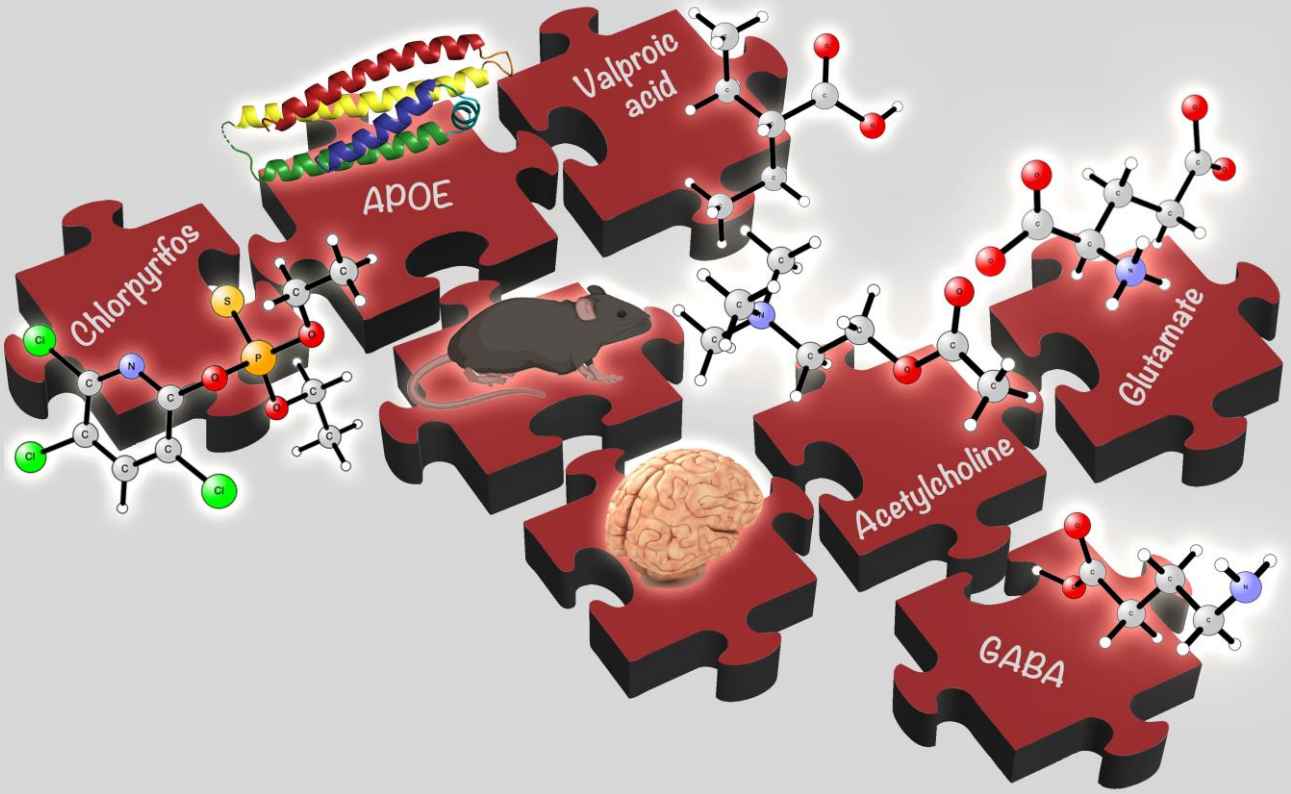
INFLUENCE OF PRENATAL CHLORPYRIFOS EXPOSURE, APOE GENOTYPE AND SEX ON NEURODEVELOPMENTAL DISORDERS: BEHAVIORAL AND BIOCHEMICAL ABNORMALITIES IN MICE

Judit Biosca Brull

UNIVERSITAT ROVIRA i VIRGILI

Influence of prenatal chlorpyrifos exposure, *APOE* genotype and sex on neurodevelopmental disorders: behavioral and biochemical abnormalities in mice

JUDIT BIOSCA BRULL



DOCTORAL THESIS
2023

Judit Biosca Brull

Influence of prenatal chlorpyrifos exposure, *APOE* genotype and sex on neurodevelopmental disorders: behavioral and biochemical abnormalities in mice

Doctoral thesis

Thesis supervised by:

Dr. Maria Teresa Colomina Fosch

Dr. Maria Cabré Bargalló

Department of Psychology

UNIVERSITAT ROVIRA i VIRGILI

Tarragona

2023

UNIVERSITAT ROVIRA i VIRGILI

I STATE that the present study, entitled "*Influence of prenatal chlorpyrifos exposure, APOE genotype and sex on neurodevelopmental disorders: behavioral and biochemical abnormalities in mice*", submitted by Judit Biosca Brull for the award of the degree of Doctor, has been carried out under my supervision at the Department of Psychology of this university.

Tarragona, 23 January 2023

Doctoral Thesis Supervisors;

Maria Teresa Colomina Fosch

Maria Cabré Bargalló

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

→ *Martí Franquès Research Grant Program – Doctoral modality. Reference: 2019PMF-PIPF-28*

→ The Ministry of Economy and Competitiveness of the Spanish Government. Reference: PSI2017-86847-C2-2-R

A la meva família

Y una vez que la tormenta termine, no recordarás como lo lograste, como sobreviviste, ni siquiera estarás seguro si la tormenta ha terminado realmente. Aunque una cosa sí es segura, cuando salgas de esa tormenta, no serás la misma persona que entro en ella.

De eso trataba la tormenta.

Haruki Murakami

ACKNOWLEDGEMENTS

Fa un parell de mesos vaig començar a escriure el que serà la meva tesi doctoral. Avui, estic escrivint una de les parts més importants, on es reflecteix tot el suport i apareixen aquelles persones que, a la seva manera, han fet que aquesta tesi sigui possible. Perquè sense vosaltres aquest camí no hagués estat tan fàcil!

La meva aventura amb la neurociència va començar fa quatre, o bé cinc anys. Durant aquest temps, he tingut la sort de comptar amb dues dones meravelloses, brillants i amb una passió per la ciència contagiosa. Elles són i seran una inspiració per mi! Gràcies a la Dra. Maria Teresa Colomina per confiar en mi i obrir-me les portes del laboratori. Gràcies per tot el que m'has ensenyat, per tots els reptes proposats i per tot el suport durant aquest procés. A la Dra. Maria Cabré, gràcies per buscar sempre la perfecció. Gràcies per aportar tranquil·litat quan els experiments no sortien com esperàvem o quan els nostres "grans amics" els congeladors feien de les seves.

Amb elles he pogut forma part d'un gran equip o bé, podríem dir que d'una gran família, ja que us asseguro que NEUROLAB sempre serà casa. A tot l'equip dir-vos que ha estat un plaer coincidir amb vosaltres. Dr. Jordi Blanco, ets alegria i pura energia! Gràcies per ensenyar-me tant i estar sempre disposat a ajudar. Et puc assegurar que has estat un pilar molt important en aquesta tesis. Las que fueron mis mentoras y con los años pasaron a ser mis amigas, Pia y Laia, o como bien nos decíamos las "ratoncitas". Gracias a las dos por enseñarme que el equipo es lo más importante. Dra. Pia Basaure, gracias por confiar en mí y guiarme desde el principio. Gracias a ti, parte de las pruebas de esta tesis se pudieron llevar a cabo. Dra. Laia Guardia-Escote, gràcies per tots els teus consells i per continuar estant. És un plaer poder seguir veure't créixer. Estic segura que arribaràs lluny! No puc deixar d'agrair a les noves incorporacions, Rocío i Sèfora. A vosaltres dir-vos que espero que gaudiu d'aquest camí que just acaba de començar. Finalment, vull agrair a les noies i nois que han realitzat pràctiques de grau o de màster al nostre laboratori. Espero haver estat a l'altura i us agraeixo infinitament el vostre suport. Sens dubte m'heu ajudat a aprendre.

El doctorat m'ha permès compartir moments i coneixements amb altres grans investigadors e investigadores. Dr. Manel Santafe, podríem dir que has estat des del primer dia que vaig tenir contacte amb la neurociència. Gràcies als teus consells les anàlisis d'aquesta tesi han estat molt més fàcils. Dra. Merche Gómez, me diste la oportunidad de trabajar en uno de tus proyectos y formar parte de tu equipo, muchas gracias. Dra. Fàtima Sabench, gràcies per estar disposada sempre a ensenya'ns la cirurgia, ha estat un plaer poder aprendre de tu. Vull agrair també a tot l'equip de TECNATOX i, en especial, al Dr. Domingo per la seva confiança i suport en els articles. Els seus consells han estat aprenentatge.

Con todo esto, no puedo dejar de mencionar a nuestro grupo de Almería. Dr. Fernando Sánchez-Santed, Dr. Cristian Pérez-Fernandez y Dr. Miguel Morales-Návas, gracias por todo vuestro apoyo y consejos. Es un placer coincidir con vosotros y hablar de nuestro amigo clorpirifós y nuestras amigas vocalizaciones. ¡Una suerte trabajar con vosotros!

M'agradaria continuar agraint el treball del personal de l'estabulari: Juan, Esperanza, Celeste i Carolina. Gracias por cuidar de la parte más importante de esta tesis, nuestros ratoncitos. Gracias por estar ahí cuando las cosas no iban del todo bien y por las charlas entre ratones. Vull agrair també a tot el Departament de Psicologia, del que he tingut la sort de formar part durant aquest procés.

I bé, aquests anys no haurien sigut tan fàcils sense el suport d'aquelles persones companyes d'etapa. Dr. Marc Bosque i els recents doctors, Dra. Laia Just i Dr. Victor Cilleros-Mañe dir-vos que sou brillants i agrair-vos tots els moments viscuts, però sobretot pel nostre viatge a Berlín. Estic ben segura que serà difícil d'oblidar.

Tots vosaltres heu estat fonamentals en aquest procés, però sense el suport d'aquells que m'han seguit a cada pas, estic segura que avui no estaria escrivint aquestes línies. Els meus pares, ells ho són tot! Gràcies per l'esforç, per recolzar-me en cada decisió i per estar-hi sempre, però sobretot gràcies pel vostre amor incondicional. A tu Lulu, gràcies per cuida'ns sense límits i tenir el millor remei per tots els problemes. Gràcies per visitar-me durant el procés d'escriptura i donar-me estones d'alegria. A Milio, gràcies per la teva ajuda incondicional i per deixa'ns disfrutar amb tu del que més ens identifica, la mar. Gràcies per ser refugui en els bons i mals moments.

A la meva doctora preferida, la Dra. Maria Biosca o la meva Teta. Gràcies per ser un gran exemple, per no ficar-me les coses fàcils i per ser la millor germana que una pot tindre. Perquè una sense l'altra no seríem el mateix. T'admiro i t'estimo infinitament. Dr. Adrian Moreno. Ets únic i inigualable. T'has convertit en una peça fonamental en la nostra família. Cunyat, amic i germà. Gràcies per aportar aquesta "xispa" d'alegria tan teva.

Amb ells voldria agrair també a la Pepi, la meva "abueli". Gràcies per seguir tots els meus passos i viure'ls intensament. Només puc dir-vos que heu fet que els dimecres siguin especials. A qui sempre em preguntava com estaven els ratolinets. Vau marxar quan aquesta aventura començava, però un trosset de vostè es va quedar en mi per viure tots els moments junts. Gràcies per ser el millor iaio que una podia tenir, per la vostra paciència i estima. Perquè heu sigut la meva força quan alguna cosa no anava com esperava i perquè allí on esteu sempre caminarem junts.

Ties i tios, però en especial tia Núria. Amb ella vam descobrir el món de la neurociència i la seva lluita em va donar les ganes d'aportar el meu granet d'arena seguint aquest camí. Per ella, avui estic escrivint aquestes línies. Cosins i cosines, fa goig veure com ens fem grans i continuem units. Padrí i

Padrina, què dir-vos? Sou únics i especials per mi. Tia Encarnación i tio Estel, però en especial a la Juani i el tio Ramonet, gràcies per cuidar-me com una neta més.

Aquests cinc anys han fet que la família Calera també fos una mica Falsetana i Darmosana. Gràcies, Maria del Carme i Salvador, per acollir-me a casa vostra com una filla més des del primer dia. A l'Eva i el Martin, gràcies per ser-hi sempre tot i els kilòmetres que ens separen. L'Andrea i l'Arnau, gràcies per totes les aventures compartides. Desitjo que en continuem compartint moltes més. I, la nostra petita Júlia, gràcies per alegra'ns els dies amb les teves rialles. A tots, gràcies pel vostre suport i caliu. Amb ells també voldria agrair a aquella persona que ens fa els arrossos més bons de tot Darmós. Oncle Joan o com ell diu Joan del Racó, gràcies per compartir amb nosaltres les vostres històries i coneixements. És un plaer poder passar estones amb vostè i aprendre de tanta experiència. Admiro les vostres ganes de continuar fent coses i desitjo que mai s'acabi aquesta energia.

No vull deixar-me la família Vallenca. Manuela i Rafael, gracias por acogerme des del primer día y por preguntar siempre por nosotros.

Ja quasi per acabar, voldria agrair a la família que es tria. Els que han estat des de ben petits: Mario, Marina i Nerea. Gràcies per continuar estant al meu costat. Les meves "floretes", el millor regal que em va fer la carrera. Tants kilòmetres ens separen, però continuem estant unides. L'Andrés, la persona que deixa marca per allí on passa. Una suerte encontrarte y que seas mi amigo. Pedro, vas ser una peça fonamental durant la carrera i ho continues sent. I la Maria Mercado. Els estius al laboratori ens van fer coincidir i la muntanya ens va unir. Gràcies per aparèixer i seguir aquí. A tots vosaltres, dir-vos que espero continuar sumant moment i que mai deixem de caminar junts. No puc deixar de donar les gràcies a la família que, de forma indirecta, em va triar: els "Conygelats" o colla Falsetana. Gràcies a tots i totes les que algun dia us heu preocupat o interessat per aquesta tesi. Entre tots formeu una gran família i és una sort formar part d'ella.

Per últim, però no menys important, voldria agrair a la persona que ha viscut el dia a dia d'aquest procés. El meu company d'aventures, i quines aventures … Jaume, gràcies per donar-me ales, per deixar-me créixer, recolzar-me en cada decisió i per creure amb mi, quan jo no em veia capaç. Gràcies per tenir el millor remei per passar les pors, però sobretot, gràcies per aparèixer i fer que tot sigui més fàcil. Perquè mai s'acabin els reptes i continuem complint somnis junts!

En definitiva, gràcies a tots per formar part d'aquesta aventura!

ABSTRACT

Organophosphate (OP) pesticides, and chlorpyrifos (CPF) in particular, are widely used in agriculture, although in the last few years environmental regulations have been drawn up to ban their use. Exposure to CPF has recently been related to an increased prevalence of neurodevelopmental disorders such as autism spectrum disorder (ASD). Even so, this disorder is associated with a wide variety of genetic factors, which can alter the effects of some chemical agents, suggesting a gene-environmental interaction. In our laboratory, we have been studying the apolipoprotein E (*APOE*) gene. We have observed that a human *APOE* genetic background confers different vulnerabilities in terms of neurobehavior, metabolism and the cholinergic system. The main objective of this thesis was to evaluate how prenatal exposure to CPF, the *APOE* genotype and sex contribute to the behavioral and biochemical effects produced in neurodevelopmental disorders. To this end, we conducted a systematic review of all the experimental evidence published on autism and pesticide exposure, with a particular focus on OP. After that, we performed an experimental study using C57BL/6J mice and humanized apoE3 and apoE4 homozygous mice. Pregnant females were exposed trough their diet to either 0 or 1 mg/kg/day of CPF between gestational day (GD) 12 and 18. For the C57BL/6J mice, we included a positive control for some autism-like behaviors. This group of females were exposed to 300 mg/kg/day of valproic acid (VPA) on two consecutive days (GD 12 and 13) via subcutaneous injection. In addition, we performed a parallel experiment with C57BL/6J mice, in which pups were orally exposed (from postnatal day [PND] 10 to 15) to 0 or 1 mg/kg/day of CPF using a micro-pipette. Dams treated with CPF or VPA were evaluated for manternal care and nest quality on PND 2, while the offpring were assessed for communication skills (PND 2, 7 and 9) by recording ultrasonic vocalizations and physical and motor development in terms of body weight, eye opening, climb ability and pull strength between PND 2 and PND 28. During adolescence, pups prenatally treated with CPF or VPA were evaluated for locomotor activity, and social and anxiety-like behaviors, as was the gene expression of elements involved in the GABAergic and glutamatergic systems. While in animals postnatally treated with CPF only social behavior was assessed. The results of the systematic review suggested that exposure to pesticides, and in particular to OP produced symptoms of autism in both clinical and preclinical studies and points to the need of analyze the interaction between pesticides and genetic factors, and how it is related to ASD development. On the other hand, our studies with C57BL/6J mice showed that gestational treatment with VPA produces two of the three core symptoms of ASD, as well as secondary symptoms. Indeed, VPA-treated mice showed an increase in the latency to emit the first call and a reduction in the average duration and the total number of vocalizations on the first and last day of evaluation (PND 2 and 9). Interestingly, we observed opposite effects in CPF-treated mice, although both treatments showed a delay in physical development. During adolescence, mice prenatally treated with VPA also showed anxiety-like behaviors, while social behavior was equally affected by both CPF and VPA treatments but only in males. Gender differences were also observed in some GABAergic and glutamatergic signaling

elements. Females treated with CPF showed an increase in the expression of GAD1 and GABA-A α1 subunit, while in males prenatal exposure to CPF or VPA increases the expression of GABA-A α2 and β3, as well as GluN2A subunit. Apart from that, differences between *APOE* genotypes were observed throughout all the experiments. ApoE4 mice emitted longer and higher USVs and showed lower body weight, but were better at eye opening and climbing. Along the same lines, apoE4 mice showed more anxious behaviors than their counterparts, while social behavior was influenced by sex and treatment. Both *APOE* females homozygous for the ε3 or ε4 allele and prenatally exposed to CPF showed a non-preference for the novel stimulus. Genotype differences were also observed in the expression of some GABAergic and glutamatergic signaling elements. The mice carrying the ε3 allele showed an increase in GABA-A α2 and α5 subunits, as well as GAD1 and the ionic cotransporter KCC2. In addition, we observed that prenatal exposure to CPF increase the expression of KCC2 in apoE3 mice, whereas the expression of GABA-A α1 was increased in both apoE3 and apoE4 females treated with CPF. The results of this thesis associate prenatal pesticide exposure with long-lasting effects during adolescence, but we not observe effects like those observed in the autistic population in the early stages of development. Likewise, *APOE* polymorphism are not observe to have different vulnerabilities to CPF after a prenatal exposure. Therefore, this doctoral thesis questions the recent association observed between the pesticide CPF and ASD, and suggests that the different apoE isoforms (apoE3 and apoE4) are a non-risk factor for the development of the disorder.

Keywords: Pesticides, Chlorpyrifos, Autism, Valproic acid, Apolipoprotein E, *APOE* genotype, Development, Anxiety-like behaviors, Social behavior, GABAergic system, Glutamatergic system.

ABBREVIATIONS

- **5-CSRTT.** Five-choice serial reaction task
- **ACh.** Acetylcholine
- **AChE.** Acetylcholinesterase
- **AChE-E.** Erythrocytic transcript
- **AChE-R.** Readthrough or R transcript
- **AChE-S.** Synaptic or S transcript
- **AD.** Alzheimer's disease
- **AMPA.** α-amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid
- **ANOVA.** Analysis of variance
- **ApoE.** Apolipoprotein E
- ***APOE.*** Apolipoprotein E gene
- **ApoER2.** ApoE receptor-2
- **ASD.** Autism Spectrum Disorder
- **Aβ.** Amyloid-β
- **cDNA.** Complementary DNA
- **ChAT.** Choline acetyltransferase
- **CNS.** Central nervous system
- **CNT.** Control
- **CPF.** Chlorpyrifos
- **CSF.** Cerebrospinal fluid
- **Ct.** Cycle threshold
- **DDT.** Dichloro-diphenyl-trichloroethane
- **DEP.** Di-ethyl phosphate
- **DETP.** Di-ethyl thiophosphate
- **E/I.** Excitatory/Inhibitory
- **EFSA.** European Food and Safety Authority
- **EPA.** United States Environmental Protection Agency
- **EU.** European Union
- **FAAH.** Fatty acid amide hydrolase
- **GABA.** γ-aminobutyric acid
- **GABA-A.** γ-aminobutyric acid receptor subunit
- **GAD.** Glutamate decarboxylase or Glutamic acid decarboxylase
- **GAPDH.** Glyceraldehyde 3-phosphate dehydrogenase
- **GD.** Gestational day
- **GFAP.** Glial fibrillary acidic protein
- **GluN2.** Glutamate ionotropic N-methyl-D-aspartate receptor subunit

- **HDL.** High-density lipoprotein
- **KA.** Kainate
- **KCC2.** Solute carrier family 12-member 5
- **KO.** Knockout
- **LDL.** Low-density lipoprotein
- **mAChR.** Muscarinic receptor
- **MAGL.** Monoacylglycerol lipase
- **MWM.** Morris Water Maze
- **nAChR.** Nicotinic receptor
- **NFTs.** Neurofibrillary tangles
- **NKCC1.** Solute carrier family 12-member 2
- **NMDA.** N-methyl-D-aspartate
- **OP.** Organophosphate/Organophosphorus
- **PCA.** Principal component analysis
- **PND.** Postnatal day
- **PNS.** Peripheral nervous system
- **PON1.** Paraxonase 1
- **PVALB.** Parvalbumin
- **qPCR.** Quantitative real-time polymerase chain reaction
- **RELN.** Reelin
- **RMANOVA.** Repeated measures analysis of variance
- **RORA.** Retinoic-acid related orphan receptor alpha
- **SD.** Standard deviation
- **TCPy.** 3,5,6-trichloro-2-pyridinol
- **US.** United States
- **USVs.** Ultrasonic vocalizations
- **VAChT.** Acetylcholine transporter
- **VGAT.** Vesicular γ-aminobutyric acid transporter
- **VGLUT.** Vesicular glutamate transporter
- **VLDL.** Very low-density lipoprotein
- **VPA.** Valproic acid

Table of contents

List of Figures and Tables

Figures

Tables

INTRODUCTION

1. INTRODUCTION

Pesticides have been used for many years to control pests. Throughout the second half of the 19th century they were used more and more intensely, and it was not until 1962 that Rachel Louis Carson raised the alarms with her book "*Silent Spring*" (Bertomeu-Sánchez, 2019; Casida and Quistad, 1998). Since then, considerable effort has been made to study these environmental contaminants in order to reduce their use and minimize the adverse risks associated to their exposure.

The first pesticide was synthesized in 1939, but it was not put on the market until the 1950s, "the golden age of pesticides". Organochloride insecticides and, in particular, dichloro-diphenyl-trichloroethane or DDT was the first pesticide to be used in agriculture because of its ability to control pests for long periods of time. However, their harmful effects on the ecosystem, biodiversity, and human health led the United States (US) to ban this chemical compound at the beginning of the 1970s (Casida and Quistad, 1998). Nonetheless, parallel researches were conducted to find similar compounds that were more efficient and less persistent. In 1937, the German chemist Gerhard Schrader discovered the insecticidal properties of organophosphorus (OP) compounds, even though it was not until 1965 that they were firsts used in agriculture. The first OP to be put on sale was tetraethylpyrophosphate (Soltaninejad and Shadnia, 2014).

