CSRTT acquisition phase

In the two-bottle choice procedure, mice of each genotype strongly preferred grape juice over water ($p<0.05$, data not shown). Importantly, we did not observe differences between genotypes in water or grape juice total intake (genotype $p>0.1$, data not shown). Notably, no differences between genotypes were observed on the total number of sessions required to acquire the task ($p>0.1$). However, we observed an effect of the genotype at stage 5 [$F(2,34)=8.920$, $p<0.01$]. A post hoc analysis revealed that apoE3 mice were significantly slower at this stage relative to apoE2 and apoE4 mice ($p<0.01$; Table 1).

5-CSRTT baseline performance

No differences between genotypes were observed in any of the behavioral variables measured, with the exception of perseverative responses [main effect of the genotype, $F(2,26)=3.542$, $p<0.05$]. A post hoc analysis revealed that perseverative responses were significantly higher in apoE4 than in apoE3 mice (Fig. 2 and Supplementary Table S2). The number of trials completed during baseline is provided in Supplementary Table S3. A main effect of the genotype was observed [$F(2,26)=4.099$, $p<0.05$]; however, the post hoc analyses failed to show significant differences between groups.

Behavioral challenge

Behavioral attributes of the three genotypes during the challenge sessions on the 5-CSRTT are depicted in Figs. 3 and 4.

ITI

A significant increase in premature responding [$F(2,26)=26.218$, $p<0.001$] (Fig. 3c), perseverative responding [$F(2,26)=4.260$, $p<0.05$] (Fig. 3d), and omissions [$F(2,26)=4.211$, $p<0.05$] (Fig. 3b) was observed when the ITI was lengthened from 5 to 7 or 10 s. We also observed a main effect

Table 1 Number of sessions to criteria per training stage of the 5-CSRTT in apoE-TR female mice

Stage	apoE2		apoE3		apoE4	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
1	4.5	3.8	4.3	2.2	3.8	1.7
2	1.1	.3	1.7	1.2	1.2	.6
3	1.3	.6	2.3	2.5	1.6	1.1
4	2.3	1.6	6.1	9.3	6.3	4.3
5	5.7	3.0	13.2*	7.7	4.8	3.3
6	7.3	5.0	9.4	10.6	10.4	8.1
7	5.6	9.9	5.0	7.5	5.0	4.4
8	3.8	3.6	3.4	3.0	3.1	2.3
9	4.8	4.8	7.6	7.2	9.8	12.9
10	5.3	5.0	9.1	8.4	8.9	11.3

The asterisk indicates that in stage 5 apoE3 mice required more sessions than apoE2 and apoE4 to reach the criteria ($p<0.01$)

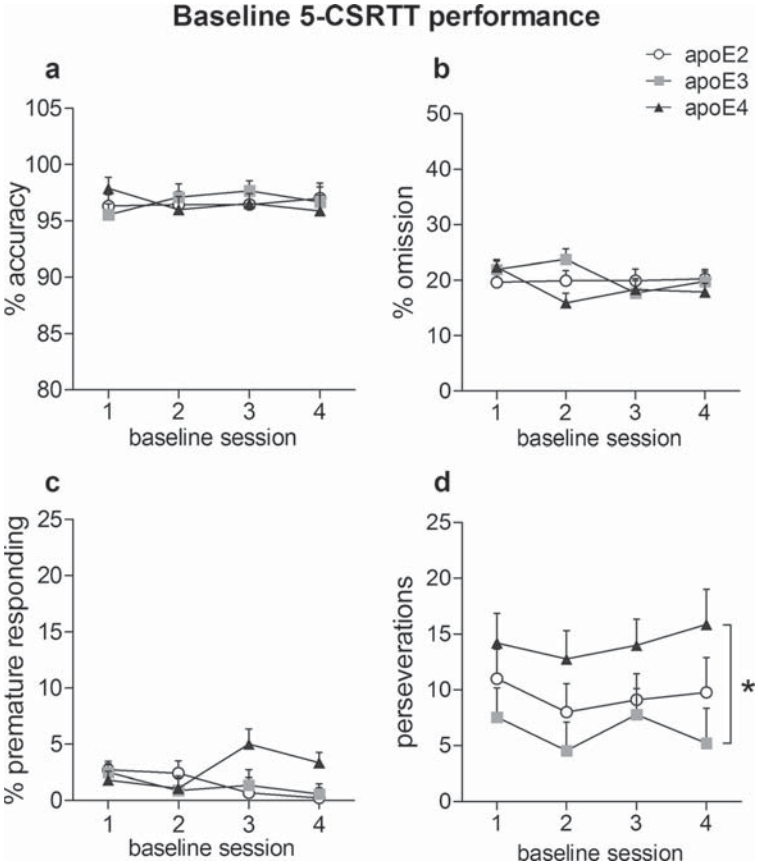

Fig. 2 Baseline performance of apoE-TR female mice in the 5-CSRTT. **a** Percentage of accuracy, **b** percentage of omissions, **c** percentage of premature responding, and **d** number of perseverative responses, during baseline sessions. Data is expressed as mean±S.E.M. The *asterisk* indicates differences between apoE4 and apoE3 at $p<0.05$

of the genotype on both premature [$F(2,26)=3.716$, $p<0.05$] and perseverative responding [$F(2,26)=3.625$, $p<0.05$]. A post hoc analysis revealed that apoE4 mice showed higher premature and perseverative responding relative to apoE2 and apoE3 mice (Fig. 3c, d).

SD

A significant decrease in accuracy [$F(2,26)=23.357$, $p<0.001$] and an increase of omissions [$F(2,26)=14.451$, $p<0.001$] were observed when the SD was decreased from 1 to 0.8 or 0.5 s (Fig. 4a, b). Furthermore, both response latency and collection latency were reduced [$F(2,26)=5.454$, $p<0.05$; $F(2,26)=4.349$, $p<0.05$, respectively] (data not shown). We also observed a main effect of the genotype on accuracy [$F(2,26)=4.089$, $p<0.05$] (Fig. 4a) and perseverative responses [$F(2,26)=3.833$, $p<0.05$] (Fig. 4d). A post hoc analysis revealed that apoE4 mice showed a steeper drop in accuracy ($p<0.05$) and increased number of perseverative responses ($p<0.05$) relative to apoE2 and apoE3 genotypes. An interaction session × genotype was also found in omissions [$F(4,26)=2.941$, $p<0.05$]. A post hoc analysis revealed a significant increase in apoE3 relative to apoE4 mice when the SD was 0.8 s (Fig. 4b). The maintenance of vigilance in the short SD session (0.5 s) was analyzed during ten-trial bins. A general effect of the trial period was observed on omissions, showing that mice performed more omissions by the end of the session [$F(5,25)=6.113$, $p<0.01$]. However, no effect of trial period or trial period × genotype interaction was observed in accuracy, which suggest that the deficit observed in apoE4 was present throughout the session (data not shown).

Pharmacological challenge

Behavioral attributes of the three genotypes during the pharmacological challenges on the 5-CSRTT are shown in Fig. 5 and Supplementary Figs. S1 and S2.

Scopolamine

Scopolamine produced a significant decrease in accuracy, an increase in omissions, and an increase in premature responding [main effect of dose, $F(2,23)=18.686$, $p<0.001$; $F(2,23)=14.456$, $p<0.001$; $F(2,23)=10.451$, $p<0.001$,

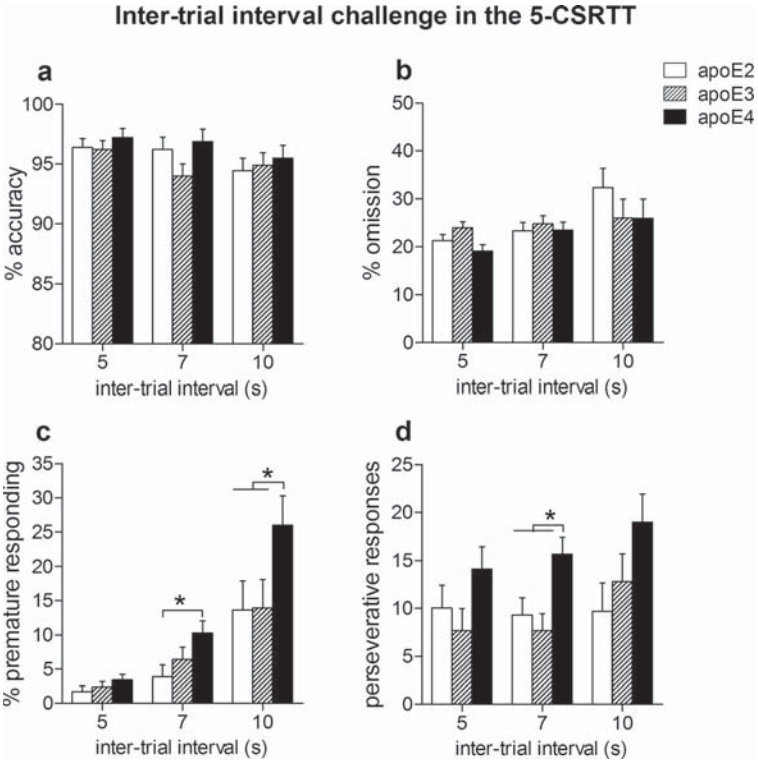

Fig. 3 Inter-trial interval (ITI) challenge in the 5-CSRTT in apoE-TR female mice. **a** Percentage of accuracy, **b** percentage of omission, **c** percentage of premature responding, and **d** number of perseverative responses, concurrent with inter-trial interval increments. Data is expressed as mean±S.E.M. The *asterisk* indicates differences between genotypes at $p<0.05$

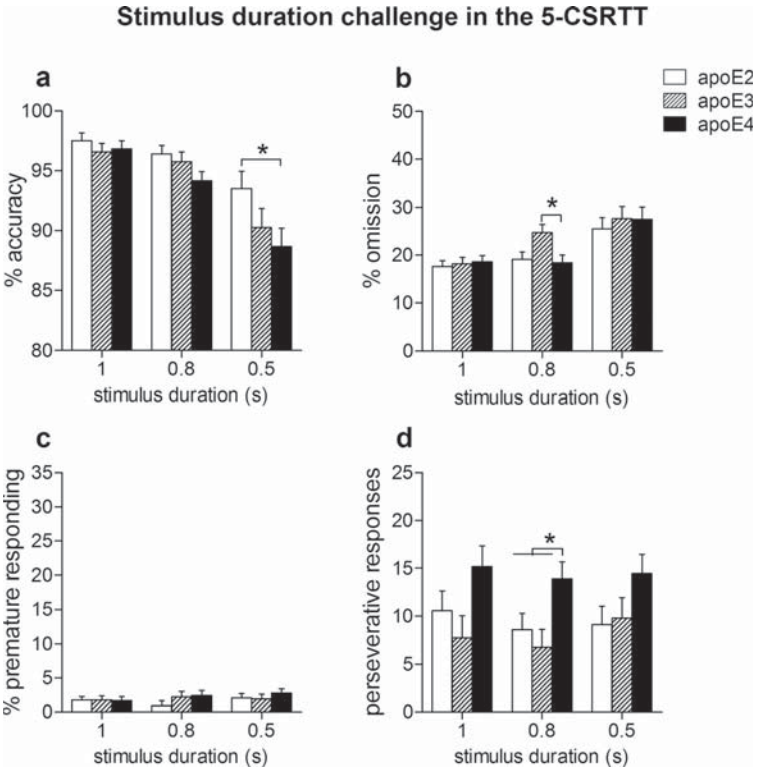

Fig. 4 Stimulus duration (SD) challenge in the 5-CSRTT in apoE-TR female mice. **a** Percentage of accuracy, **b** percentage of omission, **c** percentage of premature responding, and **d** number of perseverative responses, concurrent with stimulus duration decrements. Data is expressed as mean±S.E.M. The *asterisk* indicates differences between genotypes at $p<0.05$

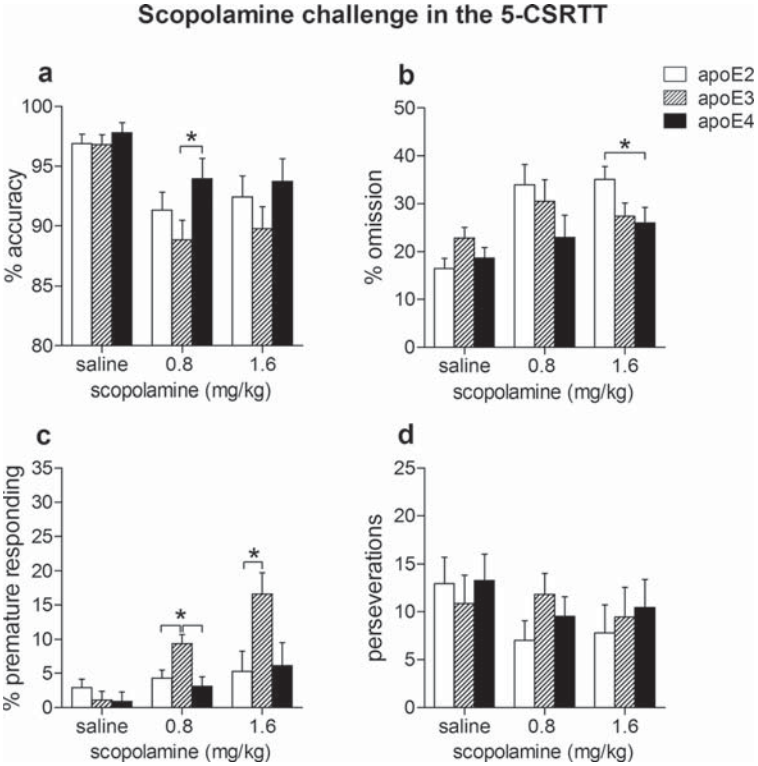

Fig. 5 Effect of scopolamine on the 5-CSRTT performance in apoE-TR female mice. **a** Percentage of accuracy, **b** percentage of omissions, **c** percentage of premature responding, and **d** number of perseverative responses, after saline and scopolamine injections. Data is expressed as mean±S.E.M. The *asterisk* indicates differences between genotypes at $p<0.05$

respectively]. A main effect of the genotype was also observed on accuracy [$F(2,22)=4.370$, $p<0.05$]. A post hoc analysis revealed differences in accuracy between apoE3 and apoE4 during the scopolamine challenge ($p<0.05$; Fig. 5a). An interaction dose × genotype was evident in omissions and premature responding [$F(4,23)=2.837$, $p<0.05$; $F(2,22)=2.768$, $p<0.05$, respectively]. Scopolamine-induced omissions were significantly higher in apoE2 relative to apoE4 ($p<0.05$), while the increase in premature responding was higher in apoE3 relative to the other genotypes ($p<0.05$; Fig. 5b, c). We also observed a main effect of the dose and a dose × genotype interaction in the latency to collect the reward [$F(2,23)=21.891$, $p<0.001$; $F(4,23)=4.381$, $p<0.01$, respectively] which increased more in apoE4 than in apoE3 at the high dose of scopolamine (data not shown). The effect of the genotype previously reported in perseverative responses was not observed during the scopolamine challenge (Fig. 5d).

Alprazolam

Alprazolam decreased omissions and increased premature responding [main effect of the dose, $F(2,23)=6.364$, $p<0.01$; $F(2,23)=5.959$, $p<0.01$, respectively] (Supplementary Fig. S1b, c). An effect of the genotype on perseverative responding was also observed [$F(2,23)=4.033$, $p<0.05$]. Post hoc analysis showed that perseverative responding was significantly higher in apoE4 mice relative to the other genotypes ($p<0.05$), as observed at baseline and during the behavioral challenge (Supplementary Fig. S1d).

Picrotoxin

Picrotoxin showed an effect on perseverative responding [dose effect, $F(2,23)=5.174$, $p<0.05$] which was reduced with the low dose. Although perseverative responses were higher in apoE4, we did not observe a significant main effect of the genotype (Supplementary Fig. S2d).

Neurochemical analyses

The amino acid and monoamine baseline levels of female apoE transgenic mice are shown in Tables 2 and 3. ApoE2 mice showed significant higher levels of GABA in the frontal cortex [$F(2,18)=4.819$, $p<0.05$] and glutamate in the striatum [$F(2,17)=4.119$, $p<0.05$] relative to apoE3 mice, as well as the highest levels of glutamate in the thalamus [$F(2,16)=9.151$, $p<0.01$]. However, no differences in the GABA/Glu ratio were observed in any brain region (Table 2).