Organophosphates are also called anticholinesterase compounds because their principal neurotoxic effect is the inhibition of acetylcholinesterase (AChE). This inhibition produces an accumulation of acetylcholine (ACh) in the synaptic cleft, which overstimulates the nicotinic and muscarinic cholinergic receptors in the central and peripheral nervous system (CNS and PNS, respectively), producing a hyperstimulation of the post-synaptic cholinergic neurons (Richardson et al., 2019). In addition, non-cholinergic targets for OPs have also been reported (Terry, 2012). These targets can elicit oxidative stress (Slotkin, 2006; Soltaninejad and Abdollahi, 2009), affect the activity of several neurotrophic molecules (Betancourt and Carr, 2003; Slotkin et al., 2007) or alter such basic neuronal processes as axonal transport (Terry, 2012).

This type of pesticides helped to improve agricultural productivity by controlling insects, nematodes and plants pathogens. However, acute exposure to OP (e.g., occupational exposure) produce such classic signs of poisoning as salivation, lacrimation, diarrhea, bronchorrhea, bradycardia or even death (Pope et al., 2005). In addition, chronic exposure below the threshold of OP toxicity has been linked to impairments in neurobehavior such as anxiety disorders and depression, learning and memory and attention deficits or problems in information processing (De Silva et al., 2006).

All OP chemical compounds are derived from phosphoric, phosphonic, phosphinic or tiophosphoric acid (Balali-Mood and Saber, 2012; Naughton and Terry, 2018). The general structure is shown in Figure 1. It is made up of R_1 and R_2 groups (akyl-, alkoxy-, alkylthio- or amido groups), whereas the R_3

is the acyl residue which is also called the "leaving group" because it dissociates from OP molecules when it reacts with their targets (Balali-Mood and Saber, 2012; Costa, 2006).

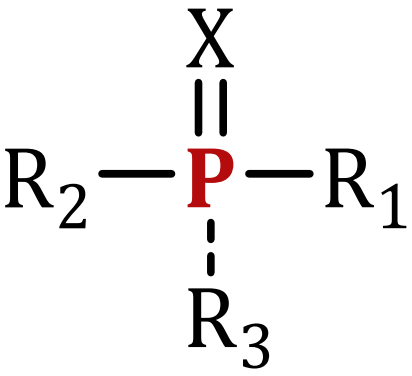


Figure 1. Representation of the general structure of an OP compound. The phosphorus atom has a double bond with sulfur or oxygen (X). Two stable bonds with organic radicals (R_1 and R_2) such as alkyl-, alkylthio-, or amino groups, and one unstable bond with an acyl residue (R_3) such as halide, cyanide, thiocyanide or any other aromatic structure.

1.1. Chlorpyrifos

Since OPs were introduced in the 1960s, one of the most commonly used pesticides worldwide is chlorpyrifos (CPF). It is manufactured by DOW chemicals and can be applied either as a spray or onto the soil before plantation. This different application methods enable CPF to be used in both agricultural and nonagricultural environments. They are used in agriculture mostly to protect important crops like cotton, citrus, corn, peaches, rice, banana, cocoa, coffee, and vegetables and occasionally to control ticks on cattle. On the other hand, they are used in the household to control insects such as cockroaches, fleas, and termites and also as a component of tick and flea collars for pets (Bose et al., 2021; Eaton et al., 2008). However, their excessive use has been related to human health problems which have led to restrictions in its use.

1.1.1. Regulation of CPF

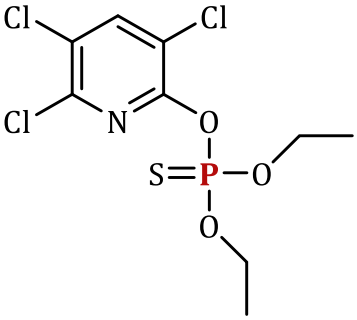
The first restriction was implemented in the US in 2001. Regulations were introduced to reduce the nonagricultural use of CPF to 3 % of total use (Eaton et al., 2008). Furthermore, the European Food and Safety Authority (EFSA) concluded that "there is no safe level of exposure to CPF" thus encouraging regulations to impose stronger restrictions or even ban the use of this pesticide (Bose et al., 2021). In 2019, the same authority stated that there were concerns about the genotoxicity and developmental neurotoxicity of CPF, which led to the withdrawal of the

authorization to use this chemical in the European Union (EU) and a complete ban in 2020 (EFSA, 2019). Along the same lines, the US Environmental Protection Agency (EPA) revoked all tolerance for CPF in 2021 and completely banned it in 2022 (EPA, 2021). Despite this, CPF is still used in developing countries so it may be present in the environment and spread around the world by routes such as sea currents (Georgieva et al., 2021).

1.1.2. Structure and chemical properties of CPF

The CPF compound [O,O-diethyl O-(3,5,6-trichloropyridin-2-yl) phosphonothioate] is a colorless to white crystalline solid which has a mercaptan-like odor. It is insoluble in water, but soluble in most organic solvents (e.g., isooctane or methanol). It is also stable under neutral and acidic conditions, so the chance of hydrolysis increases at higher levels of pH (Bose et al., 2021). CPF also binds rapidly to soil and plants and degrades quickly in the environment (Eaton et al., 2008). Table 1 summarizes other physical and chemical properties of CPF.

Table 1. Physical and chemical properties of CPF.

Molecular structure		**Molecular formula**	$C_9H_{11}Cl_3NO_3PS$
		Chemical name (IUPAC)	O,O-diethyl O-(3,5,6-trichloropyridin-2-yl) thiophosphate
		Cas number	2921-88-2
		Molecular weight	350.6 g/mol
		Density	1.44 g/cm^3 at 20 ºC
		Boiling point	No boiling point at normal pressure but decomposes at 160 ºC
		Melting point	41-42 ºC
		Vapor pressure	1.87 x 10^{-5} mmHg at 25 ºC

Adapted from National Center for Biotechnology Information (NCBI, 2022)

The structure of CPF is based on a phosphorus atom with five bonds. The pentameric phosphorus structure has a double bond with sulfur, two stable bonds with two ethyl groups and one unstable bond with an aromatic structure which is released during the biotransformation process (Karalliedde et al., 2003).

1.1.3. Degradation and exposure pathways

Although restrictions on CPF have recently been introduced, their use has significantly increased every year. CPF and its metabolites are susceptible to a wide variety of degradation pathways such as photolysis, volatilization, or abiotic hydrolysis (Barman et al., 2014; Bose et al., 2021). The half-life of CPF ranges from 3 hours to 150 days (Table 2). In air, where CPF is in contact with a considerable variety of environmental factors that contribute to its degradation, it has a half-life of 3 h, but when there is no light, water, or other factors it can be present for months (Eaton et al., 2008; Mackay et al., 2014). However, concentrations of CPF have also been also detected in air and other environmental sources far from the usual agricultural sites, indicating that CPF has potential for long-range transport (Mackay et al., 2014).

Table 2. Half-life of CPF in different environmental sources.

Environmental source	Half-life
Air	3 hours
Soil	from 7 to 30 days
Surface water	from 30 to 50 days
Sediment	from 50 to 150 days

Adapted from Mackay et al. (2014)

Agricultural workers and their families are the group with the greatest risk of exposure to OPs because of their proximity during the application of the compound. However, the general population can be exposed through three pathways: ingestion, inhalation, or dermal absorption (Suratman et al., 2015). In general, ingestion is the most common pathway accounting for 70% of total exposure trough dietary residues in food. On the other hand, dermal absorption accounts for only 3% of exposure, although it is a common occupation pathway of exposure (as is inhalation) (Albers et al., 1999; Bose et al., 2021; Fenske et al., 2012). Besides, the lipophilic property of CPF enable it to cross the placenta or be stored in breast milk, so it is the main pathway of infant exposure (Rauh et al., 2006; Sanghi et al., 2003; Saunders et al., 2012).

Although it is difficult to set an average daily exposure to CPF, in 2005 the EFSA set the acceptable daily intake at 0.01 mg/kg/day. In 2014, this was revised and modified to 0.001 mg/kg/day (EFSA, 2014). Along the same lines, the US Department of Agriculture and the US Food and Drug Administration estimated a "typical" dietary daily exposure for various age groups. The estimated average was 0.005 μg/kg/day for the adult population, 0.014 μg/kg/day for toddlers and around 0.009 μg/kg/day for infants (Eaton et al., 2008).

1.1.4. Absorption, distribution, biotransformation, and excretion

As reported above, CPF is a lipophilic compound with different routes of exposure in humans an experimental animals. Depending on the route, the pesticide can be absorbed by the skin, the intestine or the lungs (Tanvir et al., 2016). Higher concentrations of CPF are found in fat and fatty tissues such as liver, brain, kidney or adipose tissue where the pesticide has a half-life of 62 h (Eaton et al., 2008; Suratman et al., 2015; Tanvir et al., 2016). In addition, due to their high lipophilicity, pesticides can be readily absorbed through respiratory or oral routes, although less efficiently through the skin. In humans, 70 % of total CPF exposure can be recovered through the urine after oral exposure, whereas only around 1 % can be recovered after dermal exposure. However, it should be noted that a relatively large amount of CPF is recovered by washing the skin after dermal exposure (42-67 % of the dose applied) (Griffin et al., 1999; Meuling et al., 2005; Nolan et al., 1984). In this respect, dermal exposure presents a half-life of 30 h with an optimum sampling time between 17 and 24 h, while oral exposure shows a shorter half-life of 15.5 h with an optimum sampling time of 7 h (Griffin et al., 1999; Nolan et al., 1984). On the other hand, CPF also binds to proteins such as plasma albumin with a short biological half-life of approximately 18 h (Suratman et al., 2015).

Biotransformation of CPF can be through aerobic and anaerobic metabolism. However, CPF is metabolically activated, principally, in the liver by the activity of cytochrome P450, which mediates the oxidative desulfurization of CPF to the oxon form. Alternatively, CPF can also be detoxified by the activity of cytochrome to produce 3,5,6-trichloro-2-pyridinol (TCPy) and di-ethyl thiophosphate (DETP). On the other hand, CPF-oxon can be hydrolyzed by an A- or B-esterase such as paraxonase 1 (PON1) to TCPy and di-ethyl phosphate (DEP) (Figure 2) (Smith et al., 2014).

The elimination of CPF is mainly through the kidney. Once CPF has entered to the body, it is rapidly distributed and metabolized in the liver to produce lesser toxic compounds that are largely eliminated through urine. Of all the OP metabolites, 84 % are eliminated through urine, whereas only 5 % are excreted through feces (Rathod and Garg, 2017). However, the percentage of metabolites in urine varies. While TCPy accounts for 62 % of the total excretion, DETP accounts for 40 % and DEP for only 4 % (Timchalk et al., 2007). Furthermore, there are other excretion routes such as breast milk (Brahmand et al., 2019).

1.1.5. Principal targets and mechanism of action

The principal mechanism of CPF neurotoxicity is the inhibition of AChE. Before acting on the cholinergic system, CPF is metabolically converted into the active CPF-oxon form. CPF-oxon inhibits the activity of AChE by phosphorylating the serine hydroxyl group located in the active site of the enzyme. Thus, CPF-oxon loses its “leaving group” and establishes a covalent bond with

the serine hydroxyl of AChE. This phosphorylation is very stable and persists over time. In fact, in biological systems the enzyme can remain inactive for hours, days or even weeks (Mileson, 1998). Besides, OPs not only inhibit brain AChE, they also bind to red blood cells AChE and plasma butyrylcholinesterase, the second target of OP chemicals (Singleton et al., 2015).

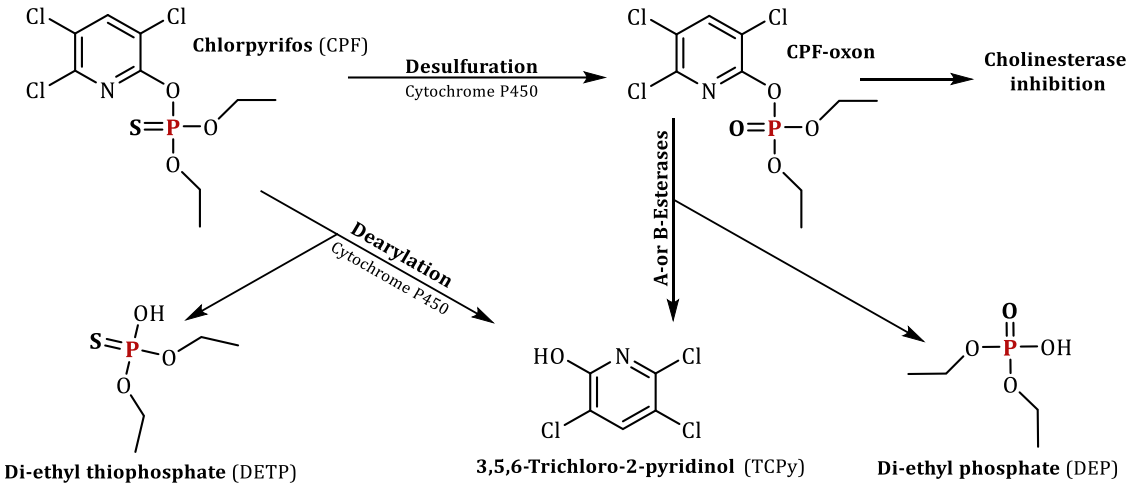


Figure 2. Biotransformation pathways involved in the degradation of CPF. Cytochrome P450 desulfurizes CPF to produce 3,5,6-trichloro-2-pyridinol (TCPy) and di-ethyl phosphate (DEP). CPF can also be dearylated to produce TCPy and di-ethyl thiophosphate (DETP). *Adapted from* Smith et al. (2014)

As mentioned above, this inhibition decreases the hydrolysis of ACh in the cholinergic synapses, leading to an accumulation of the neurotransmitter in the synaptic cleft. Consequently, this accumulation overstimulates both cholinergic receptors, and downregulates the post-synaptic membrane (Mileson, 1998; Singleton et al., 2015). It can also alter the function of somatic motor neurons, which causes seizures, brain damage or either necrosis or apoptosis (Miller et al., 2021). However, the effects of ACh accumulation depend on the distribution of CPF in the body and the cholinergic receptor type to which ACh binds.

1.1.5.1. Cholinergic system

The cholinergic system consists of several areas with high ACh density that express the different cholinergic receptors and generate neuronal projections. ACh is synthesized by the activity of the choline acetyltransferase (ChAT) enzyme which catalyzes the acetylation of choline with acetyl-CoA. Then, ACh is accumulated and released into the synaptic cleft through the vesicular acetylcholine transporter (VAChT) where it binds to both pre- and post-synaptic muscarinic (mAChR) and nicotinic (nAChR) receptors (Figure 3) (Deiana et al., 2011).

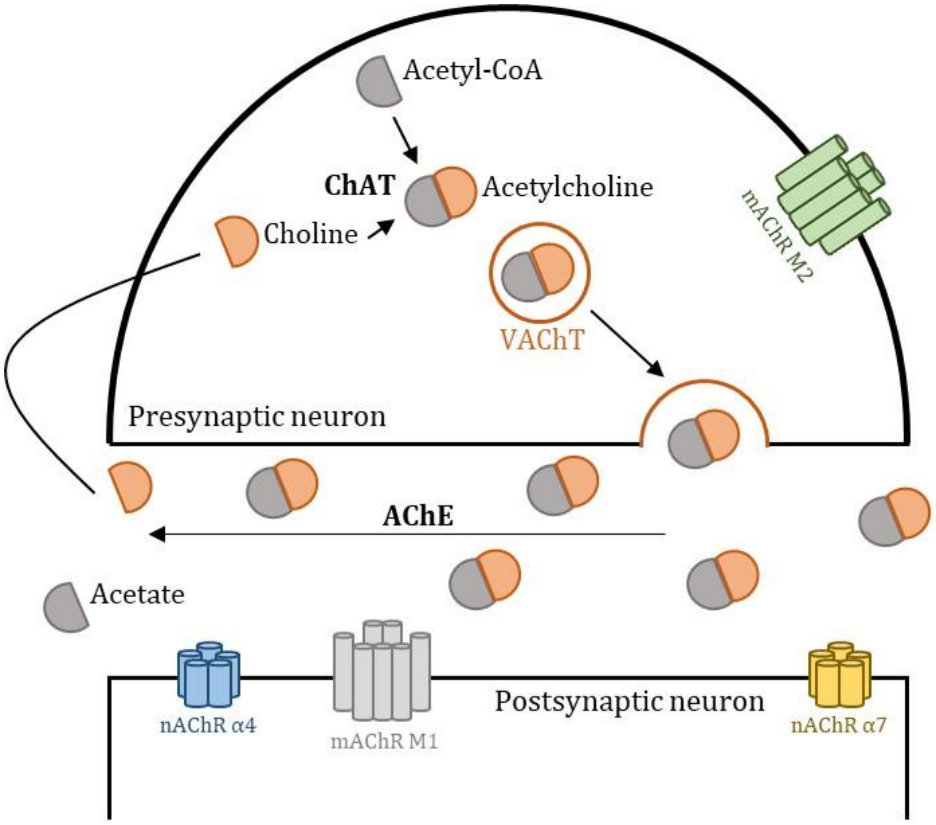


Figure 3. Schematic representation of a cholinergic synapse. In the presynaptic neurons, ACh is synthesized by the activity of ChAT, which catalyzes the acetylation of choline and acetyl-CoA. Then, ACh is released into the synaptic cleft though the VAChT and binds to mAChR and nAChR located either in the presynaptic or postsynaptic neurons. To end the transmission, the AChE enzyme hydrolyzes the ACh to choline and acetate. Abbreviations: AChE, acetylcholinesterase; ChAT, choline acetyltransferase; mAChR, muscarinic acetylcholine receptors; nAChR, nicotinic acetylcholine receptors; VAChT, vesicular acetylcholine transporter.

Muscarinic receptors belong to the family of metabotropic receptors, which has five distinct transmembrane subtypes denoted by M_1, M_2, M_3, M_4 and M_5. Three of these subtypes (M_1, M_3 and M_5) interact with G-protein and couple to phospholipase C, whereas the other two subtypes (M_2 and M_4) interact with adenylate cyclase (Deiana et al., 2011; Kruse et al., 2014). Besides, muscarinic receptors are widely distributed through the human body and play an important role in such physiological functions as heart rate regulation, smooth muscle contraction, or glandular secretion (Caulfield and Birdsall, 1998; Kruse et al., 2014). They also play a role in multiple brain signaling pathways such as neuronal excitability modulation, and ACh synaptic regulation and feedback (Abrams et al., 2006). Although mAChR subunits are expressed differently in different brain regions, all five muscarinic subtypes are found in brain. M_1 receptors are the dominant subtype in the CNS and are most abundant in the neocortex, hippocampus, and amygdala. M_2 receptors are found throughout the brain, most abundant in the hypothalamus. M_3 receptors are

expressed less in the brain, even though they are found in the hippocampus and thalamus. M_4 receptors are expressed in the cortex and striatum, whereas M_5 receptors are abundant in the hippocampus and thalamus (Abrams et al., 2006; Deiana et al., 2011).

Neuronal nicotinic receptors are a class of membrane proteins with different subtypes that form ligand-gated ion channels. The pentameric form of those ion channels consists of nine α (α2-α10) and three β (β2-β4) subunits, which are arranged symmetrically around the central pore (Changeux et al., 1998; Deiana et al., 2011; Gotti et al., 2006; Shabani et al., 2020). Two different nAChR subfamilies have been identified: the α-bungarotoxin-sensitive subfamily which includes homopentameric (α7, α8 and α9) and heteropentameric (α7α8 and α9α10) receptors, as well as the αbungarotoxin-insensitive subfamily which receptors are only heteropentameric and consist of α (α2-α6) and β (β2-β4) subunits (Gotti et al., 2006). However, this family of cationic channels regulates processes such as cellular excitability, exocytosis, motility, apoptosis or transcription (Shen and Yakel, 2009). The nAChR receptors also influence physiological functions such as sleep, fatigue or anxiety and their subunits are differently distributed within the brain (Gotti et al., 2006). Although α4, α7 and β2 are mostly expressed in the hippocampus, they are also expressed in the thalamus and spinal cord (α4), or in the hypothalamus and cortex (α7) (Deiana et al., 2011; Gotti et al., 2006; Gotti and Clementi, 2004).

Once ACh is in the synaptic cleft and is acting on the cholinergic receptors, the AChE enzyme hydrolyzes the ACh neurotransmitter to produce acetate and choline. The activity of AChE serves to terminate the synaptic transmission (Deiana et al., 2011; Lionetto et al., 2013). Therefore, AChE is mainly found at the cholinergic synapses of the CNS, and at the neuromuscular junctions. It also plays a crucial role in the normal functioning of both PNS and CNS (Lionetto et al., 2013).

AChE is made up by two distinct domains: a large catalytic domain with around 500 residues, and a small C-terminal peptide with fewer than 50 residues (Lionetto et al., 2013). AChE is the product of a single gene located in chromosome 7 in humans and in chromosome 5 in mice (Getman et al., 1992; Rachinsky et al., 1992). Besides, alternative splicing in the 3' site of primary transcripts generates several types of AChE, with the same catalytic domain but a different C-terminal, which influences the localization and molecular form (Lionetto et al., 2013; Massoulié, 2002). The different isoforms are: "synaptic" or S transcript (AChE-S), "readthrough" or R transcript (AChE-R) and AChE-E, the erythrocytic transcript. The AChE-S is typically tetrameric and is strongly expressed in all the tissues except for thymus, liver and small intestine where is poorly expressed. The AChE-R is a monomeric enzyme, and the AChE-E is a glycophosphatidylinositol-like dimer associated with red blood membranes. Both transcripts are highly expressed in all brain regions and poorly expressed or absent in testis, thymus and intestine (Grisaru et al., 1999; Massoulié, 2002; Meshorer et al., 2004).

1.1.5.2. Other CPF targets

Apart from the cholinergic system, there are other non-cholinergic targets related to other serine hydrolases. Both CPF and CPF-oxon inhibit the activity of carboxylesterases a family of enzymes that hydrolaze esters and amide bonds, as well as monoacylglycerol lipases (MAGL) and fatty acid amide hydrolases (FAAH), which metabolize two of the major endocannabinoids: the anandamide and 2-arachidonolyglycerol (Casida and Quistad, 2004, 2005, Quistad et al., 2001, 2002b). Along the same lines, OP compounds also disrupt the activity of cannabinoids by other mechanism such as the inhibition of cannabinoid receptor activity (Quistad et al., 2002a). In addition, CPF and CPF-oxon are associated with OP compound-induced delayed neurotoxicity which paralyses the lower limbs, leads to partial sensory loss, and degenerates long axons in the spinal cord and peripheral nerve. These symptoms become evident after 10 to 15 days of exposure (Johnson and Glynn, 1995). This neurotoxic effect has been associated with the inhibition of more than 80 % of neuropathy target esterase, a serine esterase involved in cell-signaling during the development of the nervous system (Glynn, 1999).

On the other hand, CPF pesticide has also been identified as an endocrine disruptor due to its activity as an androgen receptor antagonist and its suppressor activity in gene expression related to gonadotropin synthesis or steroidogenesis, as well as the association with thyroid gland changes (Otênio et al., 2022; van den Berg et al., 2011). Furthermore, CPF inhibits DNA synthesis in neuronotypic PC12 and gliotypic C6 cells (Qiao et al., 2001), disrupts the activation of distinct peroxisome proliferator-activated receptors responsible for the regulation of genes involved in lipid metabolism and energy homeostasis (Herriage et al., 2022), or induces oxidative stress by the formation of reactive oxygen species (Chiapella et al., 2013). Additionally, CPF has recently been associated with a decrease in blood glucose levels, which produces hypoglycemia (Farkhondeh et al., 2020).

1.1.6. CPF and development

Individual factors such as body weight, age or genetic background confer different vulnerabilities to pesticide toxicity. Studies on CPF exposure at distinct lifetime stages have shown that the young population is more sensitive to the toxic effects of CPF than adults (Moser, 2000). The considerable sensitivity of embryos and fetuses to toxics, as well as the higher risk observed in newborns and children, have led to study, in more detail, the exposure to neurotoxic compounds during pregnancy and developmental stages (Abdel-Rahman et al., 2002; Grandjean and Landrigan, 2014).

This differences in sensitivity between young people and adults could be due to differences in the biotransformation of CPF. Studies in mice have reported that fetuses are critically dependent on

maternal PON1 activity. Maternal PON1 increases through pregnancy, but it is at very low levels in newborn mice and it is not until postnatal day (PND) 21 that these levels increase (Costa et al., 2005). Thus, low PON1 activity levels may increase the severity of exposure to CPF, and its toxic effects (Kofman et al., 2020). In addition, exposure during pregnancy and the firsts developmental stages coincides with the development of the nervous system. According to the literature, mice are exposed to OP pesticides over a long period of time (from around gestational day (GD) 12 to PND 15), which corresponds to the second and third trimester of human pregnancy, as well as the first months of life (Azad et al., 2017; Richard and Flamant, 2018). The nervous system starts to develop with the formation of the neuronal tube which in mice takes place on GD 9.5. Neuronal tub formation coincides with the beginning of neurogenesis (GD 10) which ends before adolescence (PND 15-20) (Chen et al., 2017; Rice and Barone, 2000). However, each brain region has a different peak of neuronal production. For example, in the cerebral cortex, neurogenesis occurs from GD 12 to 17, while in the hippocampus it occurs between GD 12 and 17 (CA2 region and pyramidal cells) and from GD 17 to PND 15 (dentate gyrus) (Chen et al., 2017; Semple et al., 2013). In addition, there are other brain processes such as synaptogenesis or neuronal migration that begin around GD 11 and continue until adolescence, whereas gliogenesis and myelination start between the first and the third week after birth (Schepanski et al., 2018; Semple et al., 2013). Therefore, the period in which CPF exposure occurs could condition short- and long-term adverse effects, since prenatal and postnatal exposure cover two distinct critical windows of CNS development.

Epidemiological studies of populations living near to or far from agricultural areas provide evidence about CPF toxicity. Studies of the Chinese population have shown that prenatal CPF exposure affects gross and fine motor function in nine-month-old infants. The proper acquisition of motor skills has been associated with the correct development of cognitive and neurological processes (Silver et al., 2017), so it is not surprising that other studies that evaluate the same exposure period have observed deficits in cognitive functioning in seven-year-old children (Rauh et al., 2011). Moreover, studies conducted in an inner-city cohort from New York have shown that prenatal exposure to the pesticide CPF generates lower scores on the psychomotor and development assessment test, and a higher incidence of attention problems and attention-deficit/hyperactivity disorder in three-year-old children, whereas the same exposure has been linked to poorer levels on the Working Memory Index and Full-Scale IQ in seven-year-old children (Rauh et al., 2015, 2006). Apart from these adverse effects, in the last few years, it has been suspected that the toxics present in environment increase the risk of neurodevelopmental disorders. In line with this hypothesis, von Ehrenstein et al. (2019) found a moderately higher risk of autism spectrum disorder (ASD) in the offspring of mothers treated prenatally with CPF. Likewise, exposure during early childhood has been associated with social and motor deficits in boys, indicating that they seem to be more sensitive to CPF toxicity than girls (Guo et al., 2019).

Studies in animal models corroborate these epidemiological results. In mice, prenatal CPF exposure (from GD 12 to 15) below the threshold of fetal growth impairments, has been associated with a delay in motor development and impaired conditioned and innate social behaviors in a sex-dependent manner, being males the most affected (Lan et al., 2019). Other studies assessed prenatal exposure during late gestation (from GD 15 to 18) and exposed animals to a dose (6 mg/kg/day) that inhibits 75 % of AChE serum activity for around 24 h and reduces AChE brain activity by 60 % in pregnant dams. They showed diminished responsiveness to distress condition and a reduction in motor activity (Venerosi et al., 2009). Moreover, studies that reported both prenatal (from GD 15 to 18) and postnatal exposure (from PND 11 to 14) to CPF associated gestational exposure with an increase in motor activity during adulthood and aggressive behaviors in males, whereas late postnatal exposure enhanced maternal response in females and decreased anxiety response in both sexes. These findings indicate that the period in which CPF exposure takes place conditions the sex-specific effects (Ricceri et al., 2006). Exposure during the same late postnatal preweaning developmental stage is also associated with adult but not adolescent alterations in social novelty. Novel exploration decreased in male rats but increased in females (Perez-Fernandez et al., 2020a).

Exposure during developmental stages may also lead to changes in the normal maturation of several systems. Exposure to low doses of CPF (1 or 5 mg/kg/day) during the peak period of sexual differentiation of the brain (from GD 17 to 20) produce alterations in presynaptic serotonergic and dopaminergic activity in adolescent rats. However, no effects were observed when exposure took place during the early gestational period (from GD 9 to 12) (Slotkin and Seidler, 2007). On the other hand, postnatal exposure (from PND 10 to 16) at a dose of 0.75 mg/kg/day of CPF is associated with an alteration in the excitatory/inhibitory (E/I) balance, altering the glutamatergic and GABAergic signaling during adolescence (Alugubelly et al., 2021). Besides, late postnatal exposure to 1 mg/kg/day for six consecutive days has also been associated with medium- and long-term microbiota dysbiosis at both genus and family taxonomical levels (Perez-Fernandez et al., 2020a, 2020b), while lower exposure levels (0.5 mg/kg/day) have been associated with the inhibition of FAAH and MAGL because they increase both principal endocannabinoids and overstimulate this system, which results in inappropriate neurotransmission that can lead to neurotoxicity (Carr et al., 2020).

1.2. Neurodevelopmental disorders and autism spectrum disorder

The Word Health Organization describes neurodevelopmental disorders as behavioral and cognitive disorders that begin during the developmental period and are characterized by significant difficulties in acquiring and executing specific intellectual, motor, language, or social functions. The etiology of these disorders is complex, and in some cases unknown. However, some hypotheses

point to genetic factors or others such as lack of appropriate environment or injuries to the CNS during the developmental period, which lead to inadequate brain development (ICD-11, 2022). In addition, several disorders in this group share symptomatology, so it is more difficult for them to be diagnosed and treated. Nowadays, treatment is based on a combination of professional, therapy, pharmaceutical and home- and school-based programs. Some of the disorders that are under the umbrella of neurodevelopmental disorders are attention deficits/hyperactivity disorder, schizophrenia and ASD (Mullin et al., 2013).

1.2.1. ASD: Diagnosis, etiology, and prevalence

In 1943, autism was first defined by Leo Kanner (Kanner, 1968). Since then, it has been considered as a rare disorder of childhood onset, but nowadays, ASD is recognized as a common and heterogeneous disorder that can range from very mild to severe and which sometimes has an indirect impact on education and employment opportunities, requiring lifelong care and support (Lord et al., 2018; WHO, 2022).

ASD is a behaviorally defined disorder, characterized by difficulties in social interaction and communication, and repetitive or atypical patterns of behavior such as unusual reactions to sensations (WHO, 2022). Adverse effects of the disorder may be detected early around 18- and 24-months of age, but diagnoses are often made later because people with autism sometimes have co-occurring conditions such as anxiety, depression, epilepsy, hyperactivity or mood disorders (Lord et al., 2018; WHO, 2022). Moreover, the fact that individuals with ASD are different from one another and there are no related biomarkers makes diagnosis in the early stages of development difficult. However, nowadays autism disorder can be diagnosed by a large variety of professionals, who largely base their decisions on clinical and physical exams (Lord et al., 2018; Tidmarsh and Volkmar, 2003).

Until recently, the tools for diagnosing autism were neither precise nor reliable. In 2013, the Diagnostic and Statistical Manual of Mental Disorders 5th edition presented a validated ASD diagnostic tool, that had not been a part of previous editions (Grzadzinski et al., 2013). Even so, professionals also use other tests such as the Children's Autism Rating Scale, the Social Responsiveness Scale and the Social Communication Questionnaire to evaluate children's symptoms. Moreover, parents have to be interviewed using the Autism Diagnostic Interview-Revised, in conjunction with standard diagnostic instruments such as the Screening Tool for Autism in Toddlers and Young Children and the Autism Diagnostic Observation Schedule-Generic, which provides direct-observation measures (Tidmarsh and Volkmar, 2003). Other tests are also performed to assess co-occurring conditions. Intelligence and adaptative behaviors can be assessed using the Wechsler Preschool and Primary Scale of Intelligence-Revised, the Wechsler

Intelligence Scale for Children, the Stanford-Binet Intelligence Scale or the Mullen Scales of Early Learning. Speech and language can be assessed by the Peabody Picture Vocabulary Test-Revised or the Reynell Developmental Language Scales, whereas motor functioning and coordination are assessed by the Beery-Buktenica Developmental Test of Visual-Motor Integration. Finally, in order to understand how autism patients respond to environmental stimuli, professionals can perform the Sensory Integration and Praxis Test, which assess acoustic and tactile problems as well (Lord et al., 2018; Tidmarsh and Volkmar, 2003).

Nowadays, the etiology of autism is still unknown. Nevertheless, the prevalence of the disorder has been increasing in the last few years. Although the incidence of ASD varies from one country to another, Elsabbagh et al. (2012) reported a global prevalence of 62/10,000 in 2012, whereas a review in 2022 reported a small increase to 65/10,000 (Zeidan et al., 2022). Furthermore, autism occurs predominantly in males. For every four diagnoses only one is a female. (Chaste and Leboyer, 2012). However, this increase in prevalence leads to new research on risk factors that may explain it.

Genetic, environmental, biological or developmental factors have been associated with ASD (Currenti, 2010). Until 1980, genetic was not thought to contribute to the etiology of autism, but the co-occurrence of rare genetic ASD syndrome (e.g., fragile X chromosome) and studies conducted in twins suggest that it might (Abrahams and Geschwind, 2008). Moreover, it was observed that prenatal and perinatal environmental factors such as maternal and paternal ages, maternal gestational diabetes, hypertension, or maternal medication with an antiepileptic drug or antidepressants selective serotonin-reuptake inhibitors, were strongly related to an increased risk of ASD in offspring (Chaste and Leboyer, 2012; Lord et al., 2018).

1.2.2. ASD hypothesis and theories

As mentioned above, the etiology of autism is currently unknown, but it has been increasingly studied in recent years. ASD encompasses a wide range of symptoms, as well as theories and hypotheses. Some of these theories are based on the fact that autism is a heterogeneous and complex genetic disorder with hundreds of associated genes. Recently, it has been observed that some genetic alterations can aggravate or improve the severity of the disorder, depending on the individual. This has led some to hypothesize that genetic modifiers such as environmental factors, epigenetics, double-hit mutations, copy number variants or sex-linked modifiers may explain the heterogeneity of the disorder (Rylaarsdam and Guemez-Gamboa, 2019).

On the other hand, other hypotheses try to explain the sexual bias observed in autism through sex-specific pathways. It is well-known that there are behavioral and anatomical differences between

males and females that could be related to sex chromosomes or hormones (Ferri et al., 2018). Some theories suggest that autism is an X-linked disorder with a protective effect when both X chromosomes are present, while a Y chromosome might be a risk factor for the development of the disorder (Werling and Geschwind, 2013). In addition, it has been hypothesized that ASD may be the result of disrupted hormonal balance. Increased testosterone during the early stages of the development, for example, may produce a hypermasculinized autistic brain (Ferri et al., 2018; Ostatníková et al., 2021; Werling and Geschwind, 2013). This hypothesis can be explained by the retinoic acid-related orphan receptor alpha (RORA) which is a hormone-dependent transcription factor that regulates aromatase expression. This aromatase enzyme is responsible for the transformation of androgens into estrogens, and it is oppositely regulated by both sexual hormones. Estradiol activates aromatase activity, while testosterone inhibits it. Therefore, a RORA deficiency may increase testosterone levels and cause the hypermasculinized brain observed in ASD (Hu et al., 2015; Sarachana et al., 2011; Werling and Geschwind, 2013).

In 2003 Rubenstein and Merzenich (2003) hypothesized that ASD might be caused by alterations in the E/I balance leading to neuronal hyperexcitability due to an upregulation of the glutamatergic system and a downregulation of the GABAergic system (Nelson and Valakh, 2015; Sohal and Rubenstein, 2019; Uzunova et al., 2016).

1.2.2.1. GABAergic system

The γ-aminobutyric acid (GABA) neurotransmitter is considered to be the main inhibitory neurotransmitter in the adult brain. It exerts an important role in cell proliferation, migration and differentiation, and also in synapse formation and maturation (Di et al., 2020; Florey, 1991; Owens and Kriegstein, 2002; Wu and Sun, 2015). GABA is synthesized by the decarboxylation of glutamate through the activity of the glutamic acid decarboxylase (GAD) enzyme. Then, the vesicular GABA transporter (VGAT) releases the neurotransmitter into the synapse through exocytosis, and enables GABA to exert its effects by binding to their receptors (GABA-A, GABA-B or GABA-C) (Figure 4) (Rowley et al., 2012).

Once GABA has been released into the synapse, it binds to its ionotropic or metabotropic GABA-A, GABA-B or GABA-C receptors. The GABA-A receptors are ligand-gated chloride ion channels with a heteropentameric structure that consist of different combinations of α (α1-6), β (β1-4), γ (γ1-4), δ, ɵ, ρ (ρ1-3), π and ϵ subunits which can be localized in the post-, pre- or extrasynapse (Di et al., 2020; Wu and Sun, 2015). For example, GABA-A α1, α2 and γ2 subunits are localized in the synaptic cleft. They produce a phasic response characterized by a fast reaction to the high neurotransmitter concentrations release into the synapse. On the other hand, α4, α5, α6 and δ subunits are localized in the extrasynapse and produce a tonic response characterized by a slow

persistent reaction to the low neurotransmitter concentrations, due to the high affinity of the extrasynaptically receptors for GABA neurotransmitter (Lee and Maguire, 2014; Pizzarelli and Cherubini, 2011; Yu et al., 2018). However, Bohlhalter et al. (1996) also co-localized the α2 subunit in the presynaptic membrane, where it acted as an autoreceptor to control GABA release.

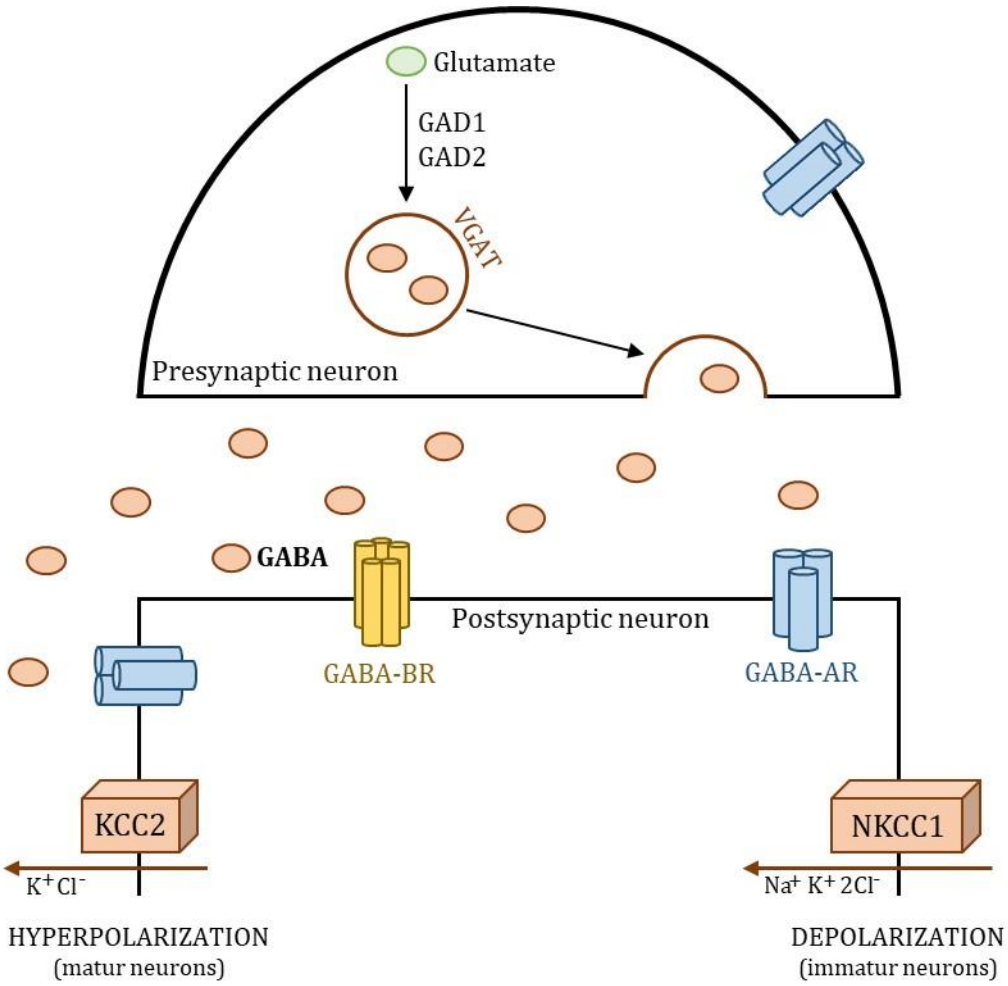


Figure 4. Schematic representation of a GABAergic synapse. GABA is synthesized in the presynaptic neuron from a pool of glutamate and by the activity of the GAD enzyme. Then, GABA is released into the synapse through the VGAT and binds to the GABA-A, GABA-B or GABA-C receptors. The binding of GABA to GABA-A receptors produces considerable import or export of chloride ions that is age-regulated by two GABA co-transporters, NKCC1 and KCC2. Abbreviations: GABA-R, γ-aminobutyric acid receptor; GAD, glutamic acid decarboxylase; VGAT, vesicular GABA transporter.

In addition to the ionotropic GABA receptor, GABA-B are metabotropic transmembrane receptors that are linked to potassium channels via a G-protein mediated pathway. They can be found in the pre- and postsynapse and are made up of two different subunits: the GABA-B1 and

GABA-B2. There are also GABA-C receptors which are made up of a homopentameric structure with three ρ subunits (Di et al., 2020; Olsen and Sieghart, 2008).

GABA circuits are formed in the embryonic stages and develop and mature further after birth. In fact, the binding of GABA neurotransmitter to GABA-A receptors produces an import and export of chloride ions that are, respectively, regulated by the GABA co-transporters, NKCC1 and KCC2. During brain maturation, GABA activity is majority excitatory and exerts a depolarizing action due to the high expression of the neuronal co-transporter NKCC1, which leads to higher chloride levels inside the neuron. Around PND 5-10, adult GABA neurons switch their activity from excitatory to inhibitory, and have a hyperpolarizing action which is a consequence of the increase in KCC2 and the decrease of NKCC1 co-transporter expression, which lead to higher chloride levels outside the neuron (Ben-Ari et al., 2012, 2007; Lemonnier et al., 2017).

Furthermore, GABA-related components are regulated differently during brain development. In rodents, it has been shown that in embryonic stages predominates the GAD1 isoform, while during postnatal maturation GAD2 isoform is more abundant (Jiang et al., 2022). Along the same lines, α1 and β3 subunits expression has been found to be low at birth, but to increase during the first postnatal week, whereas, contrarily, α2 and α5 subunits expression decreases after birth (Fritschy et al., 1994; Laurie et al., 1992).

1.2.2.2. Glutamatergic system

Unlike GABA, glutamate is the primary excitatory neurotransmitter in brain (Luján et al., 2005). It plays a central role in fundamental brain functions such as synaptic plasticity, formation of neuronal networks during development and CNS repair (Meldrum, 2000). Glutamate is released into the synapses by the vesicular glutamate transporter (VGLUT). Subsequently, the neurotransmitter can bind to its ionotropic and metabotropic receptors (Figure 5) (Luján et al., 2005).

There are three different ionotropic receptors. The first is the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor, which forms a tetrameric complex with the four GluR subunits (GluR1-4). The second is the N-methyl-D-aspartate (NMDA) receptor formed by an assembly of different GluN1, GluN2 (GluN2A-D) and GluN3 (GluN3A-B) subunits, whereas the third is the Kainite (KA) receptor, formed by five distinct functional subunits (GluR5-7 and KA1-KA2) (Hollmann and Heinemann, 1994). In addition, these ionotropic receptors are generally found in the postsynaptic membrane. However, KA subunits can be localized to both pre and postsynaptic sites (Darstein et al., 2003; Luján et al., 2005). On the other hand, metabotropic glutamate receptors, which consist of eight different subunits, are classified into three groups,

according to their sequence homology or pharmacological profile (group I (mGlu1 and mGlu5), group II (mGlu2 and mGlu3) and, finally, group III (mGlu4 and mGlu6-8)) (Luján et al., 2005).

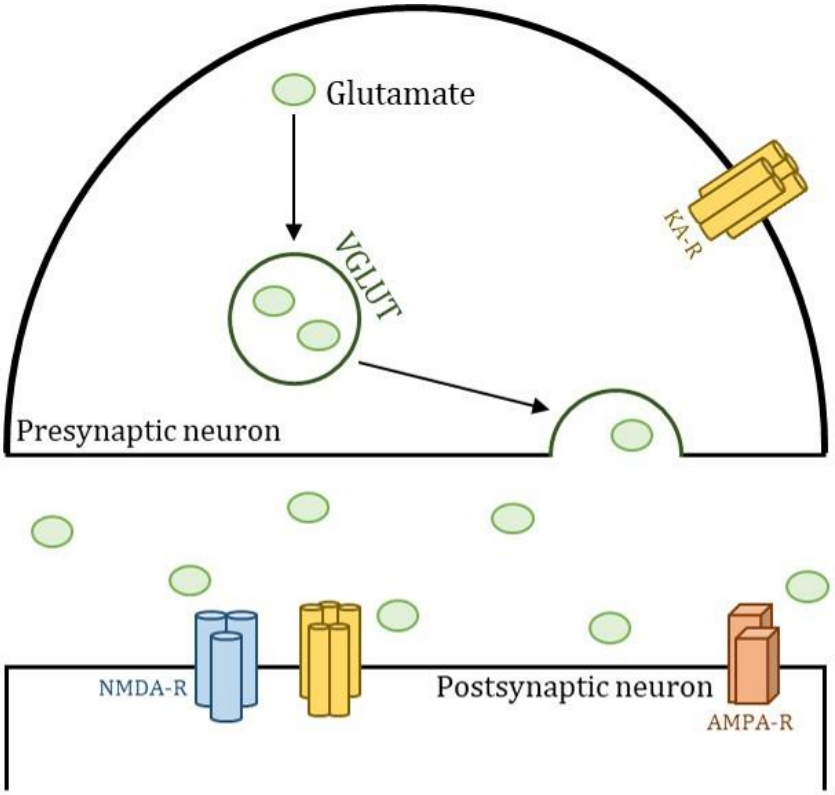


Figure 5. Schematic representation of a glutamatergic synapse. Glutamate is released into the synaptic cleft through the VGLUT and binds to either ionotropic (AMPA, NMDA or KA) or metabotropic receptors located in the presynaptic and postsynaptic neurons. Abbreviations: AMPA-R, 3-hydroxy-5-methyl-4-isoxazole propionic acid receptor; KA-R, kainate receptor; NMDA-R, N-methyl-D-aspartate receptor; VGLUT, vesicular glutamate transporter.