Genotype differences in DA and DA turnover were observed in several brain regions. Levels of DA in the frontal

Table 2 Brain amino acid levels in apoE-TR female mice

nmol/mg	Frontal cortex			Striatum			Hippocampus			Thalamus		
	apoE2	apoE3	apoE4	apoE2	apoE3	apoE4	apoE2	apoE3	apoE4	apoE2	apoE3	apoE4
GABA	2.64±0.73[a]	1.72±0.36[b]	1.92±0.46[ab]	2.47±0.64	2.46±0.90	2.43±0.78	2.04±0.39	1.74±0.34	1.85±0.44	4.13±1.77	3.24±0.41	3.32±0.93
Glutamate	3.20±0.52	2.87±0.83	2.47±0.28	2.92±0.48[a]	2.14±0.45[b]	2.45±0.46[ab]	2.90±0.30	2.74±0.31	2.71±0.49	2.57±0.13[a]	1.98±0.37[b]	1.97±0.19[b]
GABA/glutamate	0.82±0.16	0.66±0.24	0.77±0.21	0.85±0.18	1.17±0.46	1.00±0.33	0.70±0.79	0.64±0.13	0.70±0.20	1.63±0.74	1.71±0.50	1.53±0.20

Groups showing different superscript letters (a, b) differ significantly from each other at $p<0.05$. Groups sharing a letter (a, b) do not differ among them ($p>0.1$)

Table 3 Brain monoamine levels in apoE-TR female mice

pmol/mg	Frontal cortex			Striatum			Hippocampus			Thalamus		
	apoE2	apoE3	apoE4	apoE2	apoE3	apoE4	apoE2	apoE3	apoE4	apoE2	apoE3	apoE4
A	1.36±0.41	1.53±0.46	1.37±0.29	0.44±0.31[a]	1.55±0.81[b]	1.19±0.23[ab]	1.20±0.26	1.44±0.38	1.77±0.48	1.24±0.15	1.73±0.58	1.72±0.36
DA	9.95±1.81[a]	7.20±5.21[ab]	4.60±2.64[b]	34.58±16.2[a]	12.17±4.76[b]	21.66±7.32[ab]	0.26±1.23	0.61±0.80	0.79±0.61	4.91±5.82	1.67±1.33	5.75±6.70
DOPAC/DA	0.18±0.03	0.39±0.20	0.45±0.26	0.48±0.03	0.45±0.18	0.44±0.12	5.39±5.50[a]	0.89±0.71[b]	1.00±0.80[b]	1.29±1.42	0.89±0.46	0.84±0.67
HVA/DA	0.13±0.04	0.70±1.10	0.28±0.17	0.10±0.01[a]	0.27±0.08[b]	0.14±0.04[a]	0.80±0.31	1.00±0.89	0.51±0.33	0.35±0.26	0.68±0.48	0.76±0.83
DOPAC+HVA/DA	0.31±0.04	1.08±1.17	0.74±0.34	0.58±0.03	0.72±0.26	0.58±0.12	6.18±5.49[a]	1.89±1.33[ab]	1.51±0.80[b]	1.64±1.60	1.57±0.61	1.60±1.49
5-HT	2.37±0.54	1.87±1.09	1.53±0.28	1.68±0.38	1.69±0.37	1.90±0.38	2.06±0.34	1.78±0.74	1.82±0.44	2.69±0.53	2.20±0.68	2.17±0.70
5-HIAA/5-HT	0.35±0.03	0.61±0.23	0.67±0.27	0.49±0.04	1.11±0.48	0.72±0.33	0.65±0.07	1.06±0.58	0.78±0.21	0.70±0.24	1.36±0.44	1.21±0.88

Groups showing different superscript letters (a, b) differ significantly from each other at $p<0.05$. Groups sharing a letter (a, b) do not differ among them ($p>0.1$)

cortex differed between apoE2 and apoE4, being lower in apoE4 mice [$F(2,20)=3.663$, $p<0.05$]. In the striatum, the levels of DA were higher in apoE2 mice than in apoE3 [$F(2, 16)=6.683$, $p<0.001$] and the ratio HVA/DA was higher in apoE3 than in mice of other genotypes [$F(2,16)=13.744$, $p<0.001$]. In the hippocampus, the DOPAC/DA and DOPAC+HVA/DA ratios were higher in apoE2 mice [$F(2, 19)=4.848$, $p<0.05$; $F(2,19)=4.880$, $p<0.05$]. The levels of NA in the striatum were lower in apoE2 than in apoE3 mice [$F(2,17)=5.875$, $p<0.05$] (Table 3).

Discussion

In the current study, we first characterized impulsivity in the context of visuospatial attention by using the 5-CSRTT in apoE2, apoE3, and apoE4 transgenic female mice. The main finding was that apoE4 female mice showed a deficit in inhibitory control on the 5-CSRTT as revealed by the increased premature responding during the inter-trial interval challenge. Importantly, we further observed an increased number of perseverative responding under baseline conditions considered a measure of cognitive inflexibility (Dalley et al. 2002). We second investigated the role of a GABAergic agonist (alprazolam), a GABAergic antagonist (picrotoxin), and a cholinergic antagonist (scopolamine) on the 5-CSRTT performance. The second main finding was that scopolamine-induced attentional impairment was significantly less pronounced in apoE4 than in apoE2 and apoE3 female mice. We finally performed a neurochemical analysis of naïve apoE females. We found that apoE4 female mice showed lower levels of dopamine in the frontal cortex relative to apoE2 female mice.

Attention and inhibitory control performance of apoE-TR female mice on the 5-CSRTT

The 5-CSRTT has been extensively used to determine the neural basis of visuospatial attention and inhibitory control prevalently in rats (Robbins 2002). In this study, apoE-TR mice were able to learn the 5-CSRTT, as revealed by a stable performance with minimal differences among genotypes. Notably, no differences in the acquisition of the task between apoE3- and E4-TR mice were observed in a previous study (Siegel et al. 2010). This is consistent with the idea that learning and memory impairments associated to apoE4 are limited to hippocampal-dependent tasks (Acevedo et al. 2010; De Blasi et al. 2009).

ApoE4 mice showed an impaired inhibitory control in the 5-CSRTT as revealed by increased premature and perseverative responding. This is generally considered to reflect a failure of the "executive system" represented by frontal cortical areas exerting a top-down control to limbic and paralimbic areas (Dalley et al. 2011). In rodents, lesions of the ventral hippocampus, prefrontal cortex, and disconnections of the medial prefrontal cortex from the ventral striatum increase impulsivity in the 5-CSRTT (Abela et al. 2013; Dalley et al. 2008). Based on the above, it could be speculated that alterations in the fronto-temporal network associated with the *ε4* allele could account for the deficits in inhibitory control. In fact, brain imagining studies reported that human apoE4 carriers show abnormal activity in the fronto-temporal and fronto-parietal systems (Dennis et al. 2010; Filippini et al. 2009; Reiman et al. 2004). Consistently, a recent imaging study in apoE-TR mice showed a volume loss in the cortex and hippocampus associated to age in apoE4 in comparison to wild-type mice (Yin et al. 2011). Furthermore, an abnormal synaptic plasticity in the hippocampus and the amygdala of young apoE4 mice has been reported (Dumanis et al. 2013; Klein et al. 2010; Rodriguez et al. 2013).

High impulsivity is negatively correlated to attentional accuracy (Dalley et al. 2008). Likewise, apoE4 mice displayed a greater drop in accuracy when attention was challenged. Interestingly, this effect was present during the whole session, indicative of a deficit in selective attention rather than difficulty to maintain sustained attention. Similarly, in apoE-TR mice that also overexpress the human amyloid precursor protein (APP), those carrying apoE4 showed poor accuracy in a two-choice operant visual discrimination task (Kornecook et al. 2010). We observed in apoE3 mice a higher rate of omissions than in apoE4 mice when the stimulus duration was decreased. A similar finding was reported by Siegel et al. who reported a higher number of omissions in apoE3 than in apoE4 mice at baseline and after scopolamine injections in the 5-CSRTT (Siegel et al. 2010).

Comparatively, human studies have found a worse execution of apoE4 carriers in neuropsychological tests with a greater attention load (Caselli et al. 2001; Rosen et al. 2002; Wisdom et al. 2011). As far as we know, only the group of Pasuraman used a specific task to compare visuospatial attention in subjects with different apoE genotypes. They observed selective attentional deficits in apoE4 carriers with an additive effect of ε4 allele dosage, and an effect of age. While the attentional deficit was evident in middle-aged and old apoE4 individuals, it was not present in very old individuals without dementia (Greenwood et al. 2000; Greenwood et al. 2005; Negash et al. 2009). Whether the attentional and inhibitory control deficits in apoE4 individuals could be indicators of higher risk for AD is a future venue of investigation.

Effects of scopolamine, alprazolam and picrotoxin on the 5-CSRTT performance in apoE-TR female mice

The basal forebrain cholinergic system is involved in sustained attention (Paolone et al. 2013; Sarter and Paolone 2011), and the muscarinic antagonist scopolamine disrupts accuracy and increases omissions in both rats and mice in

the 5-CSRTT (Sanchez-Roige et al. 2012). In the current study, apoE4 showed a lower sensitivity to scopolamine-induced attentional impairment. Specifically, the negative effect of scopolamine on attentional performance was more pronounced in apoE2 and apoE3 than in apoE4 mice.

An interaction between apoE and the cholinergic system has been suggested to underlie the cognitive deficit associated to apoE4 in humans. In fact, several indicators of a cholinergic dysfunction have been reported in apoE4, ranging from decreased neuronal activity in the basal nucleus of Meynert, which is the main source of cholinergic projections to the cortex (Salehi et al. 1998), to decreased hippocampal and cortical choline acetyltransferase activity (Allen et al. 1997; Lai et al. 2006; Poirier et al. 1995; Soininen et al. 1995a), higher levels of acetylcholinesterase (Eggers et al. 2006; Soininen et al. 1995b), and higher levels of muscarinic receptors (Cohen et al. 2003). The presence of the *ε4* allele has also shown to modulate the response to cholinergic agents. Young and healthy apoE4 carriers benefit more of the cognitive effects of nicotine (Evans et al. 2013; Marchant et al. 2010), while the prolonged use of anti-cholinergic medications have a worse cognitive effect in non-demented apoE4 carriers (Nebes et al. 2012; Pomara et al. 2008; Pomara et al. 2004). On the other hand, anticholinesterase medications used to improve cognitive function in AD patients seem to be less effective in those carrying apoE4 (Braga et al. 2014; Farlow et al. 1996).

In rodents, the blockade of nicotinic acetylcholine receptors has shown to suppress hippocampal long-term potentiation in wild-type but not in apoE4-TR mice (Yun et al. 2005). Remarkably, decreased levels of choline acetyltransferase have also been reported in apoE4 mice (Buttini et al. 2002) while the exposure to the pesticide chlorpyrifos, a cholinesterase inhibitor, impaired memory in apoE3 mice but not in apoE4 mice (Peris-Sampedro et al. 2015). However, the alleged cholinergic hypo-function related to apoE4 remains controversial since some studies failed to find cholinergic alterations in both human apoE4 carriers (Corey-Bloom et al. 2000; Reid et al. 2001; Svensson et al. 1997; Uusvaara et al. 2009) and apoE4 transgenic mice (Bronfman et al. 2000; Siegel et al. 2010). Overall, our results suggest that the antagonism of muscarinic receptors has a less pronounced effect on visuospatial attention in apoE4 mice compared to apoE2 and apoE3 mice.

Higher anxiety in apoE4 mice has been previously reported (Reverte et al. 2012; Siegel et al. 2012). Here we inquired whether the administration of anxiolytic or anxiogenic drugs would induce a differential effect in apoE-TR mice depending on the genotype. However, we did not observe a genotype effect on the 5-CSRTT performance after alprazolam and picrotoxin administration. Accordingly, the effects of lorazepam on attention and reaction time were similar in human apoE4 carriers and non-carriers (Stonnington et al. 2009). The systemic administration of alprazolam improved attention by decreasing omissions, but slightly increased premature responding. On the other hand, picrotoxin decreased perseveration. Similarly, GABAergic agonists have shown to increase impulsivity in several mouse strains (Oliver et al. 2009). Increasing GABAergic activity in the PFC increases impulsivity, probably because of the disinhibition of downstream areas such as the ventral striatum (Hayes et al. 2014). Coupled with this, the reduction of GABA in the NAc increases impulsivity in low impulsive rats (Caprioli et al. 2014). However, the levels of GABA in the PFC and the striatum did not differ in apoE4 mice compared to the other genotypes.

Neurotransmitters in apoE-TR female mice

Cortico-striatal and cortico-limbic networks involved in attention and inhibitory control are modulated by dopaminergic, serotonergic, and noradrenergic neurons originating in the midbrain (Dalley et al. 2011). We observed lower levels of dopamine in the frontal cortex in apoE4 mice than in apoE2 and apoE3 mice. Consistently, the depletion of dopamine in the medial prefrontal cortex (mPFC) induces impulsive choice in a delay discounting task (Freund et al. 2014), and reduced cortical dopamine levels have been reported in patients with ADHD (Del Campo et al. 2011). Furthermore, reduced mPFC dopamine activity levels also correlate with poor attention outcome (Logue and Gould 2014). Taken these results together, the lower levels of dopamine in the frontal cortex found in apoE4 mice may account for both the decreased accuracy and increased premature responding.

Dopaminergic and noradrenergic alterations in the striatum play a key role in the expression of impulsivity (Caprioli et al. 2013; Caprioli et al. 2015; Dalley et al. 2007; Economidou et al. 2012; Moreno et al. 2013). In the present study though, we did not observe in apoE4 mice deficiencies in the levels of dopamine and norepinephrine in the striatum relative to apoE2 and apoE3, but quite the opposite. The reasons for these discrepancies are unclear and obviously require further investigation. A possible explanation for this discrepancy could derive from the fact that we analyzed the entire striatum (nucleus accumbens, shell and core, and caudate putamen) while in the previous studies the main differences were confined to the nucleus accumbens.

Glutamate and GABA are the main excitatory and inhibitory neurotransmitters in the brain. ApoE2 mice showed significant higher levels of glutamate in the striatum relative to apoE3 mice and in the thalamus relative to both apoE3 and apoE4 mice. GABA in the frontal cortex was also higher in apoE2, which would account for a trend toward an increased sensitivity of this genotype to picrotoxin. However, no differences in the ratio of GABA/glutamate were observed in any brain region, which indicates that the balance of brain

excitation/inhibition was not compromised in apoE2 female mice. Higher levels of glutamate in the whole brain of apoE2 mice were reported by Dumanis et al. which might be related to the neuroprotective role attributed to the apoE2 isoform (Dumanis et al. 2013).

Concluding remarks

The results from the present study demonstrate that the human apolipoprotein E isoforms impact visuospatial attention and inhibitory control as measured in the 5-CSRTT, as well as the underlying neuromodulatory brain systems. Finally, further studies are needed to determine to what extent these results generalize to male apoE-TR and human population. The current findings have relevance because they provide valuable information on the underlying neural basis of the cognitive dysfunction related to apoE4 before the onset of neurodegenerative patterns.

Acknowledgments The authors would like to thank Luis Heredia, PhD; Jennifer Calzado, MPh; Phoebe Soebagyo, MPh; Teresa Neri, MPh; and Raissa Alvear, MPh for their helpful collaboration. This research was supported by PSI2010-21743-C02-01, the Ministry of the Economy and Competitiveness (MINECO, Spain), and PI 13/01252 from the Instituto de Salud Carlos III (ISCIII, Spain), the European Regional Development Fund (ERDF), the Commission for Universities and Research of the Department of Innovation, Universities and Enterprise of the Generalitat de Catalunya (2009 FI-B 00256/2010 and BE-1 0072), and the European Social Fund.

Compliance with ethical standards

Conflict of interest The authors declare that no conflict of interest has influenced the results presented in this article.

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Publication 4 **Apolipoprotein E genotype and the pesticide chlorpyrifos modulate attention, motivation and impulsivity in female mice in the 5-choice serial reaction time task.**

Peris-Sampedro F, Reverte I, Basaure P, Cabré M, Domingo JL, Colomina MT.

Neurotoxicology 2015. Currently under review.

What is already known?