As was reported for the GABAergic system, glutamate subunit expression depends on the brain region and the developmental stage in which it is evaluated (Luján et al., 2005). All KA receptor subunits, except for KA1, seem to be expressed in the embryonic brain and downregulated during the postnatal period, as are the GluR2 and GluR3 AMPA subunits. On the other hand, KA1 and GluR4 subunits increase their expression during postnatal development and in the adult brain, whereas the expression of GluR1 is constant throughout brain development (Bahn et al., 1994; Martin et al., 1998; Métin et al., 2000). Regarding NMDA subunit receptors, both GluN2 and GluN3 presented different developmental expressions. The GluN2A and GluN2C subunits are widely expressed in brain after birth, while GluN2B and GluN2D subunits are abundantly expressed in embryonic and neonatal brain (Takai et al., 2003; Watanabe et al., 1993). Nevertheless, the GluN3 subunit has a peak of expression during late gestation and early

postnatal brain development, indicating that their expression is more abundant during early stages than during adulthood (Sun et al., 1998).

1.2.3. Autism animal models

Although autism has a strong genetic component, some cases (known as idiopathic) are related to environmental factors such as toxins, pesticides, infections or *in utero* exposure to drugs. This, lead to the generation of both, pharmacological and genetic animal models of autism, which are based on behavioral observations resembling the three core impairments of the disorder (Chaliha et al., 2020; Nicolini and Fahnestock, 2018; Silverman et al., 2010).

Rodents have been used as animal models as they make it possible to carry out experiments that cannot be carried out in humans. This experiments give greater insight into the mechanism underlying the behavioral manifestation of autism (Crawley, 2012; Silverman et al., 2010). Nevertheless, the small differences between species must be taken into account (e.g., drug metabolism, genetics, or biochemical pathways) (Crawley, 2012).

1.2.3.1. Pharmacological animal models

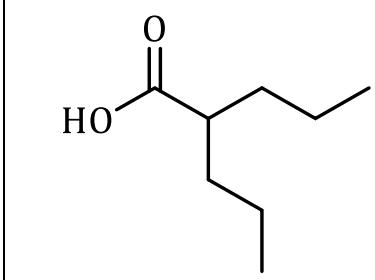
The first pharmacological animal model of autism was developed in 1987 by Vorhees (1987), who exposed pregnant rats to different doses (from 0 to 600 mg/kg) of valproic acid (VPA). His findings support the view that VPA is a teratogenic compound which at high doses can cause offspring malformations or pregnancy loss. However, it was not until 1996 that Rodier et al. (1996) described the VPA model as an animal model for ASD. Since then, a large variety of studies have used this VPA model to investigate molecular pathways and therapies associated with autism behaviors.

Valproic acid or 2-propylentanic acid is a branched-chain fatty acid widely used as an antiepileptic drug and mood stabilizer (Löscher, 2002). VPA has also been reported as an epigenetic regulator because it influence gene expression through chromatin remodeling, which inhibits histone deacetylase activity (Phiel et al., 2001). Other chemical and physical properties of this compound are summarized in Table 3.

Although dose and time of exposure vary between studies, VPA exposure during the first trimester of human pregnancy has been linked to congenital malformations that could affect the digestive, cardiovascular, dermatologic, or musculoskeletal systems, and cause spina bifida, cleft palate or craniosynostosis (Jentink et al., 2010; Meador, 2008). In addition, *in utero* VPA exposure has also been associated with an increased risk of intellectual disability, verbal and

non-verbal cognitive deficit, and a higher incidence of ASD in offspring (Roullet et al., 2013). Along the same lines, studies conducted in rodents have indicated that exposure at around GD 12 has been associated with lower social interaction, increased repetitive behaviors, and altered pattern of growth, in a sex-dependent manner, being males the most affected (Markram et al., 2008; Schneider and Przewłocki, 2005). Moreover, prenatal VPA exposure has also been linked to several comorbid non-autistic symptoms such as increased anxiety like-behaviors, altered response to eye blink or dysregulation of circadian rhythms (Mehta et al., 2011; Stanton et al., 2007; Tsujino et al., 2007). Altogether, the various studies have demonstrated that the VPA animal model of autism is considered to be valid because rodents show some of the core and secondary symptoms observed in individuals with ASD, even though most of the autistic population have not been prenatally exposed to this antiepileptic drug.

Table 3. Physical and chemical properties of VPA.

Molecular structure		**Molecular formula**	$C_8H_{16}O_2$
		Chemical name (IUPAC)	2-propylpentanoic acid
		Cas number	99-66-1
		Molecular weight	144.21 g/mol
		Density	0.904 g/cm^3 at 25 ºC
		Boiling point	From 219 to 222 ºC
		Vapor pressure	8.47 x 10^{-2} mmHg at 25 ºC

Adapted from National Center for Biotechnology Information (NCBI, 2022)

1.2.3.2. Genetic animal models

Pharmacological animal models are not the only ones used in the search for possible therapeutic targets for ASDs. In recent years, the scientific community has been looking for other factors that may explain the etiology of the disorder.

The high heritability observed in autism has led to many genetic studies that associate hundreds of genes with the behavioral phenotypes analogous to the clinical symptoms of the disorder (Banerjee et al., 2014; Vorstman et al., 2017). Most genetic animal models of autism are used to explore the duplications, deletions or mutations of the genes involved in synapse formation and maturation, particularly in postsynaptic densities, dendritic spines, and the signaling mechanism downstream of the receptors that mediate excitatory neurotransmission (Crawley, 2012). These genes include neuroligins, contactins and shanks, whereas other genes involved in transcription

or methylation such as tuberous sclerosis 1 and 2, fragile X mental retardation 1 or methyl-CpG-binding protein 2 have also been associated with the ASD phenotype (Banerjee et al., 2014; Schroeder et al., 2015; Silverman et al., 2010). However, the fact that every individual with autism has a different gene alteration hinders the search for a therapeutic target for the disorder.

1.3. Evidence on the relation between CPF exposure and ASD

A growing body of literature suggests that environmental factors and gene-environmental interactions contribute to the etiology of the autism, with particular focus on pesticides. This has led to studies on the possible association between autistic behaviors or their symptomatology with CPF, a pesticide used all over the world. However, results have been controversial. A review conducted by Williams and DeSesso (2014) indicates that exposure to CPF or its active metabolite (CPF-oxon) does not produce behaviors associated with autism. On the other hand, the reviews by Pelch et al. (2019) and He et al. (2022) have found a clear association between the pesticide and the disorder.

Exposure to high doses of CPF (6 mg/kg/day) from GD 14 to 18 appears to have a poor association with the three core symptoms of ASD (Venerosi et al., 2009, 2006). In this regard, Venerosi et al. (2009, 2006) found no effect on repetitive behaviors in young and adult mice but did find a decrese in the number and duration of pups ultrasonic vocalization (USVs) on PND 10 and an increase in unrestricted solid encounters in adults. Despite this, prenatal exposure to CPF with doses ranging from 2.5 to 5 mg/kg/day, led to a decrease in adult social interactions and an altered preference in the socially conditioned paradigm (Lan et al., 2017). These results were confirmed in a later study (Lan et al., 2019), which identified a sex bias: male mice exposed to the highest dose presented a decrease in social preference, while female mice presented the same decrease after being exposed to 2 mg/kg/day. Finally, prenatal exposure to doses below the threshold of CPF toxicity (1 mg/kg/day) showed pup USV impairments similar to those in the VPA model. In particular, the offspring of treated dams emitted fewer calls with longer latency (Morales-Navas et al., 2020). These results suggest that exposure to the pesticide during gestation plays an important role in the development of ASD-like behaviors. In contrast, drawing conclusions about postnatal exposure is more complicated. It seems that exposure to high doses of the pesticide (3 or 6 mg/kg/day) from PND 1 to 4 or PND 11 to 15 does not cause deficits in communication, social alterations or increased repetitive or stereotyped behaviors (Ricceri et al., 2003; Venerosi et al., 2008, 2006). Nevertheless, postnatal exposure to low doses of CPF (0.3 or 1 mg/kg/day) results in impaired USVs in pups and altered social behaviors in adult rats (Perez-Fernandez et al., 2020a; Berg et al., 2020).

Likewise, as reported in preclinical studies, research in humans also reveals conflicting results. Although some investigations do not associate OP exposure with ASD-like behaviors (Millenson et

al., 2017; van den Dries et al., 2019), most do (Roberts et al., 2007; Sagiv et al., 2018; Shelton et al., 2014; von Ehrenstein et al., 2019). The latest studies evaluate the presence of CPF metabolites in the urine or blood of children exposed during pregnancy. They confirm that maternal exposure can increase the risk of autism in the offspring, and that incidence is greater when the exposure occurs during the second or third trimester of pregnancy (Roberts et al., 2007; Sagiv et al., 2018; Shelton et al., 2014; von Ehrenstein et al., 2019). However, exposure to OP in clinical studies coexists with exposure to other environmental factors that are often not taken into account.

1.4. Apolipoprotein E

In 1973, apolipoprotein E (APOE) was first described by Shore and Shore (1973) as a component of very low-density lipoproteins (VLDL). At first, APOE was identified as an arginine-rich protein because of their high content. In 1975, however, Utermann (1975) suggested the current classification of APOE, based on the alphabetical nomenclature that was stating to be used in this field.

Nowadays, the apolipoprotein E (*APOE*) gene is one of the most widely studied genes, and in 2017 it was in the top ten (Dolgin, 2017). As a protein, its general function is to regulate cholesterol and lipoprotein metabolism, and to control lipid transport and redistribution in plasma and the CNS (Marais, 2019; Weisgraber, 1994). APOE is associated with VLDL, intermediate density lipoproteins, chylomicrons, chylomicron remnants and some subclasses of high-density lipoproteins (HDL) (Huang and Mahley, 2014; Mahley et al., 1984). It is synthesized in a wide variety of tissues such as skin, kidney, adipose tissue, or adrenal gland, but particularly in hepatocytes. Nevertheless, the brain is the second highest APOE-producing organ with astrocytes being the primary source (Huang and Mahley, 2014).

1.4.1. *APOE* polymorphisms, structure and functions

APOE is a polymorphic gene found in the long arm of chromosome 19 (19q13.2). It consists of four exons that encode a 299-amino acid protein with a molecular mass around 34 KDa (Bekris et al., 2010; Hatters et al., 2006; Mahley and Rall, 2000). This gene is clustered in a genomic region that encodes other apolipoproteins such as apoC1, apoC2 and apoC4 (Bekris et al., 2010). In humans, it mainly presents three alleles (ε2, ε3, ε4) with three common isoforms (apoE2, apoE3 and apoE4), which are defined by two single nucleotide polymorphisms (rs429358 and rs7412) (Figure 6) (Bekris et al., 2010; Hatters et al., 2006; Zannis et al., 1982). This gives rise to six different phenotypes: three homozygous (E2/2, E3/3 and E4/4) and three heterozygous (E3/2, E3/4 and E4/2) (Zannis et al., 1982). Nevertheless, these isoforms are differently distributed in the

population; apoE3 has a frequency of 78.3 % (SD=12.1), apoE4 a frequency of 14.5 % (SD=8.5) and apoE2 a frequency of only 6.4 % of the population (SD=5.1) (Eisenberg et al., 2010).

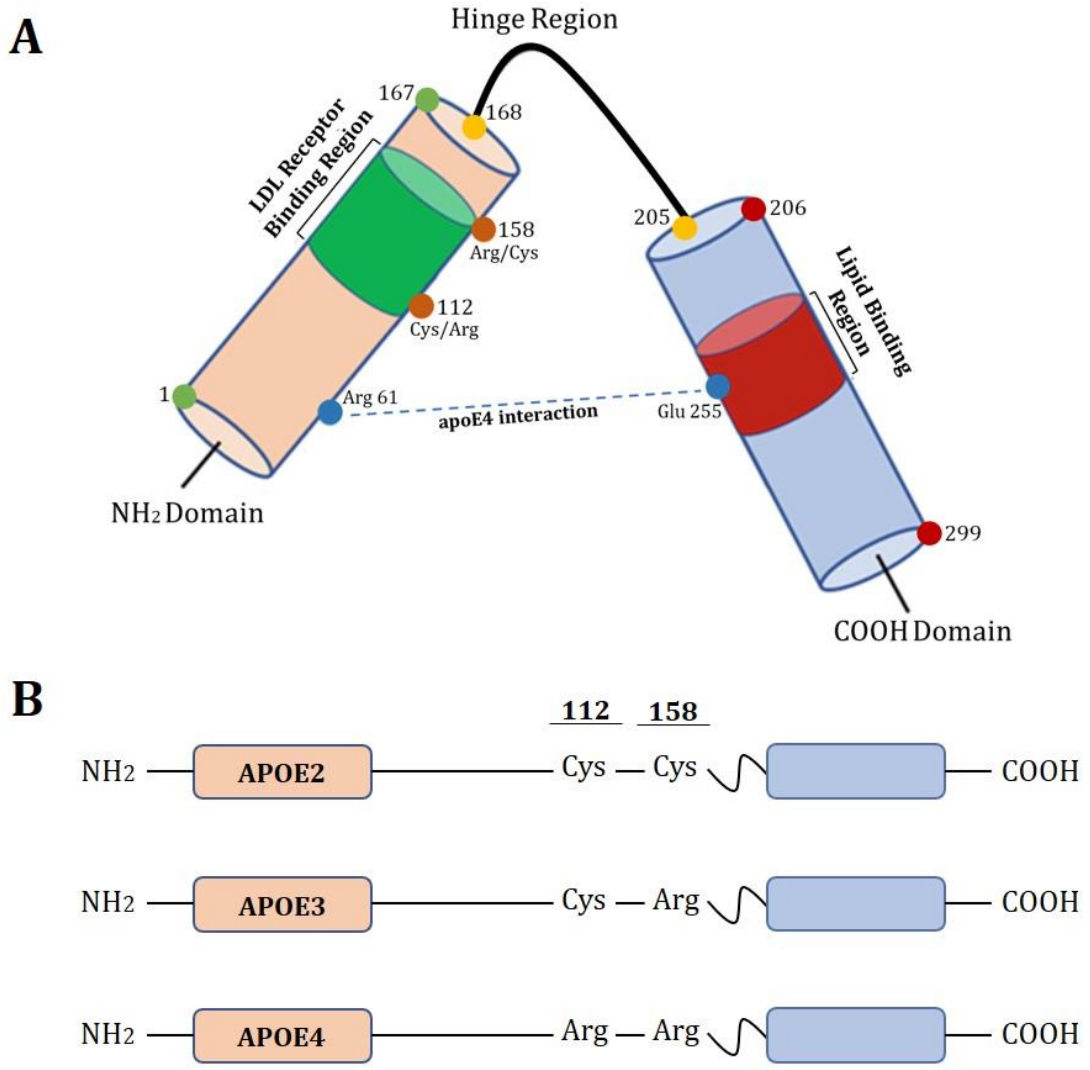


Figure 6. Representation of the human APOE protein structure (**A**) and its three major isoforms (**B**). The N-terminal domain (residues 1-167) contains the LDL receptor binding region, while the C-terminal domain (residues 206-299) contains the lipid binding region. Both domains are joined by the hinge region (residues 168-205). The three major isoforms differ from each other at two amino acid positions (112 and 158) where there can be a cysteine or an arginine. Abbreviations: Arg, arginine; Cys, cysteine; Glu, glutamic acid; LDL, low-density lipoproteins. Adapted from Fan et al. (2019)

Like other soluble apolipoproteins, all three apoE isoforms are made up of two folded structural domains at each end of the molecule (N-terminal and C-terminal domain) separated by a hinge region (amino-acid residues 168 to 205). The N-terminal domain encompasses protein residues 1

to 167 and forms an anti-parallel four-helix bundle that contains the receptor binding region. The residues in the 136-150 region mediate the interaction of APOE with the ligand binding domain of low-density lipoproteins (LDL) (Huang and Mahley, 2014; Mahley and Rall, 2000). On the other hand, the C-terminal domain (amino-acid residues 206 to 299) contains three α-helices and has a critical role in lipid binding (Mahley and Rall, 2000; Phillips, 2014). The residues in the 244-272 region form a amphipathic α-helices that mediate the binding of apoE to lipoproteins (Figure 6A) (Huang and Mahley, 2014; Mahley and Rall, 2000).

However, isoforms were observed to have differences in amino acids at position 112 and 158. ApoE3 isoform presented a cysteine at position 112 and an arginine at position 158, whereas apoE4 isoform had two arginines and apoE2 isoform two cysteines (Figure 6B) (Weisgraber et al., 1981). These differences in amino acids affect the structure, and the biophysical and pathophysiological properties of apoE isoforms. The presence of an arginine at position 158 appears to influence correct receptor binding activity, since both apoE3 and apoE4 isoforms are associated with a high affinity for LDL receptor binding. On the other hand, the presence of a cysteine at this position means that LDL receptor binding is defective, so apoE2 isoforms are associated with type III hyperlipoproteinemia, a lipid disorder that is characterized by increased plasma levels of cholesterol and triglycerides, and premature cardiovascular disease (Mahley et al., 1999; Mahley and Stanley C. Rall, 2001). In addition, an arginine at position 112 results in the Arg-61 side chain being oriented away from the four-helix bundle that enables this residue to interact with glutamic acid 255 in the C-terminal domain. This produces an alteration in the protein conformation that results in a binding preference for large VLDL and LDL particles, in the case of apoE4 isoform, and for smaller HDL particles in the case of apoE3 and apoE2 isoforms (Dong and Weisgraber, 1996; Dong et al., 1994; Mahley et al., 1999; Mahley and Huang, 2007). Table 4 summarizes all the properties of apoE isoforms.

Table 4. Key differences in apoE isoforms.

	apoE isoforms		
	apoE2	**apoE3**	**apoE4**
Functional differences			
LDL receptor affinity	Low	High	High
Lipoprotein-binding preference	HDL	HDL	VLDL/LDL
Structural differences			
Domain interaction	No	No	Yes

The main function of APOE is to transport and delivery lipids from one tissue or cell type to another. It binds to specific cell surface receptors such as LDL, VLDL receptors or the apoE receptor-2 (apoER2) and enables lipoproteins that contain APOE to be eliminated, thus maintaining lipid homeostasis (Hatters et al., 2006; Mahley and Rall, 2000). However, APOE has been associated with other biological processes such as cognitive function, immunoregulation or Alzheimer's disease (AD) (Mahley and Rall, 2000). In this regard, apoE4 isoform have been associated with an increased risk of AD in adults, particularly in homozygous individuals (Corder et al., 1993). In addition, Meyer et al. (1998) noted that the presence of the *APOE* ε4 allele can reduce the age at AD onset, while the *APOE* ε2 allele seems to exert a protective role (Corder et al., 1994).

1.4.2. Transgenic apoE animal models

Animal models are essential to gain a more complete understanding of human physiology and disease. The first apoE animal model was generated in 1992. Piedrahita et al. (1992) developed a mouse model using gene targeting to inactivate the gene encoding the APOE protein in embryonic stem cells (Plump et al., 1992; Zhang et al., 1992). Thus, the apoE knockout (KO) mice or apoE (-/-) came into existence. More specifically, this model was created by using homologous recombination to insert the neomycin resistance gene into exon 3 and part of intron 3 of the *Apoe* gene (Seitz et al., 2005). Then, animal models that lacked the murine *Apoe* gene and expressed the human isoform were generated (e.g., in astrocytes under the control of the glial fibrillary acidic protein (GFAP) promotor or in neurons under the control of the neuron-specific enolase promoter) and afterwards, matted with other mice that carried typical mutations of AD (Balu et al., 2019; Graybeal et al., 2014). However, in these mouse models the distribution of the human isoforms in the organism varied. To overcome this difficulty, Sullivan et al. (1997) developed a transgenic mice model by replacing the murine *Apoe* gene with one of the three human isoforms, without disturbing any of the known sequences. Thus, apoE transgenic remplacement (TR) mice express the human *APOE* gene at physiological levels in both temporal and spatial patterns that are similar to wild animals and humans. Therefore, it is an optimal model for research (Sullivan et al., 2004).

1.4.3. Influence of apoE isoforms on development and behavior

During the early stages of development there is a high demand for cholesterol and other lipids to cover the need of neurogenesis, synaptogenesis, and myelination. Consequently, the synthesis of lipids increases in the brain, but declines dramatically in adulthood when this processes end.

Thus, lipid distribution and elimination depend on the stage of development, and the type of apoE isoform, and so indirectly influence cognitive and behavioral functions (Dietschy and Turley, 2004; Vance and Hayashi, 2010).

The *APOE4* genotype is the greatest risk factor associated with the development of AD. It is well known that it might produce detrimental consequences in later life, but it seems to confer cognitive advantages in young adults. Caselli et al. (2009) reported an age-related memory decline in healthy *APOE* ε4 carriers. In particular, these individuals present accelerated memory loss that can begin before the age of 60. Moreover, the early development of AD is gene-dose dependent, and homozygous *APOE* ε4 carriers are at greatest risk of developing the disease. On the other hand, Wright et al. (2003) reported that *APOE* ε4 carriers showed an improved score on the Mental Development Index at very early developmental stages (24 months of age), whereas other investigations in children between the age of 8 and 16 reported, that *APOE* ε4 carriers have better verbal fluency (Oriá et al., 2005). Likewise, Marchant et al. (2010) also found greater verbal fluency in healthy *APOE* ε4 carriers between 18 and 30 years old, and improved decision making and prospective memory, although spatial memory and word recall were not affected. Moreover, other research that evaluates the same period of age associated the *APOE4* genotype with a better episodic memory and less learning- and retrieval-related brain activity, which can be interpreted as *APOE* ε4 carriers making a more efficient use of memory (Mondadori et al., 2007).

Along the same lines, preclinical studies in KO mice also suggest that the *APOE* genotype plays a major role in learning and memory processes, and deteriorates considerably over time. Champagne et al. (2002) evaluated learning and memory in a Morris Water Maze (MWM) at two different points of life (three and twelve months old). They found that old apoE KO mice presented greater learning and memory deficits than younger mice. In accordance, East et al. (2018) reported an impairment in odor habituation in apoE4-TR mice which seem to present a hyper-excited system associated with deficits in memory. Nevertheless, the *APOE* genotype does not affect only learning and memory processes; it is also associated with anxiety, impulsivity, attention and stress behaviors. A study conducted with GFAP-ApoE transgenic male mice showed higher anxiety-like behaviors in eight-month-old GFAP-ApoE4 mice than in their matched GFAP-ApoE3 (Meng et al., 2015). In addition, Zhang et al. (2021) associated the apoE4 isoform with a greater susceptibility to stress, and a higher association with depression-like behaviors in eight- but not three-month-old mice, suggesting that the apoE4 isoform increases susceptibility to stress-induced depression-like behaviors in an age dependent manner as it was observed in AD.

In our laboratory we have investigated the differences between the three human isoforms in the apoE-TR mice model. Body weight was observed to be lower in young- and old-adult apoE4-TR mice (four and twelve months old), particularly in young-adult mice. On the other hand, apoE2-TR mice showed the highest body weight (Reverte et al., 2013, 2012). Moreover, apoE4-TR mice were

associated with less activity in the elevated zero maze and the open field test and also with more anxious behaviors (Reverte et al., 2012). Subsequent studies revealed that apoE4-TR mice exhibited delayed eye-opening, while apoE2 was the most advanced isoform (Reverte et al., 2014). Differences were also observed in impulsivity and attention behaviors using a five-choice serial reaction task (5-CSRTT). Particularly, an increase in the preservative and premature response was observed in eight-months-old apoE-TR female mice (Reverte et al., 2016).

However, genotype differences are not only influenced by age; they can also be affected by sex. In fact, females carrying the *APOE* ε4 allele seem to present earlier onset and more extensive pathology than their male counterparts (Pontifex et al., 2018). In this respect, Fleisher (2005) observed memory impairments in females carrying the *APOE* ε4 allele with a consequent reduction in the hippocampal volume. Moreover, another study that evaluated more than 5,000 brain samples demonstrated that womans carrying the *APOE* ε4 allele had more neurofibrillary tangles (NFTs) and senile plaques, which results in early onset of the pathology of AD (Corder et al., 2004). Preclinical studies also report that females carrying the *APOE* ε4 allele showed higher neurocognitive impairments. In this respect, Bour et al. (2008) demonstrated that old apoE4-TR female mice (15 months old) presented spatial learning and memory deficits when they were compared with apoE4-TR male mice. Grootendorst et al. (2005) found similar results in animals between four and five months of age. Along the same lines, Reverte et al. 2012 reported that apoE4-TR mice performed worse in the acquisition phase of MWM. As far as retention is concerned, apoE4-TR mice, and especially females, were unable to remember the location of the platform. In addition, Rijpma et al. (2013) found a reduction in the density of females' presynaptic hippocampus, which was not found in apoE4-TR males. Therefore, age and sex are important factors that can modulate the differences observed between the distinct *APOE* genotypes.

1.4.4. APOE in brain

APOE is involved in neuropathology and plays an important role in normal metabolism and the functioning of the CNS. It is the major apolipoprotein in nervous systems linked to triglycerides, phospholipids and cholesterol delivery to neurons for growth, repair, and synaptogenesis processes (Mahley, 1988; Mauch et al., 2001; Pitas et al., 1998). The presence of APOE in blood and cerebrospinal fluid (CSF), suggest that APOE can cross the blood-brain barrier. However, Liu et al. (2012) states that apolipoprotein in the CSF cannot be derived from a plasma pool, so it must be synthesized in brain. As has been mentioned previously, astrocytes are the major brain APOE producers, although APOE can also be produced to a lesser extent by other glial cells such as microglia or even some types of neurons (Huang and Mahley, 2014; Pitas et al., 1987).

1.4.4.1. APOE and neurodegeneration

Alzheimer's disease is a progressive neurodegenerative disorder characterized by a progressive decline in memory, executive function, language, and other areas of cognition. Moreover, it is associated with pathological changes in the brain such as the formation of amyloid-β (Aβ) plaques and NFTs, as well as neuronal and synaptic loss and brain atrophy and inflammation. Genetically, the apoE4 isoform is the only one that has been strongly associated with early and late-onset AD (Corder et al., 1993; Kim et al., 2009). *APOE* genotype has been associated with the regulation of the Aβ metabolism in an isoform-specific manner. Indeed, apoE4 isoform decreases the clearance of Aβ plaques and accelerates the accumulation, aggregation, and deposition of plaques in brain, thus contributing to the pathology of AD (Ye et al., 2005). ApoE4 isoform has also been associated with an increase in the concentration of tau proteins, and a consequent increase in the production of NFTs (Benson et al., 2022). Furthermore, the detrimental effects of apoE4 isoform have been related to the domain-domain interaction reported above altering the protein conformation and making this isoform highly susceptible to proteolytic cleavage and the generation of truncated fragments that can produce neurotoxicity. These fragments are associated with such detrimental effects as mitochondrial dysfunction, altered cytoskeletal profiles and impaired synaptogenesis (Mahley and Huang, 2012). Moreover, it has been shown that fragments are produced by neurons in specific brain regions that are highly affected in AD, which indicates a direct role in disease onset and progression (Muñoz et al., 2019).

1.4.4.2. APOE and neurotransmission

Studies in humans and animal models have shown that *APOE* genotype has an important relationship with the cholinergic system. Poirier (1999) showed that postmortem brains of AD patients presented a reduction of several cholinergic markers in a genotype-dependent manner. More specifically, ChAT activity and the density of the nicotinic-receptor binding-site were reduced in the cortex and hippocampus, particularly in *APOE ε4* carriers. These results were confirmed by Soininen et al. (1995a) who also observed reduced ChAT activity in the frontal cortex of patients who carried the *APOE* ε4 allele. The reduction was greatest, in those with a homozygous genotype. Differences between genotypes were also observed in apoE-deficient (apoE KO) mice. Gordon et al. (1995) observed decreased ChAT levels in the hippocampus and frontal cortex of 6-month-old mice, whereas Kleifeld et al. (1998) showed reduced VAChT levels in the forebrain of 4-month-old mice. Moreover, studies carried out in our laboratory with apoE-TR animal models expressing one of the three human isoforms showed that young apoE4-TR mice presented a decrease in the expression of VAChT and an increase in the α7 nAChR subunit, whereas studies on adult mice (six months old) reported contradictory results (higher levels of

ChAT and VAChT) (Basaure et al., 2018, 2019b). These findings point to age-related differences in the cholinergic system between the *APOE* genotypes.

Nevertheless, although information is limited, the cholinergic system does not appear to be the only one to exhibit alterations related to the *APOE* genotype. Sweet et al. (2016) observed that patients with AD had a reduction in NMDA receptor subunits, which was greatest in the *APOE* ε4 carriers. Chen et al. (2010) showed a similar reduction in animal studies. On the other hand, several studies hypothesize that hippocampal GABAergic interneurons are susceptible to APOE-mediated neurotoxicity. In fact, Andrews-Zwilling et al. (2010) and Leung et al. (2012) observed a loss in GABAergic interneurons in the hilus of the apoE4-TR mice hippocampus, which correlates with adult hippocampal neurogenesis and spatial learning and memory deficits. In turn, this generates an E/I imbalance in the brain of *APOE* ε4 carriers.

1.5. Interaction between CPF exposure, *APOE* genotype and ASD

The different isoforms of the *APOE* gene are an important source of variability in the response to toxic insults. Although very few clinical studies study this association, in our laboratory we have shown that apoE isoforms confer different vulnerabilities to CPF exposure. For example, Peris-Sampedro et al. (2015a) exposed apoE-TR male mice to CPF through a supplemented chow diet which intended to deliver a dose of 2 mg/kg/day. Young adult apoE3-TR mice showed an increase in their body weight after being treated with CPF, although apoE2- and apoE4-TR mice did not. Subsequent studies reported that CPF disrupts leptin and insulin hemostasis. The *APOE3* genotype was the most affected, which suggests that it is the most vulnerable to the detrimental metabolic effects of the pesticide (Peris-Sampedro et al., 2018). The *APOE* polymorphism also elicits a different response to CPF in terms of impulsive- and compulsive-like behaviors. Peris-Sampedro et al. (2016) orally expose apoE-TR mice to 3.75 mg/kg/day of CPF. The evaluation of impulsivity and compulsivity behaviors using a 5-CSRTT showed an increase in premature and preservative responses in apoE4-TR mice. This may lead to deficiencies in inhibitory control, although they can be reversed by CPF exposure, which points to an interaction between the cholinergic agent and the apoE4 isoform (Peris-Sampedro et al., 2016). Later studies investigated the effects of early CPF exposure (from PND 10 to 15) on spatial learning and memory in 6-month-old mice, as well as the effects of an adult CPF re-exposure. Results showed that postnatal exposure disrupts the spatial search strategy and reference memory in apoE3-TR mice, whereas adult re-exposure improved the learning and memory abilities of apoE4-TR females (Basaure et al., 2019b). In addition, social behaviors were also affected in a genotype- and exposure-dependent manner. Postnatal exposure to CPF enhanced the preference for social stimulus in *APOE* ε4 carriers, whereas adult exposure had the same effect in *APOE* ε3 carriers, which suggests that there is a relationship between *APOE* genetic background and some autistic-like behaviors (Basaure et al., 2019a).

However, it is well known that the pathogenesis of ASD involves a complex interaction between environment and heredity (Fernell et al., 2013). As reported in the section 1.3 of this document, exposure to CPF is well associated with some of the core symptoms of ASD. The results on social behaviors observed by Basaure et al. (2019b) in apoE-TR mice prompted us to study the involvement of these apoE isoforms in autistic-like behaviors. The APOE protein shared biological pathways with reelin (RELN), a protein that has been implicated in autism by such evidence as position data from linkage or candidate gene studies. Both RELN and APOE protein competes to join the same LDL receptors (VLDL and apoER2 receptors) (D'Arcangelo et al., 1999). A study by Persico et al. (2004) observed an apparent distortion in the transmission of the *APOE* ε2 allele in autism families due to a fault in the *APOE* signaling pathways. Other authors associated the hypermethylation of the *APOE* gene with an increased risk of ASD (Hu et al., 2018), whereas Raiford et al. (2004) and Ashley-Koch et al. (2007) found no evidence for the linkage of *APOE* to autism. Therefore, the literature on this topic is scarce and contradictory, so further research is needed.

HYPOTHESIS & OBJECTIVES

2. HYPOTHESIS AND OBJECTIVES

In the last few years, our laboratory has focused on studying the neurobehavioral and metabolic effects of exposure to environmental toxics. More specifically, we are interested in studying the adverse effects produced by the OP pesticide CPF. Although its use has recently been restricted or banned in US and EU, it continues to be used in developing countries (EFSA, 2019; EPA, 2021). Moreover, its potential role as a long-range transport compound means that the general population can be indirectly exposed (Mackay et al., 2014). To date, our research has been aimed at studying the short-, medium- and long-term effects of postnatal exposure to CPF during the first two postnatal weeks, coinciding with third trimester of pregnancy in humans and the first months of life (Azad et al., 2017; Richard and Flamant, 2018). In addition, for many years our laboratory has also studied the effect of different human *APOE* polymorphisms on behavior, as well as the gene-environmental interaction between CPF and *APOE* genotype. Our findings show alterations in anxiety, spatial learning and memory, and social behavior (Basaure et al., 2019a; Reverte et al., 2012).

Nevertheless, the current growth of interest in the etiology of neurodevelopmental disorders has led us to investigate how environmental factors and gene-environmental interactions contribute to the development of these disorders. In particular, we have focused on two of the three core symptoms associated with ASD. Because exposure to the antiepileptic drug VPA during pregnancy is well-known to be associated with an increased risk of ASD (Chaste and Leboyer, 2012; Lord et al., 2018), we used this autism model to compare the effects of gestational CPF exposure and its possible association with autistic-like behaviors, covering another developmental window which matches the second and third trimester of pregnancy in humans (Azad et al., 2017; Richard and Flamant, 2018). Moreover, since autism is highly heterogenous between individuals and it has numerous associated genes (Banerjee et al., 2014; Vorstman et al., 2017), we also studied how the apoE3 and apoE4 isoforms contributed to the development of the disorder, and interacted with CPF.

The purpose of this thesis was to continue our research into the short- and medium-term adverse effects of the neurodevelopmental disorders produced by CPF exposure during pregnancy, as well as the contribution of sex and *APOE* genotype.

2.1. Hypothesis

Prenatal exposure to chlorpyrifos affects neurodevelopment of offspring and causes ASD-like behavioral symptoms in infant and young mice in an *APOE* genotype- and sex-dependent manner.

2.2. Objectives

2.2.1. Main objective

The main objective of this thesis is to assess the contribution of prenatal chlorpyrifos exposure and *APOE* genotype to neurodevelopmental disorders, such as ASD, by evaluating short- and medium-term physical, behavioral and biochemical endpoints in males and females.

To this end, both male and female C57BL/6J and homozygous mice for the human *APOE* alleles ε3 and ε4 were subject to two experimental phases in order to study the adverse effects of chlorpyrifos exposure during the initial stages of development and adolescence.

2.2.2. Specific objectives

I. To review the evidence published on exposure to different pesticides and the incidence of ASD, with particular focus exposure to organophosphates.

II. To determine the adverse effects of late gestational exposure to chlorpyrifos and its influence on communication skills in newborn mice and anxiety-like behaviors in adolescent mice, according to sex, strain (C57BL/6J and apoE-TR mice) and genotype (*APOE3* or *APOE4*).

- → To study the effect of prenatal chlorpyrifos or valproic acid exposure, *APOE* genotype and sex on the activity of acetylcholinesterase and maternal behavior.
- → To study the changes in physical and neuromotor development during the preweaning stages and differences between groups.
- → To study how chlorpyrifos and valproic acid exposure, *APOE* genotype and sex affects communication and anxiety behaviors and its relation to neurodevelopmental disorders.
- → To study differences between sexes.
- → To study differences between genotypes.

III. To examine changes in social behaviors in C57BL/6J mice pre or postnatally exposed to CPF and its effects on excitatory and inhibitory brain systems.

- → To study two different exposure windows.
- → To study how chlorpyrifos and valproic acid exposure and sex affects social behavior and its relation to neurodevelopmental disorders.
- → To study the alterations in GABAergic and glutamatergic systems.
- → To study the differences between sexes.

IV. To examine changes in social behaviors in apoE3- and apoE4-TR mice exposed prenatally to chlorpyrifos and its effects on excitatory and inhibitory brain systems.

→ To study how chlorpyrifos exposure, *APOE* genotype and sex affects social behavior and its relation to neurodevelopmental disorders.

→ To study the alterations in the GABAergic and glutamatergic systems.

→ To study differences between sexes.

→ To study differences between genotypes.

V. To compare the results from different animal models (C57BL/6J exposed to valproic acid and apoE-TR mice).

MATERIAL & METHODS

3. MATERIAL AND METHODS

3.1. Animals

Adult C57BL/6J mice and humanized apoE3 and apoE4-TR homozygous mice were, respectively, obtained from Charles Rivers Laboratories (Barcelona, Spain) and Taconic Europe (Lille Skensved, Denmark). After one week of quarantine, one male and a maximum of three females of the same strain were mated for 3 h. The matings were then separated and the presence of a vaginal plug was noted to assign GD 0. All the mice were housed in plastic cages containing between two and five animals. On GD 12, pregnant females were weighed to confirm pregnancy and housed individually. The day of delivery was assigned as PND 0. Pregnant C57BL/6J mice were randomly assigned to one of the three treatment groups (control [CNT], CPF or VPA), whereas the apoE-TR mice were randomly assigned to one of the two treatment groups (CNT or CPF). Animals were maintained in a 12-hour light/dark automatic cycle (light ON between 8 a.m. and 8 p.m.) with controlled temperature (22 ± 2 ºC) and humidity (50 ± 10 %). Food (SAFE® A04 diet, Panlab, Barcelona, Spain) and tap water were administered *ad libitum*. The present study was given an authorization code (10735) by the Government of Catalonia and approved by the Animal Care and Use Committee of the Rovira i Virgili University (Catalonia, Spain). It was conducted following the ARRIVE Guidelines (Percie du Sert et al., 2020) and in compliance with Spanish Royal Decree 53/103 on the protection of animals used in experiments and the European Communities Council Directive (86/609/EEC).

3.2. Chemicals, treatment, and experimental design

Pregnant C57BL/6J females were exposed to either 0 or 1 mg/kg/day of CPF [0,0-diethyl O-(3.5.6-trichloropyridin-2-yl) phosphorothioate] with a purity of 99.6 % (Sigma-Aldrich, Madrid, Spain) via supplemented diet between GD 12 and 18. In addition, a group of C57BL/6J females were exposed to 300 mg/kg/day of VPA (2-propypentanoic acid sodium) with a purity of 98 % (Sigma-Aldrich, Madrid, Spain) via a subcutaneous injection on two consecutive days (GD 12 and 13). This group of females was used as a pharmacological model for autism (Sakai et al., 2018). Pregnant apoE-TR mice were exposed to 0 or 1 mg/kg/day of CPF in the diet between GD 12 and 18. In both strains, standard food was supplemented with 15 mg CPF/kg chow (Panlab, Barcelona, Spain). The food intake and body weight of dams were monitored every day to adjust the amount of food and achieve the dose of 1 mg/kg/day of CPF. All animals were provided with regular chow *ad libitum* (Figure 7).

Another group of C57BL/6J mice was postnatally exposed to CPF from PND 10 to 15. The pesticide was dissolved in corn oil (vehicle) and adjusted to administer an oral dose of 1 mg/kg in 1 µL/g of body weight, using a micropipette. The control group received the vehicle during the same period.

During the preweaning period (from PND 0 to 28), the communication skills of C57BL/6J and apoE-TR pups of dams treated prenatally with CPF or VPA were evaluated on three PNDs (2, 7 and 9). In addition, on PND 2, and after USV assessment, maternal care and nest quality were assessed in dams. Throughout this stage, physical and motor development of the litter was also evaluated. On PND 28, the weaning day, C57BL/6J and apoE-TR mice were separated in plastic cages containing between two and five animals of the same sex, treatment and strain. During adolescence, a maximum of two animals of the same sex and litter were evaluated in the open field and in a three-chamber test. In this last test, individuals treated postnatally with CPF were also evalauted (Figure 7).

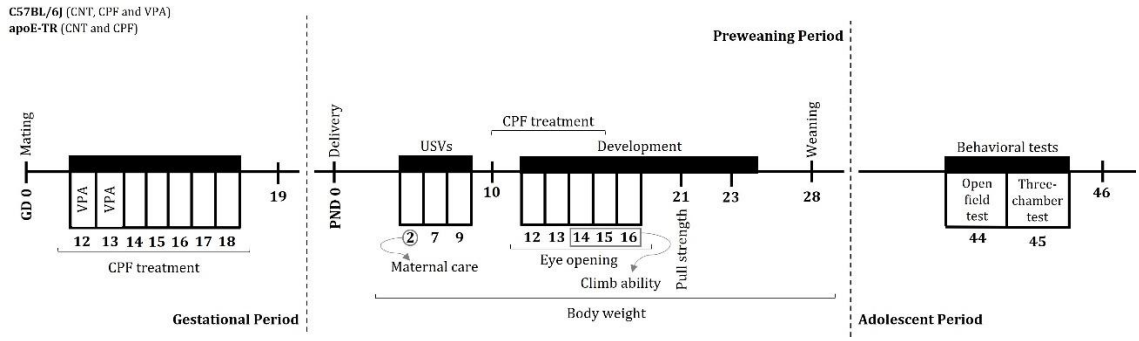


Figure 7. Experimental design of the thesis. Both strains were exposed to 0 or 1 mg/kg/day of CPF in the diet between GD 12 and 18. In addition, a group of C57BL/6J females were prenatally exposed to 300 mg/kg/day of VPA via a subcutaneous injection on GD 12 and 13, while another group of C57BL/6J mice were postnatally exposed to 1 mg/kg/day of CPF between PND 10 and 15. During the preweaning period, communication skills were evaluated assessing USVs on PND 2, 7 and 9 in C57BL/6J and apoE-TR pups prenatally treated with CPF or VPA. Also on PND 2 we evaluated the nest quality and maternal care in dams. The physical development of the litter was evaluated in terms of body weight (from PND 2 to 28) and eye opening (from PND 12 to 16). The motor development of the litter was evaluated by assessing climbing ability (from PND 14 to 16) and pull strength (PND 21). During the adolescent period, animals were evaluated in an open field (prenatally exposed) and in a three-chamber test (prenatally and postnatally exposed). Abbreviations: CNT, control; CPF, chlorpyrifos; VPA, valproic acid; GD, gestational day; PND, postnatal day; USVs, ultrasonic vocalizations.

3.3. Preweaning assessment

3.3.1. Maternal care

After assessing the USVs on PND 2, we assessed the nest quality with the following score: no nest (0), not all the pups are in the nest (1), or the nest is well-defined (2). Subsequently, maternal care was evaluated in dams. The mothers were removed from the home cage, while the pups were weighed. The pups were then placed in the corner opposite the nest and the dam was returned to the home cage. The time she took to collect the first pup (latency) and all the pups was recorded

for a maximum of three minutes. Furthermore, we assessed another variable called efficiency, which was obtained by correcting the total time for latency and dividing by the number of pups in the litter (time to collect all the pups - latency)/(number of pups in the litter) (Reverte et al., 2014).

3.3.2. Physical and motor development of the litter

As reported in the section above, the developmental timeline of mice was monitored between PND 2 and 28 with a battery of tests that allowed the evaluation of litter physical and motor development. Physical development was assessed by measuring body weight on PND 2, 7, 9, 14, 16, 21, 23 and 28, whereas eye opening was evaluated between PND 12 and 16 with the following scores: both eyes are closed (0), one eye open and one eye closed (1), or both eyes open (2). On the other hand, between PND 14 and 16, motor development was evaluated in terms of climbing ability using a metallic grid (24 x 24 cm) placed at an angle of 45º in a plastic cage and the pups were placed at the bottom (scores: no movement (0), less than half of the grid (1), or more than half of the grid (2)). On PND 21 pull strength was measured with a grip strength meter. Mice were placed near the grid and gently pulled backward by the tail (Basaure et al., 2018; Reverte et al., 2014).

3.3.3. Evaluation of communication by analyzing ultrasonic vocalizations

Communication skills were assessed by recording mice USVs in a sound-attenuated chamber using the software SeaWave (Gianni Pavan©, Pavia, Italy) and an UltraMic 250 microphone (Dodotronic, Italy).

Since the rate of USV calls increases during the first 5-6 days and decreases after PND 7, we recorded the vocalizations of mice for three minutes on different days (PND 2, 4, 6, 8 and 10) in order to obtain the complete communication profile of each strain. Data obtained by Branchi et al. (2001) and our recordings indicated PND 2, 7 and 9 as the best days to conduct the USV analysis.

USVs were recorded in one male and one female from each litter. Before the test was started, the animals were transported to the testing room in their home cage. After five minutes, we randomly selected a pup and placed it in the sound-attenuated chamber for five minutes. Subsequently, we cleaned the working place with ethanol 70 % in order to eliminate olfactory clues. Immediately, an animal from the same litter but of different sex was randomly selected and placed in the chamber for five minutes. This protocol was performed each day of evaluation and the distance between the microphone and the pup was set at 10 cm (Scattoni et al., 2008). We assessed the changes over

the five-minute period, the total number of calls and the average duration, frequency and intensity, as well as the time taken to emit the first call (latency).

3.4. Behavioral assessment

Locomotor and anxiety like behaviors were evaluated in both C57BL/6J and apoE-TR mice on PND 44 using the open field test, whereas social behaviors were assessed the day after (PND 45) with the three-chamber test. Both working places were cleaned with ethanol 70 % after each evaluation to eliminate olfactory clues. Behavioral tests were recorded by a video camera (Sony CCD-IRIS) and computerized using a video-tracking program (Etho-Vision XT 11.5, Noldus Information Technologies, Wageningen, The Netherlands).

3.4.1. Open field test

The open field test consists of an open wooden box measuring 60 x 60 cm with walls 50 cm high. The field was divided into two virtual areas: a quadrant of 30 x 30 cm in the middle of the field and the periphery, which covers the remaining space of the wooden box. As in the evaluation of USVs, the animals were transported to the testing room before the beginning of the test. After five minutes of acclimation, the animals were gently placed in the center of the box and allowed to explore freely for 30 minutes. Locomotor activity was evaluated by recording the total distance travelled in periods of five minutes. Anxiety-behaviors were also evaluated by measuring the time, the velocity and the distance that the animals spend in the center zone versus the time in the periphery (Kraeuter et al., 2019).