There is some epidemiological evidence on the impact of CPF in executive functioning and motivational status, but these relationships have been scarcely addressed in an experimental setting. The poor cognitive outcome associated with the *APOE4* genotype has sometimes been attributed to a hypothetical cholinergic dysfunction. To date, no experimental studies have evaluated the impact of CPF on attention, inhibitory control and motivation in apoE TR mice.

What this study adds?

Besides giving support to the behavioural apoE phenotypes reported in the third study, these results strengthen existing evidence, and point to protracted detrimental effects on sustained attention and motivation upon CPF exposure. Moreover, they support the conceptual premise of close links between the cholinergic system and the *APOE4* genotype.

Highlights

APOE genotype influenced the 5-CSRTT acquisition and baseline performance. The exposure to CPF increased omissions, worsened processing speed, diminished premature responding, reduced the number of trials completed and increased reward latency. Strikingly, the lack of inhibitory control inherent to apoE4 mice was reversed by the treatment.

Apolipoprotein E genotype and the pesticide chlorpyrifos modulate attention, motivation and impulsivity in female mice in the 5-choice serial reaction time task

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Abstract

It is well-established that organophosphate (OP) pesticides contribute to a wide range of neurobehavioural disorders. Among the most commonly OPs used, chlorpyrifos (CPF) has been reported to elicit learning and memory impairments. Likewise, there is some epidemiological evidence on the impact of CPF in executive functioning, but this relationship has scarcely been addressed in an experimental setting. The three isoforms of the human apolipoprotein E (apoE) confer different cognitive skills on their carriers, but research on this topic remains limited. The current study was performed to assess whether the *APOE* genotypic variability differently modulate the effects of CPF on attentional performance, inhibitory control and motivation. Human apoE targeted replacement adult female mice (apoE2, apoE3 and apoE4) were trained to stably perform the 5-choice serial reaction time task (5-CSRTT). Animals were then subjected to daily dietary doses of CPF (3.75mg/kg body weight) for 4 consecutive weeks. After CPF exposure, we established a 4-week CPF-free period to assess recovery. Although all individuals acquired the task, apoE2 mice showed enhanced learning, while apoE4 mice displayed increased premature and perseverative responding. Strikingly, this genotype-dependent lack of inhibitory control was reversed by CPF. Overall, the pesticide induced protracted impairments in sustained attention and motivation, and it reduced anticipatory responding. ApoE3 mice exhibited delayed attentional disruptions due to motivational factors throughout the wash-out period. Taken together, these findings attest potential links between the *APOE4* genotype and the cholinergic system, and provide notable evidence of the emergence of CPF-related attentional and motivational deficits.

Keywords: Chlorpyrifos, Attention, Impulsivity, Motivation, Apolipoprotein E, 5-CSRTT

1. Introduction

The onset of cognitive deficits and behavioural disorders after exposure to organophosphate (OP) pesticides – in particular to the widely-used chlorpyrifos (CPF) – has been reported in the scientific literature (Mackenzie Ross et al., 2010; Roldán-Tapia et al., 2005). In the last decade, environmental agencies have taken steps to reduce the non-agricultural uses of CPF. In 2006, however, its residues were still present in 78% of randomly-selected homes in the United States (US) (Stout et al., 2009), being also recently detected in both urban (Ccanccapa et al., 2015; Weldon et al., 2011) and rural areas (Page et al., 2014), so that implying a pervasive pattern of exposure. Although CPF may be absorbed by inhalation or through the skin, dietary intake appears to be the most common source of exposure for the general population (Lu et al., 2008). The US Environmental Protection Agency stated a reference value for a typical CPF daily intake of 0.005μg/kg/day in adults, below which no deleterious effects on human health are expected (Eaton et al., 2008). Nonetheless, the additive effect of all routes of exposure, as well as the variety of human behaviours and activities make difficult to estimate the total daily exposure to the pesticide (Saunders et al., 2012).

A constellation of epidemiological investigations has demonstrated that OPs induce deficits in cognitive processes, such as sustained attention, memory, and processing speed (De Silva et al., 2006; Mackenzie Ross et al., 2010; Miyaki et al., 2005; Roldán-Tapia et al., 2005). Consistently, data from animal models of acute or repeated CPF exposure highlighted learning and memory impairments (López-Granero et al., 2014; Peris-Sampedro et al., 2015a, 2014; Salazar et al., 2011), deficits in sustained attention (Middlemore-Risher et al., 2010; Samsam et al., 2005), destabilized inhibitory control (Middlemore-Risher et al., 2010; Montes de Oca et al., 2013), and anhedonia (Aldridge et al., 2005).

Once CPF has entered the body, it undergoes an oxidative desulfuration to its active metabolite CPF-oxon, which expresses a potent anticholinesterase activity. The inhibition of cholinesterases (ChE) elicits the accumulation of acetylcholine (ACh) at the synapses of both the central and peripheral nervous systems (CNS, PNS), leading ultimately to acute cholinergic neurotoxicity (Chen, 2012). In addition, an increasing number of reports have endorsed the involvement of other neurotransmitter systems, such as the GABAergic system, in the

neurotoxicity of CPF (Cardona et al., 2006; Montes de Oca et al., 2013; Sánchez-Amate et al., 2002). Thus, Cardona et al. (2006) reported that the administration of diazepam – a GABAergic agonist – potentiates the long-term CPF-related effects observed in a schedule-induced polydipsia paradigm in rats. Interestingly, recent data have claimed for a neglected role of GABA in impulsivity (Hayes et al., 2014).

In 1983, Robbins and co-workers designed a test to assess attentional processes in rats, which was based on the continuous performance task used for the same purpose in humans (Robbins, 2002). Nowadays, the 5-choice serial reaction time task (5-CSRTT) enables various aspects of performance to be assessed simultaneously (Bari et al., 2008; Sanchez-Roige et al., 2012). To date, only two studies have used this paradigm to evaluate the detrimental effects of CPF on cognition (Middlemore-Risher et al., 2010; Montes de Oca et al., 2013). Both studies, carried out in male rats, found disturbed inhibitory control in the short (Middlemore-Risher et al., 2010) and the long-term (Montes de Oca et al., 2013) after relatively high doses of CPF. Moreover, Middlemore-Risher et al. (2010) also reported impairments in sustained attention with no signs of altered motivation that were still evident one month after the intoxication.

Although apolipoprotein E (apoE) synthesis is largely hepatic, it is also produced in the brain, primarily by astrocytes (Poirier et al., 2014). In addition to its well-characterized role in maintaining lipid homeostasis, apoE also contributes to several neurological phenomena in the CNS (Hauser et al., 2011), and its three major isoforms (apoE2, apoE3 and apoE4) confer different neurobehavioural attributes on their carriers. Although other mammals express apoE, allelic variation (ε2, ε3 and ε4) is unique to humans. Sullivan et al., (1997) designed the apoE targeted replacement (TR) mouse model by replacing the murine apoE gene by one of the three human APOE allelic variants, thus allowing them to systemically express functional human apoE isoforms.

Learning and memory processes have widely been studied in apoE TR mice (Bour et al., 2008; Grootendorst et al., 2005; Peris-Sampedro et al., 2015a; Reverte et al., 2013, 2012). However, there is still considerable uncertainty about the extent to which APOE genotype contributes to other cognitive and behavioural processes, such as sustained attention, inhibitory control and motivation. Most studies have focused on deciphering the behavioural attributes inherent to the APOE4 genotype, since it is the largest genetic risk for Alzheimer's disease (AD) (Raber et al.,

2004). Particularly, the APOE4 genotype has traditionally been associated with poor cognitive outcome (Peris-Sampedro et al., 2015a; Reverte et al., 2012; Siegel et al., 2012), which has sometimes been attributed to a hypothetical cholinergic dysfunction (Yun et al., 2005). Furthermore, recent experimental evidence has revealed that only the APOE4 genotype confers on its carriers deficient inhibitory control and impaired attentional accuracy on the 5-CSRTT (Reverte et al., 2015). On the other hand, the most common isoform in humans, apoE3, has recently been linked to an increased risk of developing obesity and a diabetic profile upon exposure to CPF (Peris-Sampedro et al., 2015a, 2015b). In this regard, a growing body of evidence has considered both impulsivity and compulsivity as potential feeding behaviour disruptors contributing to the obesity epidemics (Schag et al., 2013; Smith and Robbins, 2013).
To the best of our knowledge, no information is available on the use of the 5-CSRTT to assess the impact of dietary exposure to CPF on attention, inhibitory control and motivation in apoE TR mice. Hence, this investigation seeks (a) to determine whether CPF alter the 5-CSRTT baseline performance of apoE TR female mice previously trained, (b) to investigate whether such CPF-related effects persist over time, and (c) to assess the extent to which human APOE genetic variations modulate the effects of both CPF and alprazolam.

2. Material and methods

2.1 Animals and care

Adult apoE TR female mice, homozygous for the human ε2, ε3 or ε4 alleles, were purchased from Taconic (Taconic Europe, Lille Skensved, Denmark). They were housed in pairs under a 12-h light-dark cycle (lights off at 8 pm) in an environmentally controlled room held at 22±2°C and at a relative humidity of 50%±10%. Food (Panlab standard rodent chow, Barcelona, Spain) and water were available ad libitum. Before the behavioural task started, mice were gradually food deprived to approximately 80-85% of their free feeding weight. These feeding conditions were maintained until the end of the study. All experiments took place five days a week and were carried out during the light phase (Reverte et al., 2015). Five animals failed to reach criterion performance and were excluded from the 5-CSRTT training (apoE2 = 1, apoE3 = 3, apoE4 = 1).

Experimental procedures were conducted in accordance with the Animal Care and Use Committee of the Rovira i Virgili University (Tarragona, Spain). Likewise, in conformity with the Spanish Royal Decree 53/2013 and the European Communities Council Directive (86/609/EEC) efforts were made to alleviate animal suffering.

2.2 Drugs

Standard rodent chow was supplemented with CPF (purity 99.5%, Sigma-Aldrich, Seelze, Germany) at a concentration intended to deliver a dose of 3.75mg/kg body weight/day (see 2.4.1 for further information about the dosing procedure). The dose of CPF was chosen on the basis of earlier work (Peris-Sampedro et al., 2015a, 2015b), being expected to be below the range of non-observed cholinergic effects. The GABAergic agonist alprazolam was supplied by Pfizer (Pfizer, S.A., Alcobendas, Spain) and was used for the pharmacological challenge at a dose of 0.12mg/kg (Reverte et al., 2015).

2.3 Five-choice serial reaction time task (5-CSRTT)

2.3.1 Apparatus

The behavioural training was carried out in two identical acrylic operant chambers (24x20x15cm) (Med Associates Inc., St. Albans VT, USA), provided with steel grid floors and enclosed in ventilated wooden sound-attenuating boxes. Each chamber consisted of a curved aluminium wall containing nine equally-spaced holes. Four of the initial round apertures were closed off with metal inserts. Thus, only five evenly-spaced 2.5cm holes were operative and equipped with infrared detectors and a bright yellow led (1.7W) at the rear. The magazine, located centrally in the opposite metallic wall, was equipped with an infrared detector and automatically delivered 0.01ml of grape juice (commercially available grape juice containing 15.13% sugar, López Morenas, SL, Spain) via a liquid dispenser. The record of the behavioural task was controlled by a Fader Control interface and MED-PC software (Med Associates Inc., St. Albans VT, USA).

2.3.2 5-CSRTT training

Pre-training and training procedures in the 5-CSRTT were performed as previously described (Reverte et al., 2015). Briefly, mice were first habituated to the liquid reinforcer and 5-CSRTT chambers. Then, two 20-min pre-training stages were established to gradually introduce the mice to the task. In these phases, animals were required to learn to poke their noses into an illuminated hole in order to trigger a reward in the magazine entry. Subsequently, they were progressively trained to detect a brief visual stimulus presented pseudo-randomly in one of the five operating holes. The stimulus duration (SD) was reduced from 30 to 1s throughout 10 acquisition stages. A nose-poke into the illuminated magazine initiated each trial, which consisted of a fixed 5-s inter-trial interval (ITI) set prior the random presentation of the visual stimulus. If the mice responded in the illuminated hole within the SD or before the end of the limited hold (LH) (5s), 0.01ml of grape juice was delivered in the magazine dispenser and a correct trial was recorded. On the contrary, they were not rewarded when incorrect responses (nose-pokes made within a non-illuminated aperture), omissions (failure to respond within the SD and/or the LH) or premature responses (responses made in any of the five holes during the ITI) were recorded. Furthermore, these responses were all punished with a 5-s brightness period (time-out, TO), in which no new trials could be started. Additional responses in a hole after a correct response and before the reinforcer collection (perseverative responses) were also recorded, but were not punished. In either case, each training session lasted for 20min or a maximum of 70 discrete trials. Once the mice reached a stable baseline performance for 5 consecutive days (final parameters: SD = 1s, ITI = 5s, LH = 5s, TO = 5s; criteria: > 50% of the total trials, > 80% accuracy, < 25% omissions), they were tested under experimental conditions involving behavioural manipulation and pharmacological challenge.

2.3.3 Behavioural manipulations

The behavioural testing was initiated after successful and stable acquisition of the task and lasted for 4 weeks. In brief, impulsivity and attentional performance were estimated once a week (i.e., Wednesday) for two consecutive weeks, by increasing the ITI (7s) and shortening the

SD (0.5s), respectively (Reverte et al., 2015; Sanchez-Roige et al., 2012). Every other day of the week, mice were trained with standard baseline parameters (for more details, please refer to Reverte et al., 2015).

2.3.4 Pharmacological challenge

Following behavioural manipulations, we tested the effects of the GABAergic agonist alprazolam on the 5-CSRTT performance. Twice a week, typically on Wednesday and Friday, alprazolam was i.p. injected 30min before the 5-CSRTT training. To provide a control injection condition, 0.9% saline was i.p. injected on Tuesday and Thursday 30min before the training started. In either case, mice were trained with standard baseline parameters.

2.4 Treatment procedures

After both behavioural and pharmacological challenges, mice were trained in standard conditions in order to restore their baseline level. A total of 20 female mice were exposed to CPF (apoE2 = 8, apoE3 = 6, apoE4 = 6). At the beginning of the treatment, the mean ages of apoE2, apoE3 and apoE4 mice were 11.19±0.53, 12.25±0.25 and 13.17±1.01 months, respectively.

2.4.1 Effects of repeated exposure to CPF on 5-CSRTT performance, impulsive condition, sustained attention and GABAergic system functioning

To investigate the effects of repeated exposure to CPF, mice were fed a supplemented rodent chow for 4 consecutive weeks. Specifically, the daily intake of CPF was estimated to be 0.075mg, which corresponds to a dose of 3.75mg/kg body weight/day. When administration of CPF began, animals were assessed daily for signs of acute cholinergic toxicity. Throughout the treatment period, mice continued to perform the task daily with standard baseline parameters, allowing the assessment of the CPF impact on their basal 5-CSRTT performance.

To determine whether CPF exposure elicited impulsive responding or impaired sustained attention, behavioural manipulations were carried out once a week during the last two weeks of

treatment, typically on Wednesday. In particular, the session in which the ITI was increased (7s) took place in the third week, while the SD was shortened (0.5s) in the fourth week.
In order to provide further insight into the interaction between the GABAergic and cholinergic systems, mice were subjected to a single i.p. dose of alprazolam, administered 30min before the last session started (i.e., Friday).

2.4.2 Assessment of the recovery period

A 4-week wash-out period followed CPF exposure. Mice were again fed standard rodent chow and subjected to daily 5-CSRTT training with standard baseline parameters. The ability of the mice to overcome the treatment with CPF was then assessed by analysing their task performance. We used the same procedure above described (see 2.4.1) to investigate whether both impulsive and attentional statuses returned to their previous state by manipulating both the ITI and the SD.

2.4.3 Cholinesterase (ChE) activity assessment

ChE activity was tested in plasma and frontal cortex in a second cohort of naïve females. Mice (n = 18) were distributed into three groups, according to the experimental conditions of the study: controls (n = 2/genotype), CPF exposure (n = 2/genotype), and wash-out (n = 2/genotype). Brain ChE activity was determined in all these individuals, while plasma ChE activity was randomly assessed in 6 mice as an indicator of acute systemic CPF effect (controls = 3, CPF-exposed = 3) (Eaton et al., 2008; Peris-Sampedro et al., 2015a). Enzymatic assays procedures, as well as detailed description of sample processing can be found elsewhere (Montes de Oca et al., 2013; Peris-Sampedro et al., 2015a, 2015b; Salazar et al., 2011). Briefly, at the end of each treatment condition, mice were anesthetized with carbon dioxide before being euthanized. Blood was obtained by cardiac puncture and immediately centrifuged to obtain plasma, which was stored at -80ºC until use. After the blood draw, mice were rapidly decapitated and the whole brains were removed, dissected, homogenized and ultimately centrifuged. In both cases, enzyme activity was determined spectrophotometrically using the Ellman method (Ellman et al.,

1961), and was calculated relative to protein concentration contained in the sample using the Bradford method (Bradford, 1976). Finally, ChE activity of exposed animals was estimated on the basis of the activity value of the control mice, and represented as a percentage.