3.4.2. Three-chamber test

The three-chamber test consists of a non-automated rectangular Plexiglas box (60 x 30 x 30 cm) with three interconnected chambers (20 x 30 x 30 cm) and two middle walls with doorways that allow the mouse to move freely between compartments. In both lateral chambers, there were two empty wire cups (7 x 7 cm). Five minutes before the start of the test, the mice were transported to the testing room for acclimation. Then, animals were placed in the central compartment and allowed to explore the space for 10 minutes. After this habituation phase, we assessed the sociability or social preference phase for six minutes. An unfamiliar mouse of the same sex and age was placed in one of the two wire cups (social chamber), while an inanimate object was placed in the other (i.e., red plastic frog 2.5 x 2.5 cm) (non-social chamber). Finally, we assessed the

novelty or social novelty preference phase for an additional six minutes. In this case, we kept the mouse from the sociability phase under the same wire cup so it was now the familiar mouse or the non-novel chamber, while the inanimate object was replaced by an unfamiliar mouse of the same sex and age (novel chamber). The preference for the social or novel stimulus was evaluated by recording the time that the subject animal spent in the social or novel chamber versus the non-social or non-novel chamber. We also evaluated other variables such as the sociability or novelty ratio (time in the social or novel chamber – time in the non-social or non-novel chamber)/(sum of time in both lateral compartments). A positive ratio indicates a preference for the social or novel stimulus (Crawley, 2004).

3.5. Sacrifice and sampling

Biological samples were obtained in two different periods. On PND 2, four animals from different litters, and of different sex, genotype, strain and treatment were randomly selected and sacrificed by decapitation, whereas in adolescence (on PND 46) six animals from different litters, and of different sex, genotype, strain and treatment were euthanized by exsanguination under isoflurane anesthesia. In both cases, we removed the brain, flash frozen it in RNAse-free cryotubes and then stored it at -80 ºC until analysis.

3.6. Biochemical analysis

3.6.1. AChE activity

AChE activity was evaluated on PND 2. Brain samples were weighed and homogenized in cold with 1 % Triton X-100 in PBS 0.1 M at pH 8. Subsequently, homogenates were centrifuged at 2,000 g for 10 minutes at 4 ºC and the supernatant was removed for analysis. AChE activity was measured in duplicates and determined spectrophotometrically using a semiautomatic COBAS MIRA analyzer (Hoffman-La Roche & Co., Basel, Switzerland) and an updated version of the Ellman method (Ellman et al., 1961; Peris-Sampedro et al., 2015b). Enzyme activity was calculated relative to the protein concentration, which was assessed by the Lowry method (Lowry et al., 1951)(see section 3.6.3). Brain AChE activity was represented as U/mg protein.

3.6.2. Gene expression analysis

RNA was extracted using the SPEEDTOOLS Total RNA Extraction Kit (Biotools, Madrid, Spain). RNA concentration and purity was measured with a Nanodrop 2000 spectrophotometer

(ThermoFisher Scientific, Waltham, MA, USA). Subsequently, we synthetized complementary DNA (cDNA) using a Maxima First Strand cDNA Kit for RT-qPCR (ThermoFisher Scientific, Waltham, MA, USA). Duplicates or triplicates of each RNA sample were included in the quantitative real-time polymerase chain reaction (qPCR), which was performed with Maxima SYBR Green/ROX qPCR Master Mix (2X) Kit (ThermoFisher Scientific, Waltham, MA, USA) and the Rotor-Gene Q Real-time Q cycler (Qiagen Inc., Hilden, Germany) or the 7900HT Fast Real-Time PCR System (ThermoFisher Scientific, Waltham, MA, USA). The qPCR analysis was used to assess the expression of some GABAergic- and glutamatergic-related genes such as glutamate decarboxylase 1 and 2 (*Gad1* and *Gad2*), vesicular GABA transporter (*Slc32a1*), glutamate ionotropic receptor NMDA type subunit 2A (*Grin2a*) and 2B (*Grin2b*), GABA-A receptor subunit alpha 1 (*Gabra1*), alpha 2 (*Gabra2*), alpha 5 (*Gabra5*) and beta 3 (*Gabrb3*), solute carrier family 12-member 5 (*Slc12a5*) and 2 (*Slc12a2*), parvalbumin (*Pvalb*) and retinoic-acid related orphan receptor alpha (*Rora*), which is an hormone-dependent transcription factor that could help understand the differences between sexes. To calculate the cycle threshold (Ct) we used the Rotor-Gene Q Real-Time PCR 2.0 software (Qiagen Inc., Hilden, Germany) or the ExpressionSuite software 1.3 (ThermoFisher Scientific, Waltham, MA, USA). Each sample was normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) (ΔCt) and standardized to the average of C57BL/6J or apoE3 control male (ΔΔCt) to assess the relative gene expression levels with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

3.6.3. Western Blot analysis

Brain samples were homogenized in RIPA buffer (Merck, Darmstadt, Germany). Homogenates were then shaken for 40 minutes and centrifuged for 15 minutes (4 ºC, 20,000 g). The proteins of the supernatant were separated by electrophoresis on 15 % acrylamide gels and transferred to Immobilon-P PVDF sheets (Millopore Corp., Bedford, MA, USA), using a transblot apparatus (BioRad, Madrid, Spain). Subsequently, we blocked the membranes for 1 h with 5 % non-fat milk dissolved in TBS-T buffer (50 mM Tris, 1.5 % NaCl, 0.05 % Tween 20 at pH 7.5). Primary monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA) against PVALB and the housekeeping protein (GAPDH) were incubated overnight. On the following day, the blots were washed thoroughly in TBS-T buffer and then incubated with peroxidase-conjugated IgG antibody for 1 h. An ImmunStar Chemiluminescence Kit (BioRad, Madrid, Spain) was used to visualize immunoreactive proteins. Digital images were obtained using the VersaDoc system (BioRad, Madrid, Spain) to perform the semi-quantification of the band intensity (Image Lab, Bio-Rad, Madrid, Spain).

3.7. Statistical analysis

Data were analyzed using the 27.0 SPSS software (IBM Corp. Chicago, IL, USA). A three-way analysis of variance (ANOVA) was conducted to assess the general effects of sex, treatment and genotype, and their interactions. In those cases, in which a variable was assessed over time, effects were evaluated by using repeated measures ANOVA (RMANOVA). The maternal care latency and physical and motor developmental landmarks were analyzed using the number of pups as a co-variable, while in the case of litter size and viability index, the co-variable was the age of the dams. The Levene test was used to study the homogeneity of variance. Consequently, in order to assess differences between groups when appropiate, non-parametric data were analyzed by the Kruskal-Wallis or Mann-Whitney U test, while parametric data were analyzed by a two-sample *t*-test or one-way ANOVA followed by a *post-hoc* Tukey or DMC. Social behavior was analyzed with a paired- or one-sample *t*-test in order to find differences between the time spent in the social/novel compartment versus the non-social/non-novel or the socialbility and novelty ratio, respectively. A principal component analysis (PCA) of **Δ**Ct was used to perform a general screening of gene expression. Correlation between genes was also assessed using Pearson or Spearman coefficients, depending on the homegenity of the sample. All data are presented as the mean $\pm$ S.E.M, with the statistical significance set at $p<0.05$.

RESULTS

4. RESULTS

This thesis includes four original articles, already available in the scientific literature or in the process of being published. The specific objective of this thesis and the corresponding publications are shown in Table 5.

Table 5. Specific objective of the thesis and corresponding publications.

Specific objective	Publications
I	Biosca-Brull J, Pérez-Fernández C, Mora S, Carrillo B, Pinos H, Conejo NM, Collado P, Arias JL, Martín-Sánchez F, Sánchez-Santed F, Colomina MT. **Relationship between Autism Spectrum Disorder and pesticides: A systematic review of human and preclinical models.** International Journal of Environmental Research and Public Health 2021; 18; 5190
II & V	Biosca-Brull J, Basaure P, Guardia-Escote L, Cabré M, Blanco J, Morales-Návas, Sánchez-Santed F, Domingo JL, Colomina MT. **Influence of gestational chlorpyrifos exposure and *APOE* polymorphism on autistic-like behaviors: differences with the valproic acid animal model**
III & V	Biosca-Brull J, Guardia-Escote L, Blanco J, Basaure P, Cabré M, Sánchez-Santed, Domingo JL, Colomina MT. **Prenatal, but not postnatal exposure to chlorpyrifos affects social behavior of mice and the excitatory-inhibitory balance in a sex-dependent manner** Food and Chemical Toxicology 2022, 169; 113423
IV & V	Biosca-Brull J, Guardia-Escote L, Basaure P, Cabré M, Blanco J, Pérez-Fernández C, Sánchez-Santed F, Domingo JL, Colomina MT. **Exposure to chlorpyrifos during pregnancy differentially affects social behavior and GABA signaling elements in an *APOE*- and sex-dependent manner in a transgenic mouse model** Environmental Research 2023, 224; 115461

PUBLICATION I

4.1. Publication I

Relationship between Autism Spectrum Disorder and pesticides: A systematic review of human and preclinical models.

Judit Biosca-Brull, Cristian Pérez-Fernández, Santiago Mora, Beatriz Carillo, Helena Pinos, Nelida Maria Conejo, Paloma Collado, Jorge L. Arias, Fernando Martín-Sánchez, Fernando Sánchez-Santed, Maria Teresa Colomina

International Journal of Environmental Research and Public Health 2021; 18; 5190
https://doi.org/10.3390/ijerph18105190

Study I overview

What do we already know?	In the last few years, an increasing number of neurodevelopmental disorders are being diagnosed. Environmental factors such as pesticides have been strongly associated with the development of some autistic-like behaviors, even though there is a wide variety of studies that show conflicting results.
What does the study add?	This is the first review to assess the effects of different families of pesticides on autism-related behaviors in both, clinical and preclinical studies. It provides a comprehensive synthesis of the information available about pesticides such as organochlorides, carbamates, pyrethroids, as well as mixed exposures, but it focuses above all OP compounds.
Highlights	Prenatal and postnatal pesticide exposure was associated with autism symptomatology in clinical studies. In animal models, pesticide exposure around GD 12 was associated with primary and secondary clinical signs of ASD. Both clinical and preclinical studies showed little evidence of pesticide exposure, genetic interactions or considering different covariates.

* No edited version of the online article

Review

Relationship between Autism Spectrum Disorder and Pesticides: A Systematic Review of Human and Preclinical Models

Judit Biosca-Brull [1,2], Cristian Pérez-Fernández [3], Santiago Mora [3], Beatriz Carillo [4], Helena Pinos [4], Nelida Maria Conejo [5], Paloma Collado [4], Jorge L. Arias [5], Fernando Martín-Sánchez [6], Fernando Sánchez-Santed [3,*] and Maria Teresa Colomina [1,2,*]

[1] Department of Pshychology, Research Center for Behavior Assessment (CRAMC), Universitat Rovira i Virgili, 43007 Tarragona, Spain; judit.biosca@urv.cat (J.B-B)

[2] Research in Neurobehavior and Health (NEUROLAB), Universitat Rovira i Virgili, 43007 Tarragona, Spain

[3] Department of Psychology, Health Research Center (CEINSA), Almeria University, 04120 Almeria, Spain; cpf603@ual.es (C.P-F.); santiagomorap@gmail.com (S.M.)

[4] Department of Psychobiology, University Institute of Research-UNED-Institute of Health Carlos III (IMIENS), National Distance Education University (UNED), 28015 Madrid, Spain; bcarrillo@psi.uned.es (B.C.); hpinos@psi.uned.es (H.P.); pcollado@psi.uned.es (P.C.)

[5] Laboratory of Neuroscience, Department of Psychology, Instituto de Neurociencias del Principado de Asturias (INEUROPA), University of Oviedo, 33011 Oviedo, Spain; conejonelida@uniovi.es (N.M.C.); jarias@uniovi.es (J.L.A.)

[6] National Scholl of Public Health, Institute of Health Carlos III, University Institute of Research-UNED-Institute of Health Carlos III (IMIENS), 28029 Madrid, Spain; fmartin@isciii.es (F.M-S)

* Correspondence: fsanchez@ual.es (F.S-S.); mariateresa.colomina@urv.cat (M.T.C.)

Abstract: Autism spectrum disorder (ASD) is a complex set of neurodevelopmental pathologies characterized by impoverished social and communicative abilities and stereotyped behaviors. Although its genetic basis is unquestionable, the involvement of environmental factors such as exposure to pesticides has also been proposed. Despite the systematic analyses of this relationship in humans, there are no specific reviews including both human and preclinical models. The present systematic review summarizes, analyzes, and discusses recent advances in preclinical and epidemiological studies. We included 45 human and 16 preclinical studies. These studies focused on Organophosphates (OP), Organochlorine (OC), Pyrethroid (PT), Neonicotinoid (NN), Carbamate (CM), and mixed exposures. Preclinical studies, where the OP Chlorpyrifos (CPF) compound is the one most studied, pointed to an association between gestational exposure and increased ASD-like behaviors, although the data are inconclusive with regard to other ages or pesticides. Studies in humans focused on prenatal exposure to OP and OC agents, and report cognitive and behavioral alterations related to ASD symptomatology. The results of both suggest that gestational exposure to certain OP agents could be linked to the clinical signs of ASD. Future experimental studies should

focus on extending the analysis of ASD-like behaviors in preclinical models and include exposure patterns similar to those observed in human studies.

Keywords: autism spectrum disorder; sociability; pesticide; organophosphate; carbamates; organochlorine; chlorpyrifos

1. Introduction

Autism spectrum disorders (ASD) are a heterogeneous group of neurodevelopmental disorders characterized by varying degrees of altered sociability, reduced communicative skills, and repetitive stereotyped behaviors unfocused on a specific goal [1]. Although these constitute the core clinical signs of ASD, it is well-known that ASD-diagnosed children also display alterations in other physical and cognitive functions such as motricity [2], attention [3], memory [4], and inhibitory control [5], amongst others.

Although the etiopathology of ASD remains unknown, there is a consensus regarding the relevance of its heritable/genetic basis, confirmed in various human twin studies [6] and animal models that show a simplified ASD-like phenotype, as in the case of the fragile X mental retardation (FMR1)-KO rodents, the BTBR models, and the neuroligin 3 (Nlgn3)-KO rodents, amongst others [7]. There is considerable empirical support for this genetic component [8], with multiple genes and loci found to be strongly associated with the diagnosis of ASD, of which the matrix metallopeptidase 12, neurotrimin, potassium calcium-activated channel subfamily *N* member 2, and the microtubule-associated protein tau are particularly noteworthy. However, the prevalence of ASD has increased significantly in recent decades [9], which can presumably be taken to indicate that heritability and genetic background are not the only causes of this set of disorders. This impressive rise in the prevalence of ASD diagnoses can be partially explained by changes in diagnostic criteria (for a food example, see [10]).

Concerning neurotoxic agents, various families of pesticides have recently been linked with ASD. Of these, Organochlorine (OC), Pyrethroids (PT), Carbamates (CM), Neonicotinoids (NN), and Organophosphates (OP) have been studied in most depth. Briefly, OC compounds are synthetic pesticides widely used globally, with important applications in both industry and agriculture and whose main mechanisms of toxicity are the regulation of the axonal sodium gates (DDT-Type) and

GABAergic regulation of Chloride ion influxes (Chlorinated Alicyclic-type) [18]. Some of the most important OC agents are DDT, DDD, Eldrin, Dieldrin, and Endosulfan, amongst others [19]. Moreover, PT agents are compounds commonly used as insecticides that induce excitatory paralysis by directly acting on the voltage-gated sodium channels [20]. Some of the most representative PT compounds are: Allethrin, Dimethrin, Tetramethin, and Alphametrin [19]. CM xenobiotic compounds generally induce their toxic profile by reversibly inhibiting the Acetylcholinesterases (AChE), thus increasing the cholinergic tone in the CNS [21]. From this chemical family, Carbaryl, Aldicarb, Pyrolan, and Carbanolate are among the most notable methyl agents [19]. Added to this, NN xenobiotic agents are a group of effective insecticides whose main use is focused on the control of sucking insects and flea control, since they act as selective agonists of nicotinic receptors [22]. Of these, Imidacloprid, Thiamethoxam, and Clothianidin are among the most used [22]. Finally, OP compounds are a wide-range group of pesticides commonly applied in industry, agriculture, and, to a less extent, used for residential purposes [23]. Their main mechanism of toxicity is the irreversible inhibition of the AChE in the CNS. Some of the most noteworthy OP agents are Chlorpyrifos (CPF), Malathion (MAL), Parathion, and Tricholorfan [19].

Both human and rodent studies have found interesting links between exposure to these various pesticides and ASD diagnosis or ASD-like behaviors [24,25]. However, results differ depending on the agent, the dose, the time of exposure, age of behavioral assessment, and the outcomes measured. Thus, whilst it appears that we have sufficient empirical results to establish solid conclusions, this is not actually the case, since the different studies have not been adequately analyzed based on the specific characteristics previously defined. Moreover, there is a lack of adequate discussion regarding the qualityof each individual study.

Given these considerations, we thought it worthwhile to conduct a systematic reviewof the most important empirical studies concerning exposure to different pesticides and their effect on ASD diagnosis and core behaviors in humans, and on ASD-like outcomes in preclinical rodent models. In doing so, the scientific community will have access to a clearer picture of the real relationship between exposure to these compounds and the incidence of ASD.

2. Material and Methods

2.1. Review Protocol

Prior to the literature search and in accordance with the ''Preferred Reporting Items forSystematic Review and Meta-Analysis Protocol" (PRISMA-P) Moher et al. [26] a detailed review protocol was created. As recommended by the PRISMA-P guidelines, and according to the suggestions of the

PROSPERO reviewers, two registrations were created on the PROSPERO database: one for animals (Prospero-ID: 145135, October 2019) and one for humans (Prospero ID: 153081, October 2019).

2.2. Eligibility Criteria

The systematic review was structured initially with the aid of the PICOS acronym (Participants, Interventions, Comparators, Outcomes measures, Study design). Participants were young humans (children or adolescents) and rodents. Interventions were prenatal or postnatal exposures to potential neurotoxic pesticides, herbicides, or insecticides. Moreover, comparison of environmental exposure (pre or postnatal) to pesticides, herbicides, or insecticides with a control/non-exposed group or comparison between groups with different levels of exposure (i.e., low, medium, high) were assessed according to the proximity to agricultural/industrial areas or the metabolite levels in blood/urine samples.

Biological outcomes such as agent exposure biomarkers (metabolite levels in blood or urine samples), hormonal alterations (enzymatic analyses in blood or urine), neurotransmitter activity alterations (protein levels in immunochemical arrays of blood and tissue samples and genetic expression in PCR), and cytokine alterations (protein levels in immunochemical arrays of blood and tissue samples) were assessed. Furthermore, behavioral measures related to autism such as cognitive and psychomotor alterations, as well as social communication impairments, were also evaluated.

In addition, experimental studies in the literature with animal models and cohort, cross-sectional and case-control human studies were considered, along with studies written in English and published within the last ten years. Exclusion criteria were defined by considering those aspects that did not meet the previously defined PICOS characteristics. Therefore, we did not select case studies, reviews, abstracts, or communications at scientific meetings, or qualitative studies. Lastly, we only included articles published in peer-reviewed journals.

2.3. Information Sources

We carried out comprehensive literature searches of Pubmed and Scopus until March 2020. The keywords used were autism spectrum disorder (ASD), pesticides, insecticides, herbicides, gestational, prenatal, and postnatal exposure, neurodevelopment, humans (plus combinations). Filters employed in the database searches were language (English) and data publication (last ten years). The search formula was: (TITLE-ABS-KEY (pesticides) AND (TITLE-ABS-KEY (prenatal

AND expos*) OR TITLE-ABS-KEY (postnatal AND expos*) OR TITLE-ABS-KEY (gestational AND expos*) AND TITLE-ABS-KEY (autism*) OR TITLE- ABS-KEY (ASD) OR TITLE-ABS-KEY (neurodevelopment*). In Scopus, an asterisk served as a substitute for any number of characters, expanding the search. Furthermore, a hand-search was performed in relevant journals and the reference lists of reviews focusing on the subject.

2.4. Study Selection and Data Collection Process

After eliminating duplicates, one reviewer examined the complete list of results for eligibility. If further relevant decisions were to be made, these were discussed among the research team until reaching a consensus. Moreover, two reviewers independently extracted the data in an unblended manner. Any disagreements were resolved until achieving consensus.

2.5. Risk of Bias in Individual Studies

Two tools were employed to assess the risk of bias: for animal studies, we used the ''SYRCLE's tool for assessing the risk of bias" [27], which is an adaptation for animal studies based on the Cochrane collaborations RoB tool [28], whilst for human studies, we employed the Newcastle-Ottawa Scale (NOS) to rate cohort and case-control studies [29].

In this regard, the SYRCLE consists of five quality parameters: selection, performance, detection, attrition, and reporting bias. It assigns a maximum of six points for selection, four points for performance, four points for detection, four points for attrition, and four points for reporting (for a total of 18 points). Therefore, the total quality index score was ranked as follows: 0 to 3, 4 to 6, 7 to 9, 10 to 12, 13 to 15, and 16 to 18, these being very low (VL), low (L), medium-low (ML), medium-high (MH), high (H), and very high (VH) quality, respectively. For human studies, the NOS uses three quality parameters: selection, comparability, and exposure/outcome assessment. It assigns a maximum of four points for selection, two points for comparability, and three points for exposure or outcome (making a total of 9 points). Hence, the total quality index score was ranked as follows: 0 to 2, 3 to 4, 5 to 6, and 7 to 9, these being L, MH, H, and VH quality, respectively.

3. Results

3.1. Selection of Studies

A flow diagram illustrates the whole search strategy (Figure 1). The first screening provided a total of 464 studies, and after removing duplicates and selecting articles based on year, language, and exclusion of reviews, a total number of 170 articles were selected. Further, 7 preclinical and 43 clinical studies were eliminated after reviewing the title and abstract. A parallel search based on words mentioned previously allowed us to find a further nine preclinical and two clinical studies. Thus, 16 preclinical and 45 clinical studies comprised the total number of studies included in this review.

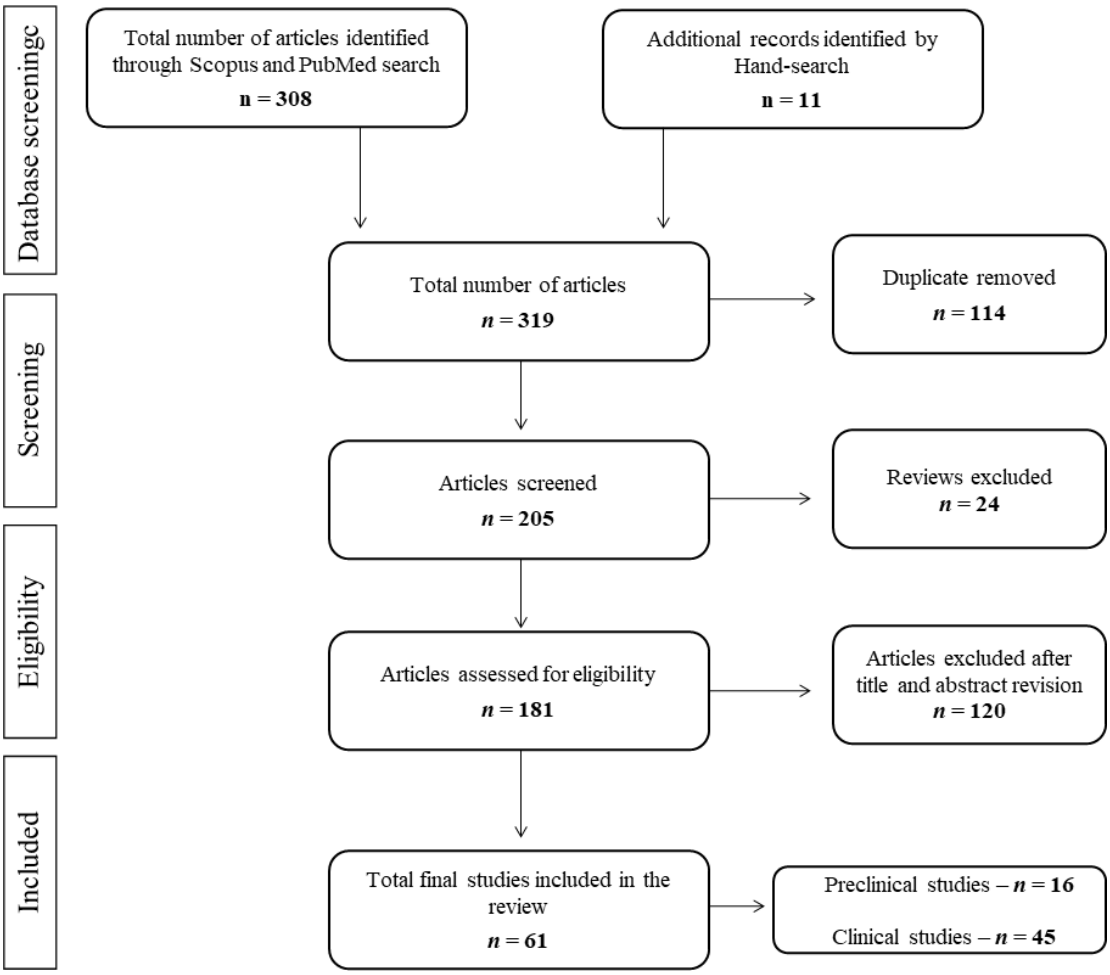


Figure 1. Flow Diagram. From top to bottom, the total number of outputs from Scopus and PubMed searches (n = 464). Selection based on year, language (n = 308) and exclusion of duplicates (n = 194), the total number of studies that successfully passed the selection checklist (n = 50), and the total number of accepted studies after parallel searching (n = 61).

3.2. Search Results and General Quality

Sixteen preclinical and 45 clinical studies were accepted. With regard to the studiesconducted in rodents, the majority exposed the animals to CPF or CPO (11 out of 16, 68.5%) [30–40]. A further two studies used other OP compounds such as CPF, MAL [41], and Phosphomedon (PMD) [42]. That is, most of the studies included in the present review are concerned with OP exposure (13 out of 16, 81.3%). Of the remaining articles,two used Glufosinate ammonium (GLA) (12.5%) [43,44], and one exposed their animals to the synthetic Pyrethroid cypermethrin (CYP) (6.3%) [45]. From all of these studies, only one [42] did not administer the pesticide during development; thus, most of the studies included in this systematic review present good examples of developmental neurotoxicity (15 out of 16, 93.8%). Interestingly, 10 out of the 16 studies (62.5%) followed a gestational exposure protocol, two used postnatal exposure (12.5%), and three employed a continuous exposure regime during both gestational and postnatal stages (18.8%). All of these studies, with the exception of the adult study, used mice models. Basaure et al. [40] also exposed their rodents to CPF during adulthood along with postnatal exposure.

Regarding the studies conducted in humans, 17 out of 45 (37.8%) studied OP exposure using urine, blood, and house-dust metabolite measures [46–62], with OP being the most studied pesticide in this review. From the remaining articles, 9 out of 45 (20.0%) studied OC exposure by DDT and/or DDE exposure [63–71], while Puertas et al. [72] and Boucher et al. [73] used other OC compounds such as Mirex and Chlordecone, respectively. Thus 11 out of 45 (24.4%) of the total studies were related to OC exposure. Further, PT exposure was assessed in 3 out of 45 studies (6.7%) [74–76], whereas exposure to Permethrin, and Piperonyl butoxide (PBO) (a synergistic component of pesticide formulation) and the metabolite of PT insecticide 3-phenoxybenzoic acid (3-PBA) exposure were reported by Horton et al. [77] and Watkins et al. [78], being five (11.1%) the total PT studies. Additionally, Zhang et al. [79] and Mora et al. [80] used Carbofuranphenol and Mancozeb compounds to assess CM exposure, whereas Keil et al. [81] assessed Imidacloprid exposure, a NN pesticide. Finally, 9 out of 45 (20.0%) studies [82–90] reported exposure to a common mix of different types of pesticides. From all these studies, 37 out of 45 (82.2%) followed a longitudinal design, while only 8 (17.8%) were case-control studies. Moreover, 32 out of 45 (71.1%) defined a prenatal exposure period, two (4.4%) a postnatal exposure period, while the remaining 11 (24.4%) considered both prenatal and postnatal periods. In human studies, exposure outcomes were assessed during childhood or adolescence.

Concerning the quality of the preclinical studies, three out of the 16 were labeled as H (18.8%) [30,37,38], 10 as MH (62.5%) [31–36,40,43–45], and the remaining three as ML- quality (18.8%) [39,41,42]. In a similar vein, 37 out of 45 human studies were classified as VH (82.2%) [46,48–

53,56–59,61,63–67,70–77,80–85,87–91], 7 as H (15.6%) [47,54,55,60,68,69,78] and only Woskie et al. [62] was classified as MH-quality.

3.3. Pesticide Exposure and ASD-Like Outcomes: Preclinical Studies

All of the preclinical studies concerned with pesticide exposure and ASD-like behaviors are summarized in Table 1.

3.3.1. Organophosphates Compounds

There were no preclinical studies labeled as VL or L-quality. Three out of the 13 OP studies were labeled as ML (23%) [39,41,42], seven were labeled as MH (54%) [31–36,40] and only three studies were classified as H-quality (23%) [30,37,38]. The OP studies were characterized as following a gestational exposure protocol (eight out of 13, 62%), although some studies used postnatal (2 out of 13, 15%) [30,40] and continuous gestational-postnatal exposure protocols (two out of 13, 15%) [33,41], with the exception of the adult study. Four of these studies used different genetic models of autism such as BTBR mice [35,36] and KO reeler mice [39] and other genes that could potentially modulate social behavior such as the different polymorphism of the human Apolipoprotein E (APOE) [40].

Gestational exposure to OPs both decreased [31,37,38] and enhanced [32,34,35] different social and communicative behaviors in mice. Lan et al. [37] exposed male mice from GD12 to 15 using a dose ranging from 2.5 to 5 mg/kg/day and found decreased social interaction and altered preference in a socially conditioned paradigm, without maternal care alterations. This result was confirmed in a later study [38] in both exposed males (highest dose) and females (lowest dose), without observing significant effects of the exposure on hypothalamic oxytocin mRNA expression. Further, Venerosi et al. [31] found that these abnormal social interaction patterns were complemented by alterations in communication skills, observing a decrease in the number and duration of the exposed pups' ultrasonic vocalizations (USVs) as well as alterations in maternal behavior (increased licking and exploration-tendency) following 6 mg/kg of CPF from GD15 to 18. Venerosi et al. [32] found a decrease in maternal aggressive behavior, increased anxiety in females, and a generally hyposensitized serotonergic system. De Felice et al. [34] exposed their mice following a similar exposure protocol and found that the exposed females showed enhanced social investigation rates. Further, De Felice et al. [35] also found an increased rate of USVs and social investigation in males using the same exposure protocol in BTBR mice. In addition to

their previous observations, De Felice et al. [36] found that the BTBR mice had, at baseline, higher levels of two of the most significant biomarkers of oxidative stress, 15-F2t-IsoP, and PGE2, both of which are associated with ASD. Interestingly, these authors found that this gestational exposure to CPF generally increased the levels of these molecules, eminently in male BTBR mice, which is congruent with their previous findings of altered neuromotor development in BTBR exposed animals [35]. Finally, Mullen et al. [39] used heterozygous/homozygous reeler mice ($Rl^{+/-}$ and $Rl^{+/+}$, respectively) and exposed them to CPO (6 mg/kg/day) from GD13 to delivery. The authors found that the exposure increased the number of USVs in the +/- male mice, but the opposite was found in the +/+ condition, whilst the females decreased USVs showed increased levels of social interaction in both genetic conditions.

Postnatal exposure to OPs was analyzed in Venerosi et al. [30] and Basaure et al. [40], both studies using CPF at the preweaning developmental stage. Venerosi et al. [30] found that 3 mg/kg from PND11 to 14 had no effects on the sociability indexes of mice but altered maternal care and social investigation whilst also reducing maternal aggression, a finding that was presumably related to a decreased state of anxiety in the exposed female rats. Further, Basaure et al. [40] used human APOE-3 or -4 mice models to characterize the presumable influences of genetic background on the social mismatches associated with preweaning CPF exposure (1 mg/kg/day from PND10 to 15). They also repeated this exposure during adulthood in both postnatal exposed and non-exposed groups. Postnatal CPF exposure increased the reaction to social novelty in APOE4 mice but reduced this reaction in APOE3 mice, this latter effect being blocked following the adult exposure protocol. The authors found that the adult exposure regime enhanced sociability regardless of the prior exposure condition in the APOE3 mice. Furthermore, the authors also analyzed various molecular markers and found that adult CPF exposure differentially modulated the hypothalamic levels of Oxytocin and Vasopressin mRNA (amongst others) depending on genotype background. Interestingly, chronic exposure (30, 45, or 60 days) to PMD (35 ppm) during adult ages did not alter sociability but produced a significant increase in locomotor activity along with multiple histological alterations in rats [42].

Finally, the studies that employed continuous gestational-postnatal exposure were those of Venerosi et al. [33] and Ouardi et al. [41], which used CPF and MAL, respectively. Venerosi et al. [33] exposed mice to 6 mg/kg of CPF from GD15 to PND14 and found a generalized enhancement in social investigation/recognition in both sexes (stronger in males) without altering motricity. Interestingly, the authors also analyzed the levels of oxytocin and vasopressin1a receptor in the amygdala and found that CPF exposure reduced the former in males and increased them latter. Ouardi et al. [41] exposed their mice to MAL (5–15 mg/kg)

from GD6 to PND21 and found a significant reduction in sociability and reaction to social novelty in the exposed animals compared with their control counterparts, along with an increased anxiety state and multiple molecular alterations in the CNS such as increased MDA levels and decreased CAT, SOD, GST and GNX in a dose and age-dependent fashion, presumably indicating an increased state of cellular oxidative stress in exposed rodents.

3.3.2. Other Potential Neurotoxic Compounds

All three studies that used xenobiotic compounds other than OPs were labeled as MH-quality [43–45]. Dong et al. [44] exposed female mice to GLA from 8 weeks before matingto delivery, whilst Laugeray et al. [43], and Laugeray et al. [45] used GLA (the former) and CYP (the latter) in a continuous gestational-postnatal exposure protocol in mice models. Dong et al. [44] exposed mice to 12 µg/mL of GLA (in water) and found a general decrease in social interaction and reaction to social novelty, along with decreased locomotor activity, increased compulsive/anxiety-like behavior, and a reduction in the mRNA expression levels of the cortical Nrxn1 gene. The authors also found a significant gut dysbiosis characterized by an increase and decrease of both Bacteroidetes and Firmicutes bacteria at the phylum taxa level and reduced biosynthesis of fatty acids, amongst other molecular changes. These effects on sociability were also found in Laugeray et al. [43], who exposed rodents to GLA from early gestational ages to PND14 in a range of doses from 0.2 to 1 mg/kg/day, finding a significant decrease in USVs and social interaction rates followingthe highest dose (without affecting anxiety-state levels), along with several molecular outcomes such as a reduction in both Pten and Peg3 brain genes, which are commonly associated with ASD. Similarly, Laugeray et al. [45] also found decreased sociability and self-grooming and increased motricity in mice exposed to 5 mg/kg/day of CYP, as well as altered maternal behaviors following a higher dosage (20 mg/kg/day), without effects on USVs and anxiety. Interestingly, the authors also found that these exposure regimens altered multiple genes.

3.4. Pesticides Exposure, Cognitive and Behavioral Alteration Related to ASD: Clinical Studies

The clinical studies included in this review evaluated different aspects related to neurodevelopmental, behavioral, and cognitive outcomes. This broad spectrum of study designs, methods, and functions evaluated added certain difficulties since each study assessed different aspects at different ages, thus hindering the possibility of drawing firm conclusions.

Table 1. ASD & preclinical studies. From left to right: Study ID, animals' strain, age at the time of behavioral/physiological assessment, sex analyzed, the xenobiotic agent used, dose, age of exposure, route of exposure, exposure measures of control, behavioral tests, physiological measures, and quality of the study.

Study, year (Reference)	Strain/age at evaluation/sex	Exposure agent/dose/age/route	Exposure control	Behavioral tests	Behavioral/pharmacological/physiological outcomes	Quality index
Organophosphate compounds						
Venerosi et al. [30]	Mice/PND>40. Mums postpartum/Both	CPF. 3mg/kg/d PND11-15 s.c.	Neuromotor battery	Three-chambers test. Maternal behavior. Nest building. Dark-light test	= sociability and reaction to social novelty. ↓ maternal care. ↓ anxiety in mums. ↓ maternal aggressive behavior. ↑ maternal social investigation. ↓ motricity	H
Lan et al [37]	Mice/PND5 (maternal care) and PND90 (sociability)/M	CPF. 2.5-5mg/kg/d GD12-15 Gavage	Weight, reflexes	Maternal behavior. Three-chambers test. Social conditioned place preference. NOR	↓ Sociability. = maternal care. ↓ preference social conditioned place. = NOR	H
Lan et al [38]	Mice/PND90/Both	CPF. 2.5-5mg/kg/d GD12-15 Gavage	N.A.	Three-chambers test. Social conditioned place preference.	↓ Social preference males vs. the rest (5mg/kg). ↓ Social preference females vs. males (2.5mg/kg). = Oxytocin mRNA levels at hypothalamus	H
Venerosi et al. [31]	Mice/PND4-15/Both	CPF. 6mg/kg/d GD15-18 Gavage	AChE activity, weight, neurobehavioral battery, reflexes	Maternal behavior. USVs. Spontaneous motricity	Altered maternal behavior in CPF exposed mums (increased wall rearing and decreased digging). ↓ USVs (calls/min and duration) only in PND10. ↓ pivoting. ↑ immobility.	MH
Venerosi et al. [32]	Mice/>PND90. Mums postpartum/Both	CPF. 6mg/kg/d GD15-18 Gavage	N.A.	Maternal aggression. Light-dark test	↓ Maternal aggressive behavior. ↑ Anxiety (Females). = Depressive-like behaviors. Serotonergic hyposensitivity (challenged with fluvoxamine)	MH
De Felice et al [34]	Mice/>PND70/Both	CPF. 6mg/kg/d GD14-17 Gavage	Pups, sex ratio, weight	Social Discrimination test	↑ Social investigation (Females). = Reaction to social novelty	MH
Venerosi et al. [33]	Mice/>PND70/Both	CPF. 6mg/kg/d GD15-PND14 Diet	AChE activity, weight, litter size, sex ratio	Social Recognition test. Open field	↑ Social investigation males (all phases) and females (second exposure to the same partner). ↓ reaction to social novelty (females). ↑ Estrogen Receptor β at Hypothalamus (Males). ↓ Oxytocin at Amygdala (males). ↑ Vasopressin receptor 1a at amygdala. = locomotor activity	MH
De Felice et al. [35]	BTBR Mice/PND4, 6, 8, 8 (USVs). >70 (Sociability and USVs)/Both	CPF. 6mg/kg/d GD14-17 Gavage	Weight, litter size, sex ratio, mortality, reflexes	USVs. Social Interaction test	↑ (trend) calls. ↑ USVs and social investigation (sniffing) (males to females). Altered developmental neuromotor functioning in exposed mice	MH
De Felice et al. [36]	Mice BTBR/PND1-21/Both	CPF. 6mg/kg/d GD14-17 Gavage	Weight, litter size	Enzyme immunoassay	↑ 15-F2t-IsoP in BTBR model (vs. wild-type). CPF both reduced (wild-type) and increased (BTBR) 15-F2t-IsoP brain levels in PND1. CPF 15-F2t-IsoP in BTBR animals (males) at PND21. CPF increased PGE2 brain levels in BTBR animals at PND21 (males) and PND70.	MH

Study, year (Reference)	Strain/age at evaluation/sex	Exposure agent/dose/age/route	Exposure control	Behavioral tests	Behavioral/pharmacological/physiological outcomes	Quality index
Basaure et al. [40]	APOE3 and 4 Mice/PNM5/M	CPF. 1mg/kg/d PND10-15 Oral 2mg/kg/d PNM5 Diet	Weight	Three chambers test	↑ Sociability in adult exposed (both preweaning exposed and not) APOE3 mice. ↓ reaction to social novelty in APOE3 mice postnatally exposed to CPF. Adult exposure blocked this effect. ↑ reaction to social novelty in APOE4 mice postnatally exposed. Hypothalamus: ↑ Oxytocin mRNA in adult exposed APOE3, ↓ in adult exposed APOE4. Adult exposure increased low expression rates of Vasopressin in APOE3. Adult exposure decreased Vasopressin and vasopressin receptor 1a mRNA levels in APOE4. Adult exposure decreased Estrogen receptor 1, Proopiomelacortina in APOE4, amongst others.	MH
Mullen et al. [39]	Reeler Mice/PND7 (USVs), PND30 (Social interaction)/Both	CPO. 6mg/Ml GD13-Delivery Pump	AChE activity	USVs. Three-chambers test. Open field. MBT	↑ USVs number in exposed $^{+/-}$Reeler (males) from its vehicle. ↓ USVs number exposed $^{+/+}$Reeler from its vehicle. ↓ USVs duration. ↑ social interaction (sniffing, females, both exposed $^{+/+}$ and $^{+/-}$)	ML
Hazarika et al. [42]	Rats/adulthood/Both	PMD. 35 ppm for 30-, 45- and 60-days Adulthood Diet	Weight	Social Interaction test. Open field	= Sociability. ↑ Locomotion (longer exposure protocol). Multiple histopathological disruptions following the different exposure protocols.	ML
Ouardi et al. [41]	Mice/PND21/Both	MAL. 5-15mg/kg/d GD6-PND21 Gavage	Weight, AChE activity	3-Chambers test. Open field.	↓ Sociability. ↓ Reaction to social novelty. ↑ anxiety (time in periphery, the highest dose). ↑ Brain MDA (PND21). ↓ brain CAT and SOD (PND5-21 for the high exposure, PND21 for the low exposure condition). ↓ brain GST (PND21) and GPX (PND15)	ML
Other families of pesticides						
Laugeray et al. [43]	Mice/PND1-5 (USVs). >PND90 (sociability)/ Both (pups) and M (adulthood)	GLA. 0.2-1mg/kg/3 times per week GD?-PND14 Intranasal	Neurobehavioral battery, weight, reflexes, litter size	Social Interaction test. Three-chambers test. USVs. Plus-maze	↓ USVs in exposed mice (highest dose). ↓ Sociability in the three-chambers test (highest dose). ↓ social interaction with females. = anxiety. ↑ relative gene expression of brain phosphatase and Pten (lowest dose). ↓ relative gene expression of brain phosphatase and Pten and Peg3 genes (highest dose).	MH
Laugeray et al. [45]	Mice/PND1-15 (USVs). >PND90 (sociability)/ Both (pups) and M (adulthood)	CYP. 5-20 mg/kg/3 times per week GD6-PND15 Intranasal	Neurobehavioral battery, weight, reflexes, litter size	Social Interaction test. Three-chambers test. USVs. Maternal behavior. Open field. Plus-maze	↓ Maternal behavior (highest dose). ↓ Sociability (lowest dose). = reaction to social novelty (lowest dose). ↓ self-grooming (lowest dose). = USVs. ↑ motricity (velocity in the highest exposed mice). = anxiety (lowest dose). Dysregulation of multiple genes	MH
Dong et al. [44]	Mice/PNW6-10/Both	GLA.12ug/mL for 8 weeks (mums before mating to delivery) Water	Pregnancy rate, litter size, weight	Social Interaction test. 3-Chambers test. Open field. MBT	↓ Locomotor activity. ↓ Social interaction, sociability, and reaction to social novelty. ↑ compulsivity/anxiety (MBT). ↓ Relative expression of cortical Nrxn1 gene. ↑ Relative abundance of Bacteroidetes bacteria in the gut. ↓ Relative abundance of Firmicutes in the gut. ↓ species diversity in the gut. Gut dysbiosis concerning multiple bacteria at genus level. ↓ Fatty acids biosynthesis.	MH

GD = Gestational day. PND, PNW & PNM = Postnatal day/week/month. s.c. = subcutaneous. F = Female. M = Male. USVs = Ultrasound vocalizations. CPF = Chlorpyrifos. CPO = Chlorpyrifos-Oxon. d = Day. PMD = Phosphomedon. MAL = Malathion. GLA = Glufosinate ammonium. CYP = Pyrethroid Cypermethrin. N.A. = Not applied. AChE = Acetylcholinesterase. NOR = Novel object recognition. MBT = Marble Burying Test. = No effects concerning exposure. ↑ Increased following exposure. ↓ Decreased following exposure. 15-F2t-IsoP = 15-F2t-isoprostane. PGE2 = Prostaglandin E2. APOE = Human Apolipoprotein. MDA = Malondialdehyde. CAT = Catalase. SOD = Superoxide dismutase. GST = Glutathione transferase. GPX = Glutathione peroxidase. Pten = Phosphatase and tensin homolog. Peg3 = Paternally expressed gene 3. H = High quality. MH = Medium-high quality. ML = Medium-low quality.

The 45 human studies included in this review are summarized in Table 2 according to the quality index. We have further categorized these studies by describing them according to the type of pesticide and period of exposure.

3.4.1. Organophosphate Compounds

We found 17 studies (37.8%) that referred to the association between OP and autism or developmental disorders. Of these, 12 were classified as VH (70.6%) [46,48–53,56–59,61], four as H (23.5%) [47,54,55,60] and only one [62] was classified as MH-quality. Twelve studies out of 17 assessed only prenatal exposure to OP, whilst five out of 17 (29.4%) [46,48,50,57,61] evaluated both prenatal and postnatal exposure and only Gonzalez-Alzaga et al. [55] studied postnatal exposure alone.

All of these studies assessed prenatal exposure to OPs by means of maternal urine or cord blood biomarkers, or child urine biomarkers (in the case of postnatal exposure). In general, the studies analyzed a set of different dialkyl phosphate metabolites (DAP) including dimethyl (DM) phosphate and diethyl (DE) phosphate metabolite [47–54,56–62]. Only two studies used other metabolites such as 3,5,6-trichloro-2-pyridinol (TCPy) as an indicator of exposure to CPF [46] or a direct estimation of pesticide exposure together with metabolites [60].