2.5 Data collection

The following variables were recorded throughout the experimental procedures. Attentional performance was assessed by the percentage of accuracy (number of correct responses divided by the sum of correct and incorrect responses x 100), the percentage of omissions (number of omissions divided by the total number of trials completed x 100) and by a measure of processing speed (correct latency: time required to respond correctly after the onset of the stimulus) (Sanchez-Roige et al., 2012). To evaluate inhibitory control, both impulsivity and compulsivity were recorded in terms of percentage of premature responses (premature responses divided by the total number of trials completed x 100) and perseverative responses (number of reiterative responses made into the holes after a correct response), respectively (Sanchez-Roige et al., 2012). The total number of trials (the sum of correct, incorrect and omitted responses), and the reward latency (time needed to retrieve the reward after a correct response) were recorded as motivational parameters (Dalley et al., 2007). The total number of trials was not analysed for the sessions in which the ITI was lengthened because the session duration was increased to 25 min to ensure mice had enough time to perform at least 50% of the trials.

2.6 Statistical analyses

Data processing was performed using the SPSS statistical package (version 20.0). We used one-way analysis of variance (ANOVA) (genotype) to determine the number of sessions required at each stage of the training. The animals performance on the 5-CSRTT during baseline, CPF exposure, and wash-out period, as well as data from the behavioural and pharmacological challenges in each treatment condition were all analysed by one-way repeated-measures (RMANOVA). The genotype was used as the between-subject factor, while weeks or days were

used as the within-subject factor. A two-way ANOVA was performed to establish the contribution of both CPF exposure and APOE genetic background to brain ChE activity. Plasma ChE activity was analysed by means of one-way ANOVA (CPF treatment). When appropriate, Tukey's post-hoc comparisons were used. Statistical significance was set at $p < 0.05$, and results are reported as mean values ±SE.

3. Results

3.1 Acquisition of the 5-CSRTT

During acquisition, all mice progressed at similar rates during the first four training levels. However, when attentional demands were higher, some differences between genotypes emerged. We observed that the genotype affected the average number of sessions required to achieve the performance criterion on training stages 5 [$F_{2, 19} = 13.778$, $p < 0.001$], 6 [$F_{2, 19} = 6.966$, $p = 0.006$], 7 [$F_{2, 19} = 12.242$, $p = 0.001$], 8 [$F_{2, 19} = 8.440$, $p = 0.003$], and 9 [$F_{2, 19} = 7.105$, $p = 0.006$] (Fig. 1). ApoE2 mice were generally faster learners than both apoE3 (stages 5 to 9, $p < 0.05$) and apoE4 (stages 7 to 9, $p < 0.05$). Ultimately, mice fully acquired the task, and there were no differences between genotypes in the total number of sessions they took (Fig. 1). Moreover, once the baseline state was reached, the animals attained and maintained high levels of accuracy (Fig. 2A).

- Insert Figure 1 over here –

3.2 5-CSRTT performance under standard conditions

3.2.1 Baseline

The baseline performance on the 5-CSRTT is illustrated in Figure 2 and Table 1. No differences between genotypes were noted for the percentages of accuracy (Fig. 2A), omissions (Fig. 2B) and premature responses (Fig. 2C), nor for the total number of trials and correct latency (data

not shown). In contrast, the genotype did affect perseverative responding [$F_{2, 19} = 5.833$, $p = 0.012$]. ApoE4 mice persevered more than apoE2 ($p = 0.011$) and apoE3 mice ($p = 0.053$) (Fig. 2D). The apoE4 group also showed an upward trend in latency to collect the reward [genotype: $F_{2, 19} = 3.435$, $p = 0.056$], which might be partly explained in terms of increased perseverative responding. Post-hoc analyses revealed that apoE2 mice were faster at retrieving the reward than apoE4 ($p = 0.054$) (Table 1).

- Insert Figure 2 over here –

- Insert Table 1 over here –

3.2.2 CPF exposure

The exposure to CPF led to a progressive reduction in both the percentage of premature responses made [weeks: $F_{3, 19} = 3.571$, $p = 0.040$] and the total number of trials completed [weeks: $F_{3, 19} = 8.631$, $p = 0.001$] (data not shown). No differences between genotypes were observed in any of the 5-CSRTT performance variables measured. Therefore, the effect of the genotype previously reported on perseverative responses during baseline was not noted during the exposure period.

3.2.3 CPF-free wash-out period

During the 4-week wash-out period there was a gradual recovery of both affected parameters: the percentage of premature responses [weeks: $F_{3, 19} = 11.919$, $p < 0.001$] and the total number of trials [weeks: $F_{3, 19} = 3.960$, $p = 0.029$] (data not shown). As previously found during CPF exposure, the genotype did not affect any of the performance variables measured.

3.3 Longitudinal characterization of the intoxication and detoxification periods

In order to assess the potential impact of exposure to dietary CPF on the 5-CSRTT baseline performance, we looked at the progression in mice performance over the three treatment

conditions (Fig. 3). The genotype did not show any significant effect on the variables analysed. Considering the three experimental phases as a longitudinal study, we found that five variables varied: omissions [$F_{2, 19} = 11.158$, $p = 0.001$] (Fig. 3B), correct latency [$F_{2, 19} = 6.876$, $p = 0.007$] (Fig. 3F), premature responses [$F_{2, 19} = 4.738$, $p = 0.024$] (Fig. 3C), total number of trials [$F_{2, 19} = 11.460$, $p = 0.001$] (Fig.3E), and reward latency [$F_{2, 19} = 16.091$, $p < 0.001$] (Fig. 3G). In agreement with the results presented in the above subsections (see 3.2.2 and 3.2.3), an interaction time x period was found for the percentage of premature responses [$F_{6, 19} = 5.700$, $p = 0.005$], and the total number of trials [$F_{6, 19} = 4.670$, $p = 0.011$].

- Insert Figure 3 over here –

As for attentional performance, post-hoc analyses revealed that CPF exposure resulted in a significant increase in omissions compared to the baseline period ($p < 0.001$) (Fig. 3B). Moreover, CPF also worsened the processing speed, manifested by higher correct latencies ($p = 0.009$) (Fig. 3F). Both parameters were not recovered during the wash-out period (Fig. 3B, 3F).
With regard to inhibitory control, the exposure to CPF significantly reduced the percentage of premature responses compared to baseline ($p = 0.005$) (Fig. 3C).
In terms of motivation, mice exposed to CPF appeared to be less likely to perform the 5-CSRTT. In particular, CPF-fed mice completed fewer trials than at baseline ($p < 0.001$) (Fig. 3E), and were slower to retrieve the subsequent reward ($p = 0.001$) (Fig. 3G). These two parameters not only remained unchanged during wash-out, but also continued to increase in the case of reward latency (CPF vs wash-out, $p = 0.031$) (Fig. 3E, 3G).

3.4 Behavioural manipulations

Figure 4, Figure 5 and Table 2 provide an overview of the behavioural effects on the 5-CSRTT described for the three genotypes throughout the challenge sessions.

3.4.1 Inter-trial interval challenge

Baseline: Significant increases in both omissions [day: $F_{1, 16} = 5.292$, $p = 0.037$] (Fig. 4B) and premature responses [ITI: $F_{1, 16} = 19.308$, $p = 0.001$] (Fig. 4C) were observed when the ITI was increased from 5- to 7-s. We also found that the genotype considerably affected premature responses [$F_{2, 16} = 6.943$, $p = 0.008$], with a trend towards a significant ITI x genotype interaction [$F_{2, 16} = 3.474$, $p = 0.060$]. Further analyses revealed that apoE4 mice generally showed higher premature responding relative to apoE2 ($p = 0.009$) and apoE3 mice ($p = 0.042$) (Fig. 4C). Specifically, after lengthening the ITI, apoE4 mice continued to display more premature responses than apoE2 mice ($p = 0.012$) (Fig. 4C).

CPF exposure: CPF counteracted the increase in omissions caused by lengthening the ITI during the baseline period (Fig. 4B). Although significant increases in premature responding were again found as a result of lengthening the ITI [ITI: $F_{1, 19} = 14.041$, $p = 0.002$], the exposure to CPF neutralized the effect of the genotype noted at baseline (Fig. 4C). Furthermore, the correct latency decreased during the ITI challenge in the treatment period [ITI: $F_{1, 19} = 9.741$, $p = 0.006$] (Table 2).

Wash-out: Just as during CPF treatment, manipulating the ITI had no effect on the percentage of omissions during wash-out (Fig. 4B). As was the case in the other two periods, significant increases in premature responding were again found after lengthening the ITI [ITI: $F_{1, 19} = 7.705$, $p = 0.013$] (Fig. 4C). Similarly to CPF exposure, mice subjected to a challenged ITI showed reduced correct latencies during wash-out [ITI: $F_{1, 19} = 12.199$, $p = 0.003$] (Table 2). We observed a main effect of the genotype on accuracy [$F_{2, 19} = 11.492$, $p = 0.001$], which was not found for the other two periods (Fig. 4A). A post-hoc analysis indicated that accuracy in apoE4 mice decreased more steeply than in apoE2 ($p = 0.001$) and apoE3 ($p = 0.010$).

- Insert Figure 4 over here –

- Insert Table 2 over here –

3.4.2 Stimulus duration challenge

Baseline: A significant decrease in accuracy [SD: $F_{1, 14} = 25.544$, $p < 0.001$] (Fig. 5A) and an increase in omissions [SD: $F_{1, 14} = 22.125$, $p = 0.001$] (Fig. 5B) were observed when the SD was reduced from 1- to 0.5-s. Moreover, correct latencies fell throughout the SD challenge [SD: $F_{1, 14} = 13.757$, $p = 0.003$] (Table 2). We also found that the genotype mainly affected accuracy [$F_{2, 14} = 4.688$, $p = 0.031$] and the total number of trials [$F_{2, 14} = 6.707$, $p = 0.011$]. Further analyses revealed a more pronounced drop in accuracy in apoE4 mice than in apoE2 ($p = 0.026$) (Fig. 5A), and generally fewer completed trials in the apoE4 group relative to the apoE2 group ($p = 0.010$) (data not shown).

CPF exposure: Overall, manipulating the SD during CPF exposure period did not affect accuracy (Fig. 5A), omissions (Fig. 5B), or latency to respond correctly (Table 2). Furthermore, CPF neutralized the effect of the genotype found at baseline on both accuracy (Fig. 5A) and the total number of trials (data not shown). The SD challenge during the exposure to CPF, however, led to a decline in perseverative responses [$F_{1, 19} = 6.172$, $p = 0.024$] (Fig. 5D).

Wash-out: As at baseline, a significant decrease in accuracy [SD: $F_{1, 19} = 11.211$, $p = 0.004$] (Fig. 5A) and an increase of omissions [SD: $F_{1, 19} = 8.354$, $p = 0.010$] (Fig. 5B) were again found when the SD was reduced from 1- to 0.5-s. The genotype significantly influenced the total number of trials performed [$F_{2, 19} = 6.756$, $p = 0.007$], and tended to do so for percentage of omissions [$F_{2, 19} = 2.944$, $p = 0.080$]. Overall, further post-hoc analyses pointed to a markedly deterioration in performance in apoE3 mice: they completed fewer trials ($p = 0.005$) (data not shown) and made more omissions ($p = 0.041$) than apoE2 mice (Fig. 5B).

- Insert Figure 5 over here –

3.5 Pharmacological challenge

Figure 6 and Table 2 summarize the behavioural effects on the 5-CSRTT described for the three genotypes throughout the administration of the GABAergic agonist alprazolam.

Baseline: Alprazolam improved overall performance. Specifically, it increased the number of trials completed [day: $F_{1, 12} = 14.980$, $p = 0.003$] (Table 2), decreased omissions [drug: $F_{1, 12} = 68.273$, $p < 0.001$] (Fig. 6B) and decreased both correct [drug: $F_{1, 12} = 3.876$, $p = 0.077$] and reward latencies [drug: $F_{1, 12} = 6.280$, $p = 0.031$] (Table 2). However, it increased premature responding [drug: $F_{1, 12} = 13.427$, $p = 0.004$] (Fig. 5C). The genotype was also observed to have an effect on perseverative responding [$F_{2, 12} = 6.436$, $p = 0.016$]: apoE4 mice persevered more than apoE2 ($p = 0.038$) and apoE3 ($p = 0.020$) mice, as already observed under standard conditions at baseline (Fig. 6D, 2D). However, no interaction genotype x alprazolam was observed.

CPF exposure: During the treatment period, alprazolam-associated improvements were more discreet. Although omissions continued to decrease [drug: $F_{1, 19} = 6.486$, $p = 0.021$] (Fig. 6B), no effect was observed on either correct or reward latencies, or on the total number of trials (Table 2). Alprazolam continued to increase premature responding in CPF-exposed mice [drug: $F_{1, 19} = 9.668$, $p = 0.006$]. As above noted, no interaction genotype x alprazolam was observed.

- Insert Figure 6 over here –

3.6 ChE activity

During the course of CPF exposure, we noticed no apparent signs of cholinergic toxicity in any group. Relative to controls, plasma ChE activity of CPF-exposed animals dropped to 22.06%. On the other hand, ChE activity in brain homogenates assessed immediately after the 4-week exposure to CPF was decreased to 76.54% of controls, and was totally recovered after the incorporation of the 4-week wash-out period. No differences between genotypes were observed in brain ChE activities.

4. Discussion

The aim of the current study was primarily to characterize the immediate and delayed effects caused by the exposure to CPF on attention, inhibitory control and motivation in pre-trained human apoE TR adult female mice. Although all the individuals eventually acquired the task, ε2 carriers learned it more efficiently than their peers. The increases in premature and perseverative responses found in the baseline performance analysis were genotype-dependent, pointing to deficient inhibitory control in apoE4 mice. Strikingly, this impulsive- and compulsive-like trait was no longer found during the CPF treatment, suggesting a specific interaction between the ChE inhibitor agent and the APOE4 genotype. Overall, the 4-week dietary administration of CPF, devoid of signs of cholinergic toxicity, gradually compromised attentional accuracy and motivation, while it reduced premature responding. These effects persisted over time, as they were mostly maintained throughout the 4-week wash-out period, even if brain ChE activities were totally recovered. Furthermore, and contrary to expectations, apoE3 mice showed attentional disruptions due to motivational factors one month after CPF exposure. The pharmacological challenges with the GABAergic agonist alprazolam, covering both baseline and CPF exposure periods, improved overall performance and generally increased impulsivity. However, interactions with the APOE genotype were not observed.

In this study, all the mice were able to cope with the 5-CSRTT. Moreover, as in previous investigations (Reverte et al., 2015; Siegel et al., 2010), there were no distinguishable differences in accuracy between genotypes after they reached the required level. Notwithstanding, throughout the acquisition process the rates of learning were different, with the APOE2 genotype being the most gifted learner. These results match those recently reported by Reverte et al. (2015), and seem to further support the idea of an enhanced learning process among apoE2 female mice in the 5-CSRTT. Nevertheless, acquisition in apoE2 male mice was also faster than in the two other human apoE TR groups in a Barnes maze spatial task (Peris-Sampedro et al., 2015a). Thus, ε2 carriers were the most frequent users of direct or serial pathways to the target hole, which implied they made a reflective choice (Peris-Sampedro et al., 2015a). In addition, apoE2 female mice exhibited the lowest reward latencies during the baseline period in the 5-CSRTT, indicating increased motivation. In a recent study carried out in our laboratory (Reverte et al., 2012), apoE2 female mice showed sustained exploratory behaviour in an open-field task. Despite the paucity of epidemiological data, the aforementioned

results suggest an advantageous cognitive outcome of the APOE2 genotype. Learning to associate specific actions with rewards and being able to remember them are higher-order executive functions mostly dependent on prefrontal cortex (PFC) areas (Puig et al., 2014), which are highly innervated by dopaminergic neurons. Indeed, dopamine (DA) is involved in motor and reward systems and it contributes to adaptive behaviours, such as attention, learning and motivation (Nieoullon, 2002; Tye et al., 2012). It has been further shown that its depletion from PFC triggers poor attentional outcome and impulsivity (Puig et al., 2014; Puumala and Sirviö, 1998). Recently, Reverte et al. (2015) found that DA levels in the frontal cortex were higher in apoE2 than in apoE4 mice. Hence, increased basal levels of DA in certain brain areas intrinsic to the APOE2 genotype may account for its privileged cognitive condition.

In agreement with our recent study (Reverte et al., 2015), the APOE genotype strongly influenced the baseline period in the 5-CSRTT. ApoE4 mice displayed impaired inhibitory control, manifested by higher levels of premature and perseverative responses, and also a decrease in accuracy when attention was challenged. In recent years, and despite some controversy, the hypothesis that a cholinergic dysfunction explains some of the cognitive deficits associated with the APOE4 genotype has gained strength. In relation to this, higher levels of AChE (Eggers et al., 2006), a greater number of muscarinic receptors (mAChRs) (Cohen et al., 2003) and a reduced activity of cholinergic neurons (Salehi et al., 1998) have been considered as potential contributors to their cognitive failure. The current results suggest that CPF was able to match the attentional ability of the three genotypes, thereby mildly improving the deteriorated attentional condition of the APOE4 genotype. Besides, existing research recognizes the critical role played by the cholinergic system in impulsivity (Cardona et al., 2006; Middlemore-Risher et al., 2010; Montes de Oca et al., 2013), but the underlying mechanisms are not yet fully understood. In the present investigation, apparently unprecedented, we found that CPF was surprisingly able to restore the impaired inhibitory control inherent to apoE4 mice. Similarly, being carrier of the ε4 allele has been shown to condition the response to other cholinergic agents. Thus, ChE inhibitors used in the treatment of AD seem to be less effective in APOE4 patients (Braga et al., 2014), and CPF itself impaired memory in apoE3 but not in apoE4 mice (Peris-Sampedro et al., 2015a). In our recent study (Reverte et al., 2015), apoE4 mice were the least affected by scopolamine - a muscarinic antagonist -, while ε3 carriers showed increased

premature responding upon its administration. Recently, Potter et al., (2012) pointed out that the inhibitory behaviour of highly impulsive human subjects was improved by the interaction of nicotine with nAChRs. Interestingly, carriers of the apoE4 isoform benefit more from the improving effect of nicotine on cognition (Evans et al., 2013). Based on the above, it could be hypothesized that CPF improve the poor inhibitory control of apoE4 mice upon interacting with nAChRs.

The anxiogenic character of the APOE4 genotype has extensively been addressed (Reverte et al., 2014; Siegel et al., 2012), and may partly explain its impulsive condition in the 5-CSRTT (Loos et al., 2009). Several studies have used GABAergic pharmacological approaches to test the hypothesis that impulsivity is associated with anxiety (Sanchez-Roige et al., 2012). However and just as previously reported (Reverte et al., 2015; Stonnington et al., 2009), ε4 carriers did not show higher response to benzodiazepines (e.g., alprazolam and lorazepam).

A review of the literature shows that the 5-CSRTT is a useful tool for assessing not only attentional and motivational processes but also inhibitory behaviours (Robbins, 2002; Sanchez-Roige et al., 2012). A considerable amount of epidemiological data has confirmed that CPF contributes to boosting neuropsychological and psychiatric impairments (Mackenzie Ross et al., 2010; Roldán-Tapia et al., 2005). However, experimental studies on attention, motivation and inhibitory control using the 5-CSRTT are scant and rather conflicting. As for attention, while Montes de Oca et al. (2013) found no variations seven months after a single dose of 250mg/kg CPF, Middlemore-Risher and co-workers (2010) revealed deficits during and after prolonged treatment with CPF. In the present investigation, repeated dietary exposure to CPF produced protracted attentional disturbances in apoE TR female mice that had stable baseline performance, as revealed by increased number of omissions and deteriorated processing speed. Despite differences in experimental protocols, our results partially agree with those reported by Middlemore-Risher et al. (2010) who found decreased accuracy and increased omissions throughout both 14-day and 30-day every other day exposures to 18mg/kg CPF. In line with our results, the effects they observed were maintained over a 30-day period of detoxification. The mechanisms by which CPF exerts its detrimental effect on attention may be multifactorial. For instance, several studies have attributed these effects to the ability of CPF to inhibit AChE (Middlemore-Risher et al., 2010; Samsam et al., 2005), but they also emphasized a possible role

of nAChRs (Hoyle et al., 2006; Middlemore-Risher et al., 2010). With regard to anticipatory behaviour, an array of research has demonstrated that CPF increases premature responding (Cardona et al., 2011, 2006; López-Granero et al., 2013; Middlemore-Risher et al., 2010). The current findings though, suggest that it has the opposite effect: the exposure to CPF gradually reduced premature responses in apoE TR female mice. It is worth noting that basal premature responses were notably low in these individuals. Accordingly, the level of premature responding in mice has been suggested to be lower than that seen commonly in rats (Humby et al., 1999). Nonetheless, the reasons for these discrepancies are not clear and deserve further investigation. One explanation may be the substantial differences between protocols. Indeed, all these studies were performed using male rats, while we used female mice. In addition, all but one (López-Granero et al., 2013) are based on single high (Cardona et al., 2011, 2006) or repeated relatively high (Middlemore-Risher et al., 2010) CPF doses. Furthermore, there are some differences between our dosing schedule and the others: CPF-related effects on the impulsive behaviour of the mice were evaluated once they had reached a stable baseline performance and they were followed up in the 5-CSRTT during exposure.

To date, little research has attempted to clarify the role of OPs in inducing motivational deficits. Epidemiological data on the subject reported emotional impairments (i.e., anxiety, depression, and irritability) as a consequence of occupational exposure to OPs (Bazylewicz-Walczak et al., 1999; Mackenzie Ross et al., 2010). In the present study, although food was restricted and a liquid reinforcer was used to prevent mice from becoming satiated, the reduction in the number of trials completed, as well as the increase in reward latencies here found indicate a loss of motivation in mice treated with CPF.

According to Middlemore-Risher et al. (2010), almost all the behavioural features described during the exposure to CPF in the 5-CSRTT were still apparent throughout the 4-week wash-out period. Strikingly, the APOE3 genotype developed delayed attentional impairments, probably due to motivational factors, during the course of the detoxification. In previous studies, we found that being carrier of the ε3 allele increases vulnerability to developing obesity and related metabolic dysfunctions after CPF exposure (Peris-Sampedro et al., 2015a, 2015b). Furthermore, apoE TR mice that had been exposed to CPF ate more than their control counterparts (Peris-Sampedro et al., 2015a, 2015b). It is difficult to establish whether there are any links between

the present and the previous results, and therefore they must be examined more closely in further investigations.

In recent years, there has been renewed interest in ascertaining the role of GABA in impulsivity (Hayes et al., 2014). Pharmacological interventions that target GABA receptors (e.g., benzodiazepines) have been shown to increase impulsive behaviour (Oliver et al., 2009). Moreover, alprazolam improves cognitive function in human volunteers (Bentué-Ferrer et al., 2001). The current results show that alprazolam improved the overall mice performance in the 5-CSRTT, notably by increasing the total number of trials and decreasing not only omissions but also both correct and reward latencies. Furthermore, the GABAergic agonist increased the percentage of premature responses. However, administering the drug during CPF treatment did not have any relevant effect.

In summary, the present study demonstrates that the APOE genotype affects attentional performance and inhibitory behaviour in the 5-CSRTT. Together with recent data (Peris-Sampedro et al., 2015a, 2015b), the current findings attest that the three apoE isoforms respond differently to a CPF challenge, highlighting the fact that genetics of the population must be taken into account in epidemiological studies. According to the current results, a fruitful area for further research should be to assess whether the three genotypes differ in terms of the brain expression and distribution of mAChRs and nAChRs. It would also be interesting to assess whether such genotype-related differences could elicit an array of therapeutic responses upon cholinergic treatment. The results of the present investigation provide some support for the conceptual premise of potential close links between the cholinergic system and the APOE4 genotype. Overall, the results described strengthen existing evidence, and point to protracted detrimental effects on sustained attention following repeated exposure to CPF. Furthermore, this research provides a framework for the exploration of motivational deficits that emerge after the administration of CPF, which could subsequently lead to appetite and emotional disturbances.

Acknowledgements

The authors would like to specially thank Jennifer Calzado and Phoebe Soebagyo for their helpful collaboration throughout the behavioural assessment. Likewise, the authors thank Laia Guàrdia for having skilfully helped in enzymatic determinations. This research was supported by the Ministry of the Economy and Competitiveness (MINECO, Spain) (grants number PSI2010-21743-C02-01 and PSI2014-55785-C2-2-R) and the Commission for Universities and Research of the Department of Innovation, Universities and Enterprise of the Generalitat de Catalunya (grant number 2013 FI_B00170).

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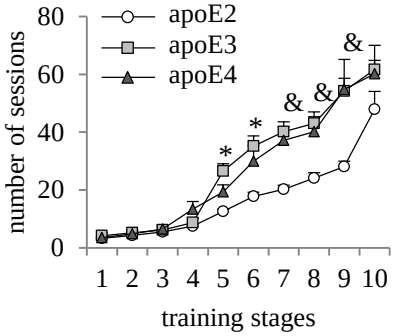
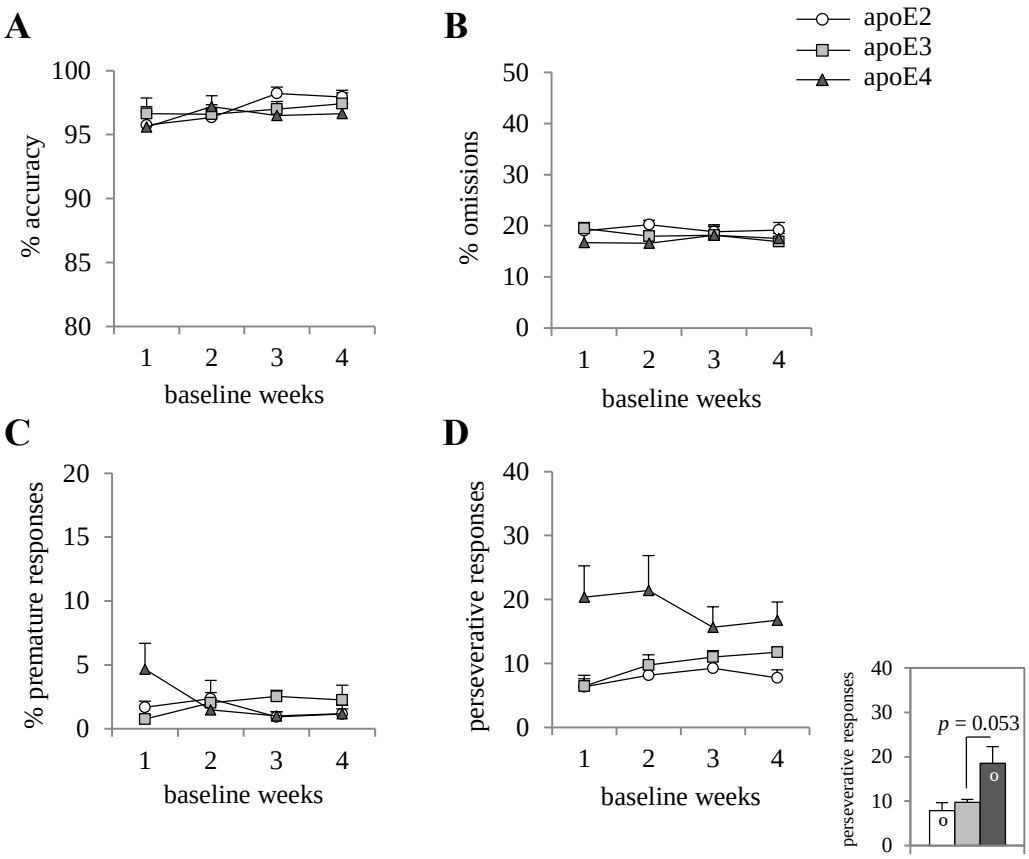
Figure captions

Figure 1



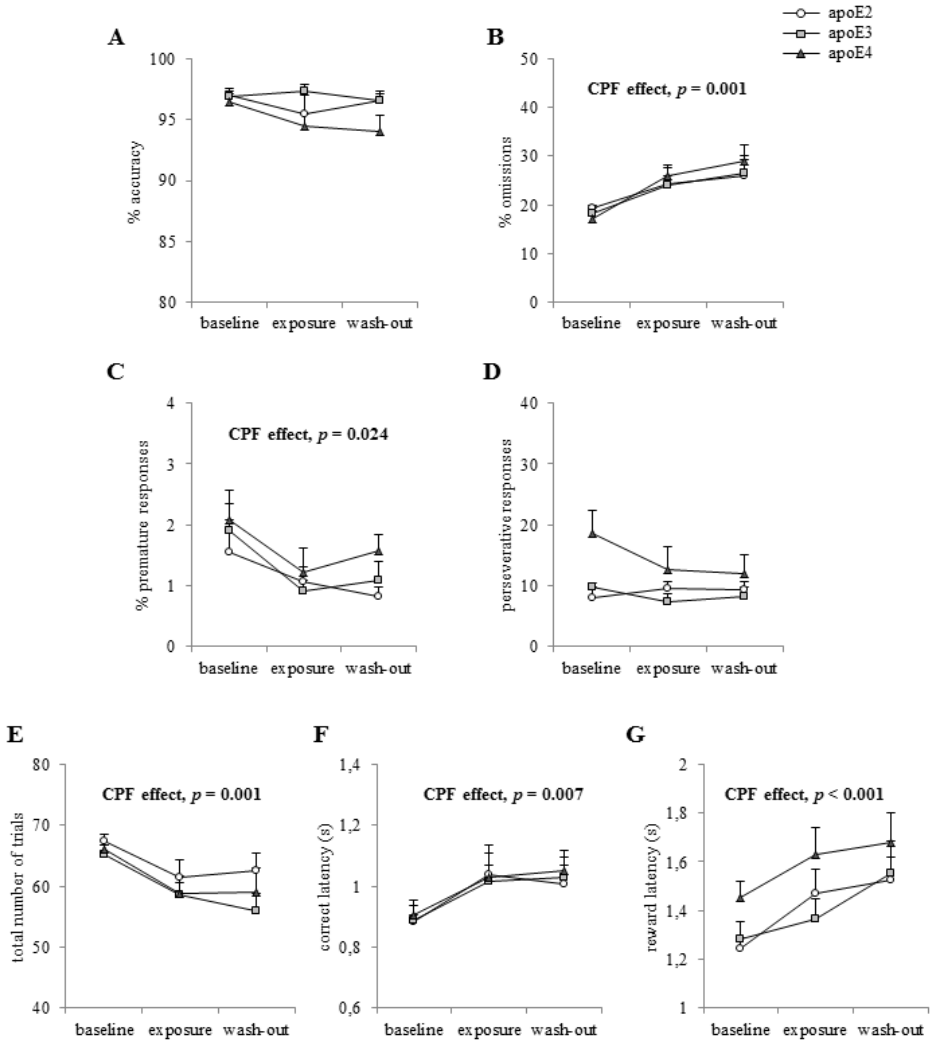
Cumulative representation of the total number of sessions required to successfully learn the 5-CSRTT throughout the 10 acquisition stages for each *APOE* genotype. Symbols indicate: apoE2 differs from apoE3 mice (*), and apoE2 differs from apoE3 and apoE4 mice (&) at $p < 0.05$.

Figure 2



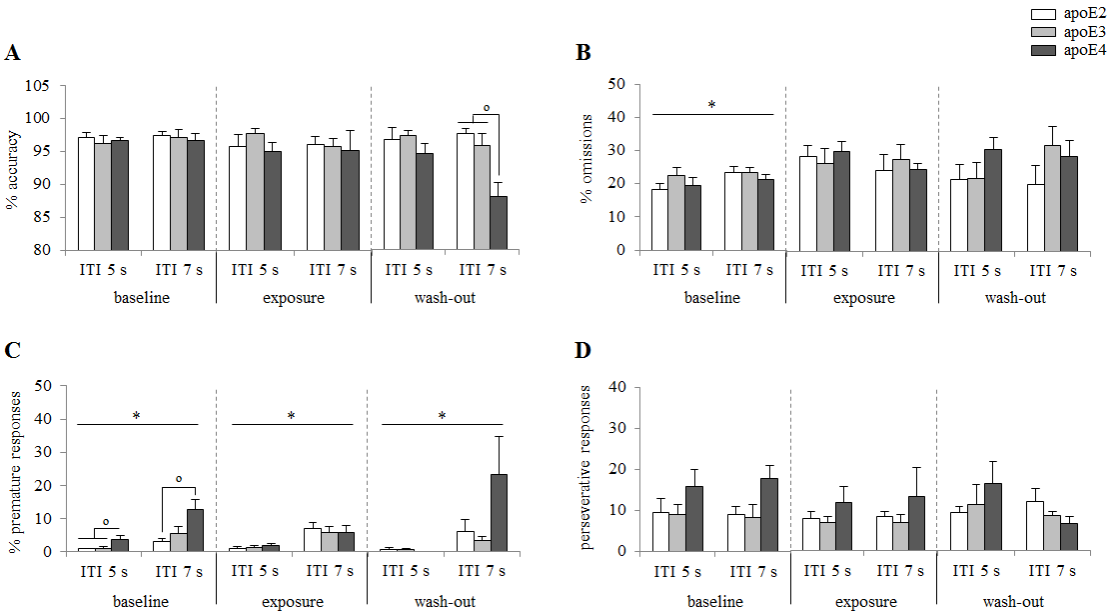
Baseline performance of apoE TR female mice on the 5-CSRTT under standard conditions. Percentage of accuracy (A), percentage of omissions (B), percentage of premature responses (C), and number of perseverative responses (D) are depicted. The symbol ° indicates differences between apoE2 and apoE4 mice at $p < 0.05$.

Figure 3



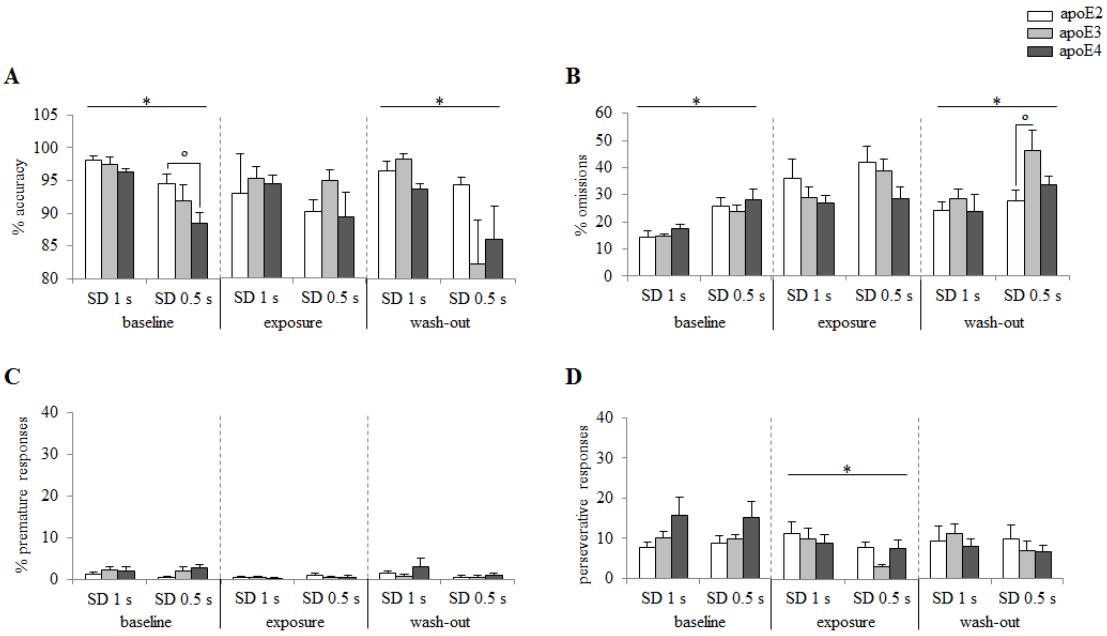
Performance progression of apoE TR female mice on the 5-CSRTT over the three experimental periods: baseline, CPF exposure, and wash-out. Percentage of accuracy (A), percentage of omissions (B), percentage of premature responses (C), number of perseverative responses (D), total number of trials (E), and both correct (F) and reward (G) latencies are illustrated.

Figure 4



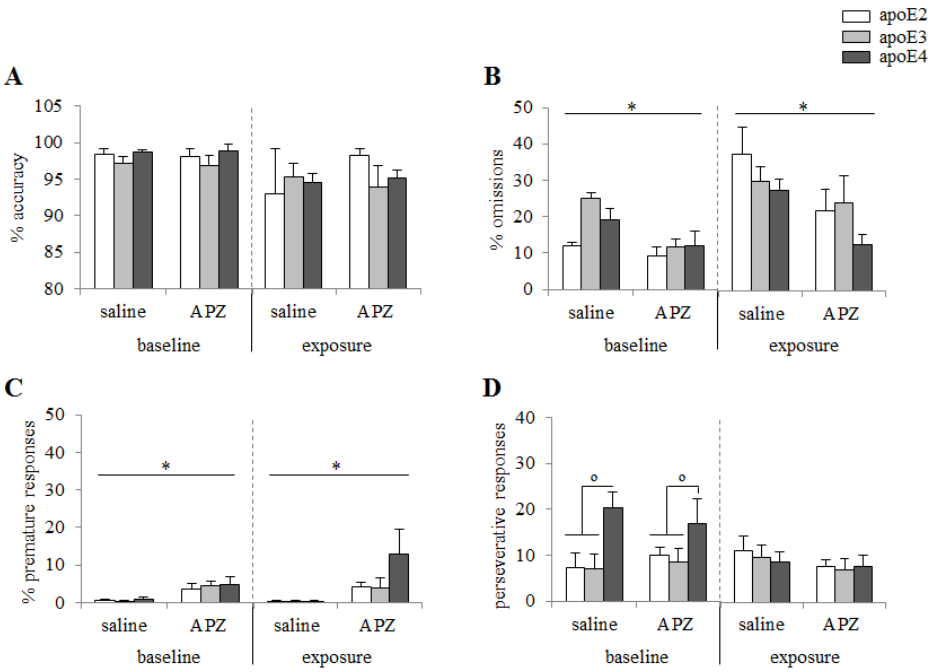
Effects of inter-trial interval (ITI) manipulation on the 5-CSRTT performance of apoE TR female mice for the three experimental periods: baseline, CPF exposure, and wash-out. Percentage of accuracy (A), percentage of omissions (B), percentage of premature responses (C), and number of perseverative responses (D) are depicted. Symbols indicate: differences between genotypes (°), and effects of the ITI increase (*) within each period at $p < 0.05$.

Figure 5



Effects of stimulus duration (SD) manipulation on the 5-CSRTT performance of apoE TR female mice for the three experimental periods: baseline, CPF exposure, and wash-out. Percentage of accuracy (A), percentage of omissions (B), percentage of premature responses (C), and number of perseverative responses (D) are depicted. Symbols indicate: differences between genotypes (°), and effects of the SD reduction (*) within each period at $p < 0.05$.

Figure 6



Effects of alprazolam administration on the 5-CSRTT performance of apoE TR female mice for the two periods considered: baseline and CPF exposure. Percentage of accuracy (A), percentage of omissions (B), percentage of premature responses (C), and number of perseverative responses (D) are illustrated. Symbols indicate: differences between genotypes (°), and effects of the GABAergic agonist (*) within each period at $p < 0.05$.

Tables

Table 1. Total mean number of trials and both correct and reward mean latencies in apoE TR female mice on the 5-CSRTT throughout the baseline period

	apoE2	apoE3	apoE4
total number of trials	67.47 ± 1.11	65.10 ± 1.62	66.13 ± 0.66
correct latency (s)	0.88 ± 0.02	0.89 ± 0.05	0.91 ± 0.05
reward latency (s)	1.24 ± 0.05	1.28 ± 0.07	1.45 ± 0.07†

† $p = 0.054$, indicating a tendency *vs.* the apoE2 group in reward latency.

Table 2. Motivational status and processing speed during behavioural and pharmacological challenges on the 5-CSRTT for the three experimental periods

		total number of trials			correct latency (s)			reward latency (s)		
		baseline	exposure	wash-out	baseline	exposure	wash-out	baseline	exposure	wash-out
ITI (s)										
	5	-	-	-	0.90 ± 0.03	**1.02 ± 0.06**	**1.04 ± 0.04**	1.37 ± 0.06	1.53 ± 0.10	1.65 ± 0.12
	7	-	-	-	0.85 ± 0.03†	**0.91 ± 0.04***	**0.91 ± 0.04***	1.32 ± 0.05	1.53 ± 0.12	1.50 ± 0.07
SD (s)										
	1	67.6 ± 0.8	55.3 ± 3.1	65.1 ± 1.5	**0.93 ± 0.03**	1.12 ± 0.13	1.03 ± 0.09	1.33 ± 0.04	1.58 ± 0.11	1.56 ± 0.11
	0.5	66.3 ± 1.2	51.4 ± 2.5	62.8 ± 2.3	**0.82 ± 0.04***	0.91 ± 0.05	1.08 ± 0.11	1.36 ± 0.05	1.40 ± 0.05	1.51 ± 0.06
APZ (mg/kg)										
	sal	**64.3 ± 1.9**	55.3 ± 3.1	-	1.09 ± 0.05	1.12 ± 0.13	-	**1.45 ± 0.07**	1.58 ± 0.11	-
	0.12	**68.2 ± 0.8***	60.5 ± 3.6	-	0.98 ± 0.04†	0.98 ± 0.05	-	**1.30 ± 0.06***	1.50 ± 0.13	-

Statistically different changes or tendencies *vs.* the basal value (5 s, 1 s or saline, respectively) are indicated as * $p < 0.05$ or † $p = 0.054$ and $p = 0.077$, respectively.

DISCUSSION

5. DISCUSSION

The main objective of the present project was to assess the impact of *APOE* genotype on the one hand, and the pesticide CPF on the other, on both metabolism functioning and cognitive performance during adulthood. In addition, it also attempted to shed light whether there are human subpopulations particularly susceptible to the detrimental effects of the toxic. To meet these objectives, adult apoE TR mice were subjected to repeated dietary doses of CPF, under a pattern of moderate dosing, being expected to cause any cholinergic sign of toxicity. In this chapter, the results are discussed as a whole, offering a logical sequence of events regarding metabolic findings first, a global perspective on the neurobehavioural effects found later, and finally, an overview of the implications of these findings. Ultimately, the main limitations of the study and future perspectives of work are further discussed.

5. 1. GENERAL DISCUSSION

When we addressed for the first time the approach of the objectives of the current doctoral thesis, we relied on an initial idea evolved around the *APOE4* genotype and its vulnerability to neurodegeneration. Earlier research by our group *(Peris-Sampedro et al., 2014; Salazar et al., 2011)* had already demonstrated back then that high doses of CPF, either acutely or subchronically administered, exacerbated some of the signs observed in the course of AD. In particular, these investigations, which were performed in Tg2576 transgenic male mice carrying the Swedish mutation responsible for a familial form of AD, revealed increases in β-amyloid levels *(Salazar et al., 2011)*, and delayed memory impairments *(Peris-Sampedro et al., 2014)* after exposure to the pesticide.

With these findings as background, we considered the possibility of studying the neurobehavioural effects of CPF in individuals with different vulnerability to AD. As it has been repeatedly stated throughout this dissertation, *APOE* gene is polymorphic and its three major alleles confer on their carriers increased vulnerability to AD following the ε*4* > ε*3* > ε*2* rank order *(Roses, 1996)*. Therefore, we decided to include the apoE TR mouse model in our studies,

aiming at approaching the worsening of the disease from a different perspective. Furthermore, we were also encouraged by the fact that, at that time, there was no study investigating the behavioural response of apoE TR mice to CPF. On the other hand, we also believed it appropriate to reduce the dose of treatment, with the intention of matching experimental exposures to what would be a more or less real everyday exposure in humans. That was how the first experiment comprised within this thesis dissertation arose, in which we sought to assess the effects of repeated dietary doses of CPF on spatial learning and memory in apoE TR male mice.

During the course of treatment, we proceeded to routine weight control as an essential procedure in any toxicological study. To our surprise, we noted that apoE3 mice considerably fattened upon CPF exposure and, moreover, were the only ones to do so. This fortuitous discovery made us setting a later study aimed at determining more in-depth the metabolic effects of repeated exposure to CPF in these animals. To assess the net contribution of the *APOE3* polymorphism, we decided to include its background model (i.e., C57BL/6N mice) in the study. Overall, the results of this second experimental phase stressed that both mice strains were metabolically affected by CPF. Nonetheless, in agreement with the results of the first study, apoE3 mice were more prone than C57BL/6N to become overweighed upon repeated dietary CPF, and exhibited increased levels of leptin. In addition, we also found that exposed apoE3 mice displayed a sharper diabetic profile, as indicated by higher insulin levels and HOMA-IR values. Intriguingly, CPF generally increased food intake. Having succeeded in replicating the previous findings in this second study, we were able to categorically affirm that the possession of the *ε3* allele conferred increased vulnerability to develop obesity and related metabolic disorders following CPF exposure. In parallel, in our laboratory we were tuning up the 5-CSRTT in order to initiate a series of experiments aimed at characterizing attention, inhibitory control and motivation in apoE TR female mice, and at evaluating the effects of dietary CPF on such cognitive domains and behaviours. Within these experimental phases, we took advantage of the strength of this task to explore whether the *APOE3*-dependent CPF-induced weight gain and general increases in food intake could be due to a deficient inhibitory control. However, the

results found failed to associate the exposure to CPF in apoE3 mice with increased impulsive and/or compulsive behaviour.

The ε*3* has become the most frequent *APOE* allele in all human populations, but appears to be particularly common (>87%) in those that have had a long-established agricultural economy, such as those of the Mediterranean region *(Corbo and Scacchi, 1999; Egert et al., 2012)*. It is well-documented that the transition from hunting-gathering to agriculture promoted prolonged episodes of mass starvation due to recurrent crop failures and diminished dietary diversity *(Prentice et al., 2005)*. There is also widespread evidence that starvation and famines must have exerted a strong selection effect on the human genome, even if that effect has been occasionally considered exaggerated *(Speakman, 2007)*. Therefore, an advantage for individuals carrying a thrifty *APOE* allele favouring fatty acid deposition and curbing fatty acid mobilization and energy dissipation - such as ε*3* – may be reasonable. It could be then argued that such advantageous condition against the other two *APOE* genotypes may have contributed to the worldwide expansion of the ε*3* allele. Notwithstanding, Western countries are presently facing the biggest epidemic of obesity in history. Hence, beyond the advantageous evolutionary condition provided by this allele, it stands to reason that the worldwide frequency of the *APOE3* genotype may be currently aggravating the global prevalence of obesity.

A limited number of experimental studies have inquired about the increased vulnerability of apoE3 TR mice to diet-induced obesity and related metabolic dysfunctions *(Arbones-Mainar et al., 2008; Huebbe et al., 2015; Karagiannides et al., 2008)*. Consistently with the results derived from the first two experimental phases, apoE3 mice fed either normal *(Huebbe et al., 2015)* or western-type diet *(Arbones-Mainar et al., 2008; Karagiannides et al., 2008)* were phenotypically more obese and exhibited increased fat depots than apoE4 mice. Coupled with this, Huebbe and collaborators empirically demonstrated that apoE3 mice had reduced fatty acid mobilization relative to apoE4, while apoE4 mice showed increased fatty acid oxidation *(Huebbe et al., 2015)*. Karagiannides and co-workers also described hyperglycaemia, hyperinsulinemia, hyperleptinemia, glucose intolerance and insulin resistance following a 24-week high-fat regime *(Karagiannides et al., 2008)*. Despite the existing experimental evidence, human data are scarce.

Indeed, only few studies have attempted to link human *APOE* genetic variability to obesity and related metabolic dysfunctions, and findings are sometimes conflicting. Nonetheless, a recent case-control study has associated being homozygous for the *APOE3* polymorphism to an increased risk of type 2 diabetes in Turkish patients (i.e., *ε3/ε3* frequency: 81% in diabetic patients *vs*. 38% in healthy subjects) *(Mehmet et al., 2015)*.

Even though there has been renewed interest in ascertaining the contribution of environmental hazards to the global epidemics of obesity and type 2 diabetes; to date, very little research has focused on OPs. Epidemiological studies, although being scarce, have successfully linked exposure to OPs to type 2 diabetes outcome *(Montgomery et al., 2008; Raafat et al., 2012; Saldana et al., 2007)*. However, there remains a paucity of human data on the role played by OP pesticides in the aetiology of obesity. Similarly, the same trend is observed in investigations involving experimental animals: while research on type 2 diabetes has been more or less recurrent *(Lasram et al., 2014)*, the obesogenic effect of OPs has been scarcely addressed in an experimental setting. Furthermore, as it has been highlighted in the introductory chapter, evidence is even scarcer when focusing solely on CPF. It is important to bear in mind that, once presumed that OPs act beyond their ability to inhibit cholinesterases, it can no longer be assumed that they will all act alike, since the various compounds belonging to this class may diverge in their actions mediated by other mechanisms. Unfortunately, current knowledge of adulthood exposure to CPF is limited to four highly-dosed studies carried out in male rats *(Acker and Nogueira, 2012; Ehrich et al., 2004; Elsharkawy et al., 2013; Meggs and Brewer, 2007)*, being the bulk of the research focused on early life stages and/or on other OP compounds.

Overall, the results of the current project expand the scanty existing literature on the obesogenic effect of OP exposures, and further demonstrate their contribution to the development of metabolic diseases. In particular, besides exacerbating genotype-dependent insulin and leptin increases, body weight gain, and insulin resistance, subchronic exposures to CPF broadly induced hyperglycaemia and hypercholesterolemia in adult male mice. These results are in line with those from previous studies *(Acker and Nogueira, 2012; Ehrich et al., 2004; Elsharkawy et al., 2013; Meggs and Brewer, 2007)*. Data reported by Meggs and Brewer attested increased body weight and

fat depots upon chronic exposure to moderate CPF, although they failed to confirm CPF-induced adipocyte differentiation *(Meggs and Brewer, 2007)*. On the other hand, the investigations conducted by Acker and Nogueira first, and Elsherkawy and co-workers after, also revealed disturbances of both glucose and lipid homeostasis in exposed animals *(Acker and Nogueira, 2012; Elsharkawy et al., 2013)*. Nevertheless, without underestimating the relevance of these three experimental works, none of them examined the hormonal changes elicited by adulthood CPF administration. Indeed, only two studies explored the imbalance caused by CPF in hormones related to energy homeostasis, but both involved neonatal CPF exposures *(Lassiter and Brimijoin, 2008; Slotkin et al., 2005)*. It is important to mention that health consequences of environmental insults are time dependent, entailing a great difference between effects observed and structures affected depending on the age at which exposure occurs. It is well-known that some chemicals induce developmental toxicity at doses not affecting adult individuals *(Grandjean and Landrigan, 2014, 2006)*. Indeed, it has been suggested that the developing brain is more vulnerable to toxic injuries, as it is within this period that CNS structures differentiation occurs. Surely this is the reason why most scientific research has focused on establishing the impact of early-life exposures to toxicants on health, rather than adulthood exposures. By the same token, however, assessing the detrimental effects of these substances later in life must also be regarded as crucial to elucidate the full range of mechanisms of action inherent to these hazardous agents.

Increased food intake was observed following subchronic exposure to CPF in both, apoE3 and C57BL/6N male mice. Accordingly, male rats subjected to a single acute dose of CPF showed enhanced food intake *(Carvajal et al., 2014)*. Additionally, the results from the 5-CSRTT indicate that these feeding disruptions were not due to altered inhibitory control. In particular, pre-trained apoE TR female mice subchronically administered CPF did no manifest increased premature and/or perseverative responses in the 5-CSRTT. However, it should be taken into account that behavioural assessment in the 5-CSRTT involved female mice, whilst male mice were used for the first two experimental studies of this thesis. Moreover, as it is commonly seen in experimental approaches using the 5-CSRTT, females were food deprived in order to stimulate their motivation to perform the task. Consequently, these remarkable differences between experimental protocols imply this assumption needs to be interpreted with caution,

deserving to be more accurately addressed. It is worth noting that, even if apoE3 female mice did not show impaired inhibitory control upon CPF exposure, they did so after being treated with scopolamine, a mAChRs antagonist.

For the first time, the results of the present project underscore a markedly vulnerability of the *APOE3* genotype towards the metabolic-disruptor role of CPF. As it has been previously discussed throughout this chapter, the *APOE3* polymorphism has been recently associated to confer increased efficiency at harvesting dietary energy on their carriers *(Huebbe et al., 2015)*. Despite the fact that this inherent characteristic may have accounted for the worldwide expansion of this phenotype, it can be now playing a detrimental role to the current prevalence of obesity. Although there remains a lack of empirical research concerning the impact of CPF on the development of this health condition, the existing evidence points to increased adiposity in treated subjects. In the light of the above, it is reasonable to expect an additive effect upon combining *APOE3* and CPF. Hence, it could be speculated that individuals carrying the apoE3 isoform and being susceptible to be exposed to CPF would constitute a subpopulation at risk. Besides, as most noticeable findings encompassed a worsening of both, insulin and leptin statuses in apoE3 mice upon CPF exposure, it is thereby likely to suggest that insulin and leptin pathways certainly stand as a future venue of investigation. Although underlying mechanisms may be diverse and obviously remain to be elucidated, some hypotheses can be raised, including: genotype-dependent CPF-induced differences in adipocyte differentiation, β-cell functioning, or key enzymes activities involved in fatty acid uptake, among others.

Returning to the initial approach of this dissertation, three studies were designed to characterize the effects of *APOE* genotype on several cognitive functions, including spatial learning and memory, attention, and inhibitory control, as well as its contribution on motivational status. Furthermore, we also attempted to assess the impact of dietary CPF in all these behavioural processes. Finally, we wanted to establish potential interactions between exposure to the pesticide and the three *APOE* genetic variants, as there was no single study inquiring about the response of these genetic polymorphisms to the toxic. When we raised the

first study, aimed at assessing spatial learning and memory in apoE TR male mice, previous research had already reported back then the anxiogenic character of the *APOE4* genotype *(Reverte et al., 2014; Siegel et al., 2012)*. Therefore, we decided to replace the MWM for the BM to avoid stress induced by swimming. Although references alluding to the use of this task were lower than was the case for the MWM, we felt this protocol update was appropriate in view of the animal model we were going to use. Subsequently, we became interested in other cognitive and behavioural processes, of which there were little published data on: attention, inhibitory control and motivation. Thereafter, we designed a set of experiments in female mice that were performed using the 5-CSRTT. Besides mere behavioural assessment, we sought to determine the neurochemical and neuropharmacological bases of the potential behavioural differences among the three *APOE* genotypes. Thus, we carried out pharmacological challenges using GABAergic and cholinergic agents, and we analysed the levels of brain amino acids, monoamines, and their metabolites in several brain regions.

It is worth briefly recalling on why the use of different sexes in this three studies. Needless to say, experimental investigations involving male individuals are to date much more abundant that those using females; does not matter the scientific scope they are focusing on. Female's estrous cycle is often singled-out as the driving reason researchers prefer to use male subjects, but this selective discrimination is to blame for the lack of empirical data regarding the differences between both sexes. Nowadays, it is well-recognized that they differ in such several behavioural processes as emotion *(Girbovan and Plamondon, 2013)*, impulsivity *(Bayless et al., 2012; Weafer and de Wit, 2014)*, basal activity *(Simpson and Kelly, 2012)*, learning and memory *(Jonasson, 2005; Li and Singh, 2014)*, or attention *(Bayless et al., 2012)*. In addition, as it has been suggested on many occasions, sex differences are evident when analysing the prevalence and severity of AD. In fact, clinical and pre-clinical studies have shown that women not only are more prone to develop AD than men, but also show significantly age-related faster decline and greater deterioration of cognition than they actually do *(Cornutiu, 2015; Li and Singh, 2014; Raber et al., 2004)*. On the other hand, it is well-founded that AD patients frequently exhibit deficits on executive function and attention, being even proposed as the earliest features of the disease *(Belleville et al., 2007; Nedjam et al., 2004)*. Interestingly, a growing body of reports have pointed out

the existence of *APOE4* – sex interactions, suggesting a detrimental additive effect on the course of the disease by combining both the fact of being a woman and carry the ε*4* allele *(Raber et al., 2004; Ungar et al., 2014)*. In the light of the above, we believed it suitable to undertake the 5-CSRTT experimental phases with females, instead of males. Nonetheless, we are aware of the constraints posed by this decision, and thus consider they merit to be further discussed in ***section 5.2***.

In general, behavioural differences among genotypes were noted at the three experimental stages, in both male and female mice. This observation supports the existing data *(De Blasi et al., 2009; Greenwood et al., 2005; Suri et al., 2013; Wilson, 2002)*, and strengthens the idea that the three *APOE* polymorphisms not only condition metabolism, but also cognition in the absence of disease.

On the basis of the current results, it could be asserted that the three apoE phenotypes strongly modulated cognitive performance during adulthood. Specifically, both spatial learning and memory in the BM task and attention in the 5-CSRTT were dependent upon *APOE* polymorphisms. In particular, apoE4 mice displayed the worst acquisition of the BM, and exhibit poor attentional abilities in the 5-CSRTT. On the contrary, apoE2 mice showed the best performance in the BM, and acquired the 5-CSRTT faster than the other two groups. In between the two extremes, the *APOE3* genotype appears to be basally favouring the expression of an intermediate phenotype. Similarly, previous studies carried out by our group have come to the same pattern of findings *(Reverte et al., 2012)*. However, it is noteworthy the paucity of research simultaneously evaluating these processes in the three genotypes.

Sometimes referred to as the forgotten allele *(Suri et al., 2013)*, ε*2* has often been attributed to confer neuroprotection and better cognitive function on their carriers. Accordingly, healthy older individuals carrying this polymorphism show reduced cognitive decline, and faster processing of information *(Suri et al., 2013; Wilson et al., 2002)*. Nevertheless, underlying mechanisms through which the apoE2 isoform acts to improve cognitive performance remain poorly understood. The results provided by this thesis, however, offer a valuable contribution

into this field. As a matter of fact, adult apoE2 female mice showed higher levels of DA than apoE4 in the frontal cortex, which could at least partly explain their privileged cognitive outcome. Conversely, the *APOE4* genotype has been thoroughly examined because of its implication in AD. In this context, its negative influence on several cognitive processes, including spatial learning and memory, and attention, has been widely endorsed by a large amount of experimental *(Pfankuch et al., 2005; Reverte et al., 2013, 2012; Rodriguez et al., 2013)* and epidemiological reports *(De Blasi et al., 2009; Kukolja et al., 2010; Reitz and Mayeux, 2009; Wolk et al., 2010)*. When we adapted the protocol of the BM, we decided to rotate the maze 90° between each trial in order to avoid any proximal cue in the maze that could potentially facilitate the finding of the escape box by non-spatial strategies. This procedure also forced the mice to discriminate between relevant and irrelevant cues, thereby adding difficulty to the acquisition of the task. With this protocol update, we conditioned the attentional demand, as it was essential for the animals to rule out strategic searches to focus on a mapping strategy, by detecting distal visual cues. Considering the results found, it is reasonable to expect that the poor attentional ability inherent to the *APOE4* genotype observed in the 5-CSRTT could be a trigger for the impaired learning of the BM task. Similarly, the attentional deficits observed in AD patients are sometimes predictors for the disease *(Stopford et al., 2012)*.

Inhibitory control was also differently modulated by *APOE* polymorphisms. Under basal conditions, apoE4 female mice showed increased premature and perseverative responding in the 5-CSRTT. In line with this finding, few epidemiological investigations revealed deficits in behavioural inhibition in non-demented humans carrying the ε*4* allele *(Wetter et al., 2005)*. Furthermore, AD patients also display a lack of inhibitory control, but causal factors leading to this behavioural deficit remain speculative *(Crawford et al., 2013)*.

Over recent years, an escalating body of research has identified a great number of neurobehavioural deficits, including cognitive and motivational disturbances in humans exposed to OPs *(Farahat et al., 2010; Mackenzie Ross et al., 2010; Rohlman et al., 2015; Ross et al., 2013; Stephens and Sreenivasan, 2004)*. At the experimental level, most of the research has focused on the contribution of CPF on boosting deficits in spatial learning and memory. Notwithstanding, more

and more evidence is pointing to a disruptor role of CPF in attentional processes *(Bushnell et al., 2001; Middlemore-Risher et al., 2010)*. In support of this tendency, the current results indicate that subchronic exposure to CPF impaired attention in adult apoE TR female mice specifically by increasing omissions, and reducing processing speed in the 5-CSRTT. However, the results concerning inhibitory control are much more controversial. Indeed, while most studies report a diminished inhibitory control upon acute CPF administration *(Cardona et al., 2011, 2006; López-Granero et al., 2013a)*, our results in the 5-CSRTT seem to point to an improvement of this behaviour. The basis of this controversy may lie in the substantial differences between experimental protocols. On the one hand, in these studies, animals were behaviourally tested long-term after the administration of the pesticide. On the other hand, all of these investigations have been performed in males, whereas we used females. In this regard, it has been confirmed that both sexes differ in terms of impulsiveness. In particular, it has been suggested that males have increased waiting impulsivity than females *(Weafer and de Wit, 2014)*, and thus, comparatively, they would show higher premature responding in the 5-CSRTT than females. Considering all results, it is plausible that CPF could be differently modulating impulsivity according to sex: while it would be contributing to further increase impulsivity in males, it would be conversely acting in females. However, supplementary research needs to be carried out in order to validate this hypothesis and to assure better understanding of sex differences in pathways controlling impulsivity.

Although more discreetly, the scientific literature also reflects the onset of affective disorders upon exposure to OPs both in humans *(London et al., 2005; Mackenzie Ross et al., 2010; Roldán-Tapia et al., 2006, 2005; Steenland et al., 2000)* and experimental animals *(Aldridge et al., 2005; De Felice et al., 2014; Venerosi et al., 2015, 2010)*. Following the same trend, the results of the current thesis dissertation prove the emergence of motivational deficits after subchronic exposure to CPF in adult apoE TR female mice. Comparisons with previous works lead us to state that the observed effects were not due to general malaise or deficits in locomotor activity *(Middlemore-Risher et al., 2010; Montes de Oca et al., 2013; Samsam et al., 2005)*. Consistently with our observations, male and female rats postnatally exposed to 1 mg/kg/day CPF showed anhedonia later in life in a chocolate milk preference test *(Aldridge et al., 2005)*. Authors attributed such blunted behaviour to

a reduced serotoninergic synaptic function. Intriguingly, apoE3 female mice exposed to CPF showed delayed attentional impairments surely due to motivational effects, as revealed by an increase in omissions and diminished number of trials completed in the 5-CSRTT across the wash-out period.

The results of the present doctoral dissertation demonstrate that the three *APOE* polymorphisms confer on their carriers varying cognitive performance during adulthood. Furthermore, the three *APOE* genotypes have been proved to differently modulate the toxicity produced by dietary CPF. Indeed, this *APOE*-dependent modulation of toxicity might ultimately being masking potential effects of OPs in epidemiological approaches not including genetic polymorphisms as variables of study. Interestingly, some of the most striking effects reported here are observed in those individuals conceptually protected by an apparently healthy intermediate phenotype, apoE3. Coincidentally, this phenotype appears to be the most frequent in all human population. It should be highlighted that there is a vast number of potential chemicals that are currently widespread in the environment, being able to produce by themselves both metabolic and neurobehavioural disturbances in humans. Therefore, this fact represents an added value, as these toxicants may exacerbate the adverse effects caused by the exposure to CPF. Although I am aware of the limitation imposed by the doses used in the current study, I firmly believe that, collectively, these results support the imperative need to review policies on the use of pesticides and other environmental hazardous agents in view of the increasing emergence of mental and metabolic disorders.

5. 2. LIMITATIONS OF THE STUDY

Despite the relevance and strengths of the results provided by this thesis, several limitations need to be addressed.

The first one deals with the doses of CPF we used. The FDA issued a reference value for CPF daily intake in adults of 0.005 μg/kg body weight/day, being even higher when toddlers and infants are concerned *(Eaton et al., 2008)*. Nonetheless, even if our dosing schedule is relatively

low relative to that employed in other similar studies, we must admit that it is still too high to directly compare these experimental exposures with what would be the average daily intake in humans. However, given the wide range of sources exposure, much uncertainty still exists about what would be the total daily exposure to CPF. Therefore, it is not so unreasonable to expect that our selected dose itself may reflect to some extent the total human exposure to the pesticide.

The second one lies in the use of animals of different sexes throughout the experimental phases. As it has been mentioned in more than one occasion, in the first two experiments of this thesis we used male mice, whereas in the last two we used female mice. Actually, when we started working on the idea of evaluating the behaviour of apoE mice and their responses to CPF, we decided to start working with males, because of the benefits that entails. However, as we progressed in that way, we decided to embark on a parallel investigation on the effects of the pesticide on different cognitive processes, beyond the already exploited spatial learning and memory. In this second phase, we decided to use females in view of the reasons already stated above. The chance finding of increased weight in apoE3 male mice slightly destabilized the initial approach, but we still believe it necessary to delve into the mechanisms by which CPF differentially acted in these individuals. So, we decided to continue using male mice in the metabolic study, in order to be able to replicate the earlier findings. However, given the strength of the 5-CSRTT, we considered the possibility of investigating whether the *APOE3* genotype had poorer inhibitory control, although females were evaluated. Certainly, the ideal scenario would have been running all the tests in both males and females. However, this meant much economic cost and animal lives, and unfortunately by that time this was not viable.

The third one refers to the imperative condition of food deprivation in the 5-CSRTT. Although it seems logical to use this restrictive condition to ensure animals' motivation to perform the task, I do not know to what extent this may have influenced the results, or may have interacted with either the OP agent or *APOE* genotype.

5. 3. FUTURE PERSPECTIVES

The results of the current investigation stress the importance of studying genetic vulnerability to toxic agents, and point to a more vulnerable phenotype – apoE3 -, which has been so far considered as the healthy one. Nonetheless, these findings have thrown up many questions in need of further investigation. Basically, research should henceforth focus on the following points: a) to provide more face-validity to the experimental exposure to CPF compared to that seen in humans, b) to delve into the neurobehavioural and metabolic effects observed, c) to elucidate the underlying mechanisms through which CPF acts, d) and to assess whether these results can be epidemiologically extrapolated.

The following proposals offer some direction for the development of future research that will substantially extend the knowledge provided by the results of this thesis.

· The doses of CPF used in these studies, albeit cholinergic symptom-free, were relatively high when compared with those that would be expected for typical non-occupational exposures in humans. Therefore, I propose reducing the treatment dosing in order to shed light into what CPF doses would be exempt from behavioural and/or metabolic effects.

· Furthermore, potential metabolic alterations were assessed immediately after exposure to the pesticide, period during which there was still plasma ChE inhibition. Certainly, it would be very interesting to include a wash-out period to better characterize the net contribution of acute ChE inhibition on CPF-related metabolic consequences. On the other hand, the results of this thesis attest differences between genotypes, as well as *APOE* – CPF interactions at punctual life stages, but they fail to follow their time course. Bearing in mind the lack of consistent data concerning long-term behavioural effects of CPF exposures, I consider it crucial to evaluate them in a further study involving the three *APOE* genotypes.

· The current results confirm a set of behavioural differences between the three *APOE* genotypes: spatial learning and memory, inhibitory control, and attentional processes depended upon *APOE* polymorphisms. To go beyond, additional experimental investigations are needed to appraise the involvement of *APOE* genotype in other behavioural processes, of which little

evidence is actually available, such as aggressiveness, social hierarchy, social motivation, or feeding behaviour.

· The effects of dietary CPF on metabolism and especially the increased vulnerability of the *APOE3* genotype towards them constitute one of the cornerstones of this thesis. Notwithstanding, these results need to be further corroborated by using *in vivo* approaches. In particular, it would be worthwhile to examine both glucose and insulin sensitivities by means of oral glucose and insulin tolerance tests, respectively. Additionally, it is necessary to clarify whether there is a direct relationship between the weight gain observed and a greater amount of fat deposited. I thereby suggest a parallel follow-up study in which animals could be non-invasively subjected to whole body composition analysis by means of periodic scans using nuclear magnetic resonance technology. This system is extremely useful as it enables lean, fat, and total body water to be assessed simultaneously. Besides, to the extent possible, it would be compelling to include the three apoE TR mice groups within these studies, in order to investigate the different metabolic responses of the three genotypes after CPF exposure.

· Coupled with the latter, increased leptin levels inherent to apoE3 male mice seem to further support the idea of genotype-dependent increases in adiposity upon CPF exposure. Nevertheless, this statement must be further validated by *in vitro* studies. In this regard, it is well-established that apoE induces *in vitro* adipocyte differentiation. However, the extent to which the different protein isoforms modulate this process has not yet been demonstrated, nor is the case for CPF, implying that it is a promising area for future research.

· Further on, it has been suggested that developmental exposures to some neurotoxicants could contribute to a subsequent preference for a high-fat diet later in life *(Slotkin, 2011)*. On the other hand, a recent cross-sectional study revealed that diary dietary patterns (i.e., processed energy-dense food, rich in refined carbohydrates, sugar and fat *vs.* balanced diet) are associated with cognitive performance in children *(Park et al., 2012)*. On the basis of the above, it could be interesting to inquire about potential interactions between *APOE* genotype, CPF exposure, dietary choices and learning disabilities.

· Emotional disturbances ensued upon subchronic exposure to CPF in female mice. I therefore propose to investigate these outcomes more thoroughly. This intention requires the implementation of a battery of tests mainly aimed at evaluating the depressive component of CPF exposure, such as the hotplate (i.e., a simple test to grade pain response in mice), forced swim test (i.e., an established paradigm to measure the tendency to give up on attempts to escape from an unpleasant environment), or saccharine/chocolate preference test (i.e., a test to estimate anhedonia, a depressive-like behaviour). Moreover, considering the growing body of studies supporting the CPF impact on other neurotransmitter systems, including the serotoninergic system, it would be of interest to assess whether the three *APOE* genotypes basally differ in the functioning of this system.

· Finally, it should not be forgotten that it is essential to relate the findings in experimental animals with observations at the population level. In regard to this, it has been shown that carriers of the *1914G* allelic BChE variant, besides having a lower enzyme activity, exhibit the highest rates of obesity and TG levels, which may cause an imbalance in lipid metabolism, and ultimately lead to an increased predisposition to obesity and to a lower ability to maintain metabolic homeostasis *(Lima et al., 2013)*. At the same time, it is widely accepted that CPF inhibits BChE. So, it would be of interest to study whether the obesity-like phenotype imposed by the genetic *1914G* variant is aggravated by the fact of being carrier of the ε*3* allele within a population highly exposed to CPF.

CONCLUSIONS

6. CONCLUSIONS

APOE genotype effect

1 *APOE genotype affects spatial learning and memory during adulthood. ApoE4 male mice display the worst acquisition of the BM task, followed by apoE3, and apoE2, which show the best acquisition performance. On the other hand, apoE2 male mice exhibit the worst retention of the BM task.*

2 *APOE genotype conditions the acquisition of the 5-CSRTT during adulthood. ApoE2 female mice acquire the task faster than apoE3 and apoE4 female mice.*

3 *APOE genotype modulates inhibitory control and sustained attention during adulthood. ApoE4 female mice show a lack of inhibitory control and an impaired sustained attention in the 5-CSRTT.*

4 *Dopamine levels in the frontal cortex of adult apoE2 female mice are higher than those found in apoE4.*

5 *The muscarinic antagonist scopolamine rises premature responding in adult apoE3 female mice, but not in apoE2 or apoE4.*

CPF effect

6 *Subchronic dietary exposure to CPF during adulthood alters metabolic functioning in male mice. Specifically, it elicits hyperglycaemia and hypercholesterolemia, increases insulin levels and impairs HOMA-IR index scores.*

7 *Subchronic dietary exposure to CPF during adulthood enhances food intake, and tends to increase acyl ghrelin levels in male mice.*

8 *Subchronic dietary exposure to CPF during adulthood induces protracted attentional and motivational deficits in female mice.*

9 *Subchronic dietary exposure to CPF during adulthood reduces waiting impulsivity in female mice.*

***APOE* – CPF interaction effect**

10 *Chronic and subchronic dietary exposures to CPF during adulthood lead to significant body weight gain in apoE3 male mice.*

11 *Chronic dietary exposure to CPF during adulthood impairs spatial memory in apoE3 male mice.*

12 *Adult apoE3 male mice are more vulnerable than C57BL/6N to the metabolic-disruptor role of CPF. In particular, apoE3 mice have higher insulin and leptin levels, as well as higher HOMA-IR index scores.*

13 *Subchronic dietary exposure to CPF during adulthood reverses the lack of inhibitory control inherent to apoE4 female mice.*

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7. REFERENCES

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SCIENTIFIC CONTRIBUTIONS AND OTHER MERITS

ORIGINAL RESEARCH ARTICLES

Publications derived from the present work:

· **Peris-Sampedro F**, Basaure P, Reverte I, Cabré M, Domingo JL, Colomina MT. *Chronic exposure to chlorpyrifos triggered body weight increase and memory impairment depending on human apoE polymorphisms in a targeted replacement mouse model.* Physiol Behav 2015; 144:37-45. DOI: 10.1016/j.physbeh.2015.03.006. PMID: 25747767.

· **Peris-Sampedro F**, Cabré M, Basaure P, Reverte I, Domingo JL, Colomina MT. *Adulthood exposure to a common pesticide leads to an obese-like phenotype and a diabetic profile in apoE3 mice.* Environ Res 2015; 142:169-76. DOI: 10.1016/j.envres.2015.06.036. PMID: 26162960.

· Reverte I, **Peris-Sampedro F**, Basaure P, Campa L, Suñol C, Moreno M, Domingo JL, Colomina MT. *Attentional performance, impulsivity and related neurotransmitter systems in apoE2, apoE3 and apoE4 female transgenic mice.* Psychopharmacology (Berl) 2015. DOI: 10.1007/s00213-015-4113-9.

· **Peris-Sampedro F**, Reverte I, Basaure P, Cabré M, Domingo JL, Colomina MT. *Apolipoprotein E genotype and the pesticide chlorpyrifos modulate attention, motivation and impulsivity in female mice in the 5-choice serial reaction time task.* Neurotoxicology 2015. Currently under review.

Other publications:

· **Peris-Sampedro F**, Salazar JG, Cabré M, Reverte I, Domingo JL, Sánchez-Santed F, Colomina MT. *Impaired retention in AβPP Swedish mice six months after oral exposure to chlorpyrifos.* Food Chem Toxicol 2014; 72:289-94. DOI: 10.1016/j.fct.2014.07.036. PMID: 25106752.

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PARTICIPATION IN NATIONAL AND INTERATIONAL CONFERENCES, AND SEMINARS

Conference: 8th Federation of European Neuroscience Societies (FENS) Forum of Neuroscience, Barcelona, July 14 – 18, 2012.
Authors: Colomina MT, Salazar JG, **Peris-Sampedro F**, Margalef JR, Reverte I, Cabré M, Domingo JL, Sánchez-Santed F.
Title: *Repeated chlorpyrifos exposure impairs memory and increases BACE-1 expression in a transgenic model of Alzheimer's disease.*
Format: Poster
Publication: Programme book; p.490

Conference: Seminars in Neuroscience, Reus, March 8, 2013.
Authors: **Peris-Sampedro F**
Title: *Neurobehavioral effects resulting from repeated exposure to chlorpyrifos in transgenic mice carrying the APPswe mutation.*
Format: Oral communication

Conference: 14th International Neurotoxicology Association (INA) meeting, Egmond aan Zee, Nederlands, June 9 – 13, 2013.
Authors: **Peris-Sampedro F**, Reverte I, Cabré M, Domingo JL, Sánchez-Santed F, Colomina MT.
Title: *Chronic oral exposure to low doses of chlorpyrifos differentially affects physical and behavioural endpoints in apoE2, apoE3 and apoE4 transgenic mice.*
Format: Poster
Publication: Neurotoxicology 2014; 43:143-59.

Conference: XX Congreso Español de Toxicología y IV Iberoamericano, Salamanca, June 26–28, 2013.
Authors: Reverte I, **Peris-Sampedro F**, Cabré M, Domingo JL, Colomina MT.
Title: *Efectos neuroconductuals de la exposición crónica a bajas dosis de clorpirifos en ratones transgénicos apoE2, apoE3 y apoE4.*
Format: Poster
Publication: Revista de Toxicología 2013, 30(1): 1-128.

Conference: 45th European Brain and Behaviour Society (EBBS) meeting, Munich, Germany, September 6 – 9, 2013.
Authors: Reverte I, **Peris-Sampedro F**, Basaure P, Moreno M, Domingo JL, Colomina MT.
Title: *Effects of GABAergic and cholinergic agents in anxiety, attention and impulsivity in apoE2, apoE3 and apoE4 mice.*
Format: Poster

Conference: 15º Congreso Nacional de la Sociedad Española de NeuroCiencia (SENC), Oviedo, September 25 – 27, 2013.
Authors: **Peris-Sampedro F**, Reverte I, Basaure P, Cabré M, Domingo JL, Colomina MT.
Title: *Long-term behavioural effects after a chronic oral exposure to low doses of chlorpyrifos in apoE2, apoE3 and apoE4 transgenic mice.*
Format: Poster
Publication: Communications book; p.275

Conference: Jornadas de Verano en Neurotoxicología y Psicofarmacología, Reus, June 18, 2014.
Authors: **Peris-Sampedro F**
Title: *Organophosphates and chronic diseases*
Format: Oral communication

Conference: 9th FENS Forum of Neuroscience, Milan, Italy, July 5 – 9, 2014.
Authors: **Peris-Sampedro F**, Reverte I, Cabré M, Neri T, Basaure P, Domingo JL, Colomina MT.
Title: *Subclinical oral exposure to chlorpyrifos differentially affects physical and behavioural endpoints in apoE2, apoE3 and apoE4 transgenic mice.*
Format: Poster
Publication: Programme book; p.341

Conference: 9th FENS Forum of Neuroscience, Milan, Italy, July 5 – 9, 2014.
Authors: Reverte I, **Peris-Sampedro F**, Basaure P, Moreno M, Campa L, Suñol C, Domingo JL, Colomina MT.
Title: *Differences in attention and impulsivity and related neurotransmitter systems in apoE2, apoE3 and apoE4 mice*
Format: Poster
Publication: Programme book; p.343

Conference: 9th FENS Forum of Neuroscience, Milan, Italy, July 5 – 9, 2014.
Authors: Colomina MT, Basaure P, **Peris-Sampedro F**, Reverte I, Domingo JL, Cabré M.
Title: *Neurobehavioural effects associated with cholinesterase inhibitors chlorpyrifos and rivastigmine.*
Format: Poster
Publication: Programme book; p.288

Conference: XXIII Setmana Psicològica, Tarragona, November 17, 2014.
Authors: **Peris-Sampedro F**
Title: *Interaccions genètiques i ambientals en el desenvolupament de trastorns neurodegeneratius i obesitat.*
Format: Oral communication
Publication: http://psico.fcep.urv.cat/setpsico14/programa.html

Conference: 16º Congreso Nacional de la SENC, Granada, September 23 – 25, 2015.
Authors: **Peris-Sampedro F**, Cabré M, Basaure P, Neri T, Reverte I, Domingo JL, Colomina MT.
Title: *The metabolic-disruptor role of chlorpyrifos: from feeding behaviour to hormonal imbalance.*
Format: Poster

GRANTS, AWARDS AND RECOGNITIONS

Grant: PhD fellowship (grant number: *2013 FI_B00170*), January 2013 – January 2016.
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Award: Elsevier Poster Award, 2º best poster presentation at the 14th INA meeting, Egmond aan Zee, Netherlands, June 9 – 13, 2013.
Funding entity: Elsevier
Publication: Neurotoxicology 2014; 43:143-59.

Recognition: Recognition of the quality of teaching activities, Tarragona, November 10, 2014.
Issuing entity: Universitat Rovira i Virgili

Grant: Travel grant to attend the 16th Congress of the SENC. Granada, September 23 – 25, 2015.
Funding entity: SENC
Publication: http://www.senc2015.com/docs/list-of-awardess-senc2015.pdf

Recognition: Recognition of the quality of teaching activities, Tarragona, October 9, 2015.
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"A black cat crossing your path signifies that the animal is going somewhere."

~ Groucho Marx