Two of these studies, conducted in Thailand and China, evaluated the effects of prenatal exposure to OPs during the first postnatal week [49,62]. In a pilot study, Woskie et al. [62] found a significant positive relationship between maternal urinary DM phosphate metabolites levels and the Bazelton Neonatal Behavioral Assessment (NBAS) habituation cluster score, along with a significant positive relationship between total DE phosphate metabolites and the NBAS range of state cluster score. However, Zhang and coworkers [49] reported a consistent negative association between neurodevelopmental scores and OPs metabolites in maternal urine, that is, they found an association between DEs and lower scores on the behavior scale whilst DMs concentrations were associated with poorer scores in passive tone, active tone, and primary reflex [49].

Another two studies evaluated children during the first year of life, between 6 weeks and 9 months of age [60] and at 5 months of age [56]. The former study measured metabolites and pesticides in cord blood samples from a Chinese population, and whilst they found no effects in six-week-old children, a significant negative association was found between exposure to Naled and CPF and motor function among girls aged nine months [60]. Moreover, Kongtip and coworkers [56] studied a population from Thailand and found an

association between concentration of DEs in maternal urine during the third trimester of gestation and a decrease in cognitive and motor function at 5 months of age, as well as a significant relationship between prenatal total DAP levels and motor scores.

In addition, three studies evaluated children aged from one to two years [48] and at two years of age [53,57]. Wang and coworkers [48], studying a population from China, found a significant negative association between prenatal urine levels of DEs and DAPs and social scores (among boys) at two years of age. In addition, in this study, postnatal urine levels of DAPs and DMs were also associated with increased scores on the adaptive domain in children at two years of age [48]. A study conducted in California, with the CHAMACOS cohort, found that maternal DAPs were negatively associated with cognitive and mental abilities as well as with child PON1 polymorphism, whilst no sex differences were reported [53]. Moreover, in a study of a cohort living in Shenyang (China), Liu et al. [57] reported that prenatal DEs levels were associated with an increased risk of developmental delay (in boys), while postnatal DAPs and DEs levels were associated with delays in development, particularly in motor and social areas among boys [57].

Two more studies evaluated children at three years of age [46,47] with the former finding no association between prenatal maternal levels of TCPy metabolite, although the authors reported an association between postnatal levels of the metabolite TCPy and social development, mainly in boys. Philippat et al. [47], evaluated a population of mothers from California at high risk of having a child with autism (the MARBLES cohort) and found a positive association between exposure to OPs and risk of autism (clinical diagnostic), but only in girls. However, it is necessary to interpret these results with caution since the sample size for girls was very small.

Moreover, a set of four studies longitudinally evaluated outcomes in children aged from one to five years [51], from one to nine years [52,54], and from one to fourteen years of age [59]. Donauer and coworkers [51] did not find any effect at any age on cognition and neurodevelopment in a population from Ohio. However, in another study conducted in a cohort from New York (80% black or Hispanic women) where metabolites of OPs were measured in cord blood samples, the levels of DAPs were negatively associated with mental development at one and two years of age, but no association was observed between DAPs and psychomotor development. In this study, certain associations were also found in relation to DAPs and race/ethnicity at one year of age. In this cohort, prenatal levels of DEs were negatively associated with IQ, perceptual reasoning, and working memory in children from seven to nine years, associations that are influenced by PON1 Q192R QQ genotype, which affects CPF metabolism [52]. In a similar vein, and in the same cohort from New York, Furlong et al. [54] found that levels of DEs were associated with poorer

social responsiveness in black participants, with a stronger effect found in boys, although in this study only social functioning was evaluated in children from seven to nine years [54]. Similarly, in a study conducted in California (CHAMACOS), maternal DAPs were associated with an increase in autism-related traits in childhood and adolescence, but no association was observed with deficits on specific facial recognition tests for children aged nine and twelve years [59].

Evaluations conducted with prenatally exposed cohorts aged six [61], seven [50], and eight years [58] reported different outcomes. A study conducted in a cohort from the Netherlands found no association between DAPs measured in urine during pregnancy and autism traits [61]. In a Californian cohort (CHAMACOS), prenatal exposure to DAPs was associated with lower cognitive scores, particularly in IQ, verbal comprehension (DAPs), and processing speed (DEs). However, postnatal urinary concentrations of DAPs were not associated with cognitive scores, and in this study, autism traits were not evaluated [50]. In the HOME cohort from Ohio, levels of DAPs were not associated with autism symptoms and no evidence was found to suggest that PON1 polymorphism modified prenatal DAPs exposure or autism risk [58].

Only one study evaluated postnatal exposure, in a cohort of children between the age of six and eleven years from Andalusia (Spain) [55]. In this study, the authors reported that urine DAPs levels of the children were associated with a decrease in verbal comprehension, processing speed, and IQ (primarily in boys). Information about prenatal exposure and the postnatal period until evaluation was estimated according to the proximity of their residence to agricultural areas, with the authors concluding that postnatal exposure to pesticides can negatively affect children's neuropsychological performance while prenatal exposure was weakly associated with neurodevelopment impairment.

3.4.2. Organochloride Compounds

Only 11 out of 45 studies carried out in humans reported an association between OC pesticides and autism. Of these, nine were classified as VH (81.8%, [63–71]), and Kao et al. [68] and Lyall et al. [69] were classified as H-quality. Prenatal exposure was the most frequently studied exposure for OC, except Kao et al. [68] who studied postnatal exposure, while prenatal and postnatal exposure was reported by Kim et al. [65] and Boucher et al. [73].

Table 2. ASD & clinical studies. From the left to the right: Study ID, Study design/region, age at evaluation/sex/sample size, type, agent and source of exposure assessment, neurobehavioral or neuropsychological assessment in children, behavioral/physiological outcomes or Diagnostic and quality of the study.

Study, year (Reference)	Study design Region	Age at evaluation / Sex / Sample size	Type, agent and source of exposure assessment	Neurobehavioral / neuropsychological assessment in children	Behavioral, Physiological outcomes / Diagnostic	Quality index
Organophosphate compound						
Guo et al. [46]	SMBCS Cohort / Shenyang (China)	3 yo Both / N=377	Env; OP (TCPy) Prenatal (prior to delivery) and postnatal (3 yo) urine samples	Gesell Developmental Schedules	No relationship between prenatal TCPy exposure and neurodevelopment alterations. ↓ Motor and social development related to postnatal exposure mainly in boys	VH
Wang et al. [48]	LWBC Cohort / Shandong (China)	1-2 yo Both / N=262	B; OP (DAPs) Prenatal (delivery) and postnatal (1 and 2 yo) urine samples	Gesell Developmental Schedules	No association between prenatal or postnatal exposure was found in children at 1 yo. Prenatal exposure to DEs and DAPs was associated with a ↓ in social scores (among boys), while postnatal exposure to DMs and DAPs ↑ adaptive domain in children 2 yo	VH
Zhang et al. [49]	Chinese Cohort / Shenyang (China)	3 do Both / N=249	Env; OP (DAPs) Prenatal urine samples (prior to delivery)	Neonatal Behavioral Neurological Assessment	↓ Overall neurodevelopment scores after prenatal exposure to OP measured by urine DAPs metabolites. DAPs concentrations, specially DEs was associated with lower scores in behavior scale and DMs was associated with poorer scores in passive tone, active tone and primary reflex	VH
Bouchard et al. [50]	CHAMACOS Cohort / California (USA)	7 yo Both / N=329	Env; OP (DAPs) Prenatal (13 and 26 gw) and postnatal (6 mo, 1, 2, 3.5, 5 yo) urine samples	Wechsler Intelligence Scale for Children – 4th edition	Prenatal DAPs exposure was associated with lower cognitive scores, especially, in IQ, verbal comprehension (DAPs) and processing speed (DEs). Postnatal urinary DAPs concentrations were not associated with cognitive scores	VH
Donauer et al. [51]	HOME Cohort / Ohio (USA)	Annually from 1 to 5 yo / Both / N=327	Env; OP (DAPs) Prenatal urine samples (16 and 26 gw)	Bayley Scales of Infant Development – 2nd edition / Clinical Evaluation of Language Fundamentals – Preschool, 2nd edition / Wechsler Preschool and Primary Scale of Intelligence – 3rd edition	No effect on cognitive and neurodevelopmental performance	VH
Engel et al. [52]	Mount Sinai Environmental Health Cohort / New York (USA)	1, 2 yo and 6-9 yo Both / N=169	Env; OP (DAPs) Maternal blood, cord blood and prenatal urine samples (between 26 and 28 gw)	Bayley Scales of Infant Development – 2nd edition / Wechsler Psychometric Intelligence Test / Wechsler Preschool and Primary Scale of Intelligence – 3rd edition / Wechsler Intelligence for Children – 4th edition	↓ mental development by DAPs (1 and 2 yo) and DMs (1 yo, race/ethnicity). No association in DAPs and psychomotor development. DEs negatively associated with IQ, perceptual reasoning and working memory in children 7-9 yo	VH
Eskenazi et al. [53]	CHAMACOS Cohort / California (USA)	2 yo Both / N=353	Oc; OP (DAPs) Prenatal urine samples (during pregnancy)	Bayley Scales of Infants Development – 2nd edition	Maternal DAPs were negatively associated with cognitive and mental abilities as well as with PON1 polymorphism	VH

Study, year (Reference)	Study design Region	Age at evaluation / Sex / Sample size	Type, agent and source of exposure assessment	Neurobehavioral / neuropsychological assessment in children	Behavioral, Physiological outcomes / Diagnostic	Quality index
Kongtip et al. [56]	Cohort / Thailand	5 mo Both / N=50	B; OP (DAPs) Prenatal urine samples (around 28 gw)	Bayley Scales of Infants Development – 3rd edition	DEs exposure during 3rd trimester were associated with ↓ in cognitive and motor function, while DAPs maternal levels only affected motor scores	VH
Liu et al. [57]	Chinese Cohort / Shenyang (China)	2 yo Both / N=310	B; OP (DAPs) Prenatal (prior to delivery) and postnatal (2 yo) urine samples	Gesell Developmental Schedules	Prenatal DEs exposure associated with ↑ risk of being developmentally delayed (in boys). Postnatal DAPs and DEs exposure showed delays in development, specially, in motor and social area among boys	VH
Millenson et al. [58]	HOME Cohort / Ohio (USA)	8 yo Both / N=224	Env; OP (DAPs) Prenatal urine samples (between 13 and 19 gw)	Social Responsiveness Scales	DAPs exposure was not associated with autism symptoms after adjusting for covariates. No evidence that PON1 polymorphism modified prenatal DAPs exposure and autism risk / ASD	VH
Sagiv et al. [59]	CHAMACOS Cohort / California (USA)	1, 2, 5, 7, 9, 10.5, 12 and 14 yo / Both N=333	Env; OP (DAPs) Prenatal urine samples (13 and 26 wo)	Social Responsiveness Scales / Behavioral Assessment Scales for Children, Version 2 / Infant Neuropsychological Evaluation Facial Expression Recognition Test / NEPSY-II Affect Recognition Test	Maternal DAPs were associated with an ↑ in autism-related traits in childhood and adolescence. However, no association was observed on facial recognition test in children 9 and 12 yo / ASD	VH
Van den Dries et al. [61]	Generation R Cohort / Rotterdam (Netherlands)	6 yo Both / N=622	Env; OP (DAPs) Prenatal (early, mid- and late pregnancy) and postnatal (6 yo) urine samples	Social Responsiveness Scales	No association between DAPs and autism symptomatology / Autistic traits	VH
Philippat et al. [47]	MARBLES Cohort / California (USA)	3 yo Both / N=203	Env; OP (DAPs) Prenatal urine samples (1st, 2nd, 3rd trimester)	Autism Diagnostic Observation Schedule / Social Communication Questionnaire / Mullen Scales of Early Learning	OP exposure assessed by DMTP metabolite concentrations tended to ↑ the risk of autism only in girls. No association were observed without sex-stratification / ASD	H
Furlong et al. [54]	Mount Sinai Environmental Health Cohort / New York (USA)	1, 2, 4, 6, 7-9 yo Both / N=136	Env; OP (DAPs) Prenatal urine samples (3rd trimester)	Social Responsiveness Scales	DEs levels were associated with poorer social responsiveness in black participants with stronger effect on boys. No association was found with DAPs and DMs concentrations /ASD	H
González-Alzaga et al. [55]	Cohort / Andalusia (Spain)	Between 6 and 11 yo Both / N=256	Env; OP (DAPs) Postnatal urine samples (between 6 and 11 yo)	Wechsler Intelligence Scale for Children – 4th edition	DAPs levels associated with a ↓ in verbal comprehension, processing speed and IQ among boys	H

Publication I

Study, year (Reference)	Study design Region	Age at evaluation / Sex / Sample size	Type, agent and source of exposure assessment	Neurobehavioral / neuropsychological assessment in children	Behavioral, Physiological outcomes / Diagnostic	Quality index
Silver et al. [60]	Chinese Cohort / Fuyang (China)	6 wo and 9 mo Both / N=199	Env; OP Prenatal cord blood samples	Peabody Development Motor Scales	No significant findings observed at 6 wo. Naled and CPF exposure associated with deficits in motor function, among girls at 9 mo	H
Woskie et al. [62]	Cohort / Thailand	Between 0 and 4 do Both / N=82	B; OP (DAPs) Prenatal urine samples (7 gm and prior to delivery)	Brazelton Neonatal Behavioral Assessment Scale	↑ Score in the Range of state cluster score associated with maternal DEP metabolite levels and ↑ urinary DMP metabolite levels was associated with ↑ scores in Habituation cluster	MH
Organochlorine compounds						
Bahena-Medina et al. [63]	Morelos Cohort / Mexico	1 mo Both / N=265	Env; OC Prenatal blood samples (each trimester)	Brazelton Neonatal Behavioral Assessment Scale / Graham – Rosenblatt Scale / Bayley Scales of Infant Development	No effects on reflex, neurological or psychomotor development at 1 mo	VH
Brown et al. [64]	FiPS-A Case-Control / Oulu (Finland)	0-7 yo / Both N=1,556	Env; OC Prenatal blood samples (each trimester)	Autism Diagnostic Interview – Revised	DDE ↑ odds of autism / ASD	VH
Kim et al. [65]	CHECK Cohort / Seoul, Anyang, Ansan and Jeju (Korea)	13-24 mo Both / N=140	Env; 38 OC Prenatal blood (pregnancy) and breast milk (30 days after delivery) samples	Bayley Scales of Infant Development – 2nd edition	No specific results related to OC pesticides exposure	VH
Puertas et al. [72]	INMA Cohort / Granada (Spain)	4 yo Both / N=255	Env; OC (Mirex) Placenta samples (at delivery)	McCarthy Scales of Children's Abilities	↓ Cognitive performance, especially, working memory and quantitative area (numerical memory, counting and sorting). No effects were observed in perceptual-performance, verbal and motor areas	VH
Boucher et al. [73]	Timoun Cohort / Guadeloupe	18 mo Both / N=204	Env; OC (Chlordecone) Cord blood and breast milk (3 mo) samples	Ages and Stages Questionnaire / Bayley Scales of Infant Development – 2nd edition	Prenatal exposure was associated with poorer motor ability among boys Postnatal exposure: no significant association with personal-social, communication, problem-solving, fine and gross motor scores	VH
Braun et al. [66]	HOME Cohort / Ohio (USA)	4 and 5 yo Both / N=175	Env; OC Prenatal and blood samples (2nd trimester and at delivery)	Social Responsiveness Scales	Maternal trans-nonachlor ↑ autistic behaviors / ASD	VH
Jeddy et al. [67]	ALSPAC Cohort / England	15-38 mo Girls / N=400	Env; OC Prenatal blood samples (pregnancy)	Adapted versions of the MacArthur Communicative Development Inventory	No association between β-HCH or DDE and communication scores (15 and 38 mo). HCB ↓ vocabulary comprehension and production (15 mo) and ↓ intelligibility scores (38 mo). DDT was associated with a ↓ in communication scores (38 mo)	VH

Study, year (Reference)	Study design Region	Age at evaluation / Sex / Sample size	Type, agent and source of exposure assessment	Neurobehavioral / neuropsychological assessment in children	Behavioral, Physiological outcomes / Diagnostic	Quality index
Hamra et al. [70]	EMA Case-control / California (USA)	4-9 yo Both / N=864	Env; OC Prenatal blood samples (2nd trimester)	Diagnostic and Statistical Manual of Mental Disorder – 4th edition	No association between pesticides exposure and odds of autism / ASD	VH
Torres-Sanchez et al. [71]	Morelos Cohort / Mexico	42-60 mo Both / N=203	Env; OC Prenatal blood samples (each trimester)	McCarthy Scales of Children's Abilities	DDE exposure during the 3rd trimester was associated with ↓ cognition, verbal comprehension and memory	VH
Kao et al. [68]	FiPS-A Cohort / Taiwan	8-12 mo Both / N=55	Env; 20 OC Postnatal breast milk (between 2 wo and 1 mo) samples	Bayley Scales of Infant Development – 3rd edition	DDT and trans-chlordane ↓ cognitive, language, social-emotional and motor scores	H
Lyall et al. [69]	EMA Case-Control / California (USA)	3-10 yo Both / N=1,144	Env; 46 OC Prenatal blood samples (2nd trimester)	Diagnostic and Statistical Manual of Mental Disorders – 4th edition, Text Revision	No clear evidence that higher levels of prenatal exposure to p,p'-DDE and trans-nonachlor increased risk of ASD / ASD	H
Pyrethroids compounds						
Viel et al. [75]	PELAGIE Cohort / Brittany (France)	6 yo Both / N=287	Env; PT Prenatal (6-19 gw) and postnatal (6 yo) urine samples	Wechsler Intelligence Scale for Children – 4th edition	No effect on neurocognitive scores after prenatal exposure ↓ Verbal comprehension and working memory associated with postnatal exposure to 3-PBA and *cis*-DBCA	VH
Viel et al. [76]	PELAGIE Cohort / Brittany (France)	6 yo Both / N=287	Env; PT Prenatal (6-19 gw) and postnatal (6 yo) urine samples	Strengths and Difficulties Questionnaire	No significant association between maternal urinary PT metabolites and child neurobehavioral deficits. Childhood urinary levels of 3-PBA and trans-DCCA associated with ↑ odds of behavioral disorders	VH
Furlong et al. [74]	Mount Sinai Children's Environmental Health Cohort / New York (USA)	1, 2, 4, 6, 7-9 yo Both / N=162	Env; PT Prenatal urine samples (3rd trimester)	Behavior Assessment System for Children / Behavior Rating Inventory of Executive Functioning	3-PBA associated with depression, somatization, behavioral and emotional deficits. Cis-DCCA exposure was associated with behavioral regulation, emotional and externalizing problems, while, trans-DCCA was not associated with adverse effects	VH
Horton et al. [77]	CCEH Cohort / New York (USA)	3 yo Both / N=342	Env; PT (PBO and Permethrin) Prenatal air (3rd trimester), maternal or cord blood (delivery) samples	Bayley Scales of Infant Development – 2nd edition	No association between permethrin air and blood samples with mental or motor development. ↓ in mental development after prenatal PBO exposure, while no association was found in motor development	VH
Watkins et al. [78]	ELEMENT Cohort / Mexico	2-3yo Both / N=187	Env; PT Prenatal urine samples (3rd trimester)	Bayley Scales for Infant Development - Spanish version, 2nd edition	Lower mental development in 1 yo children, being stronger in girls. No association between maternal 3-PBA and motor development at 2 or 3 years of age	H

Study, year (Reference)	Study design Region	Age at evaluation / Sex / Sample size	Type, agent and source of exposure assessment	Neurobehavioral / neuropsychological assessment in children	Behavioral, Physiological outcomes / Diagnostic	Quality index
Carbamates compounds						
Zhang et al. [79]	SMBCS Cohort / Shenyang (China)	3 yo Both / N=337	Env; CM (Carbofuranphenol) Prenatal (prior to delivery) and postnatal (3 yo) urine samples	Gesell Developmental Schedules	Prenatal exposure associated with ↓ in social and adaptative behaviors Postnatal exposure associated with language and social behavior deficits	VH
Mora et al. [80]	ISA Cohort / Matina (Costa Rica)	Pregnancy and 1 yo Both / N=355	B; CM (Mancozeb) Hair, blood and prenatal urine samples (19, 30 and 33 gw)	Bayley Scales of Infants Development – 3rd edition	↓ Cognitive abilities in girls, while language and fine motor development were affected in boys. ↓ social-emotional scores in both sexes	VH
Neonicotinoids compounds						
Keil et al. [81]	CHARGE Case-control / California (USA)	3 and 4 yo Both / N=669	Env; NN (Imidacloprid) Prenatal household by maternal interviews	Autism Diagnostic Interview – Revised / Autism Diagnostic Observation Schedules / Mullen Scales of Early Learning / Vineland Adaptive Behavior Scales / Child Development and Social Communication Questionnaire	Association between autism and Imidacloprid exposure / ASD	VH
Mixture: pesticides and other potential neurotoxic						
Andersen et al. [82]	Cohort / Denmark	Between 6 and 11 yo Both / N=177	Oc; Insecticides, fungicides and plant growth regulators Prenatal (1rst trimester) exposure No biomonitoring, estimation of exposure	BAEP / Finger Tapping Test / Conner/s Continuous Performance Test II / Wechsler Intelligence Scale for Children – Revised / Woodcock Intelligence Tests of Cognitive Abilities / Copying Test of the Stanford – Binet, 4th edition	↑ Brainstem evoked potential (BAEP) latency (boys and girls). Impairment in neurobehavioral, language, motor speed and short-term memory functions, only in girls	VH
Gunier et al. [91]	CHAMACOS Cohort / California (USA)	7 yo Both / N=283	Env; 15 OP, 6 CM, 2 Mn-fungicide, 8 PT and 1 NN Prenatal house-dust samples	Wechsler Intelligence Scale of Children – 4th edition	OP associated with IQ and verbal comprehension deficits. OP and CM associated with ↓ IQ. NN, PT and Mn-fungicides associated with ↓ in IQ, perceptual reasoning and verbal comprehension	VH
Schmidt et al. [89]	CHARGE Case-Control / California (USA)	2 and 5 yo Both / N=516	B; OP, PT and CM Prenatal household (3 mo before conception and during pregnancy)	Autism Diagnostic Observation Schedule / Social Communication Questionnaire / Mullen Scales of Early Learning / Vineland Adaptive Behavior Scales	Exposure to OP, PT and CM ↑ autism risk, while FA intake ↓ the risk / ASD	VH

Study, year (Reference)	Study design Region	Age at evaluation / Sex / Sample size	Type, agent and source of exposure assessment	Neurobehavioral / neuropsychological assessment in children	Behavioral, Physiological outcomes / Diagnostic	Quality index
Eskenazi et al. [84]	VHEMBRE Cohort / Limpopo (South Africa)	1-2 yo Both / N=705	B; OC and PT Blood and urine (prior and post-delivery) samples	Bayley Scale of Infant Development – 3rd edition	(1 yo) No effect of DDT/DDE exposure and neurodevelopment. Cis-DCCA, trans-DCCA and 3-PBA were associated with socio emotional deficits. (2 yo) Motor problems associated with DDT, while DDE ↓ in communication and language. Cis-DBCA were associated with a ↓ in communication and language, among girls (1 yo) and both sexes (2 yo)	VH
Furlong et al. [85]	Mount Sinai Children's Environmental Health Cohort / New York (USA)	1, 2, 4-7, 9 yo Both / N=404	Env; OP and PT Prenatal (between 25 and 40 gw) urine samples	Behavior Rating Inventory of Executive Functioning / Behavior Assessment System for Children / Wechsler Preschool and Primary Scale of Intelligence – 3rd edition / Wechsler Intelligence Scales – 4th edition	DMs levels associated with worse internalizing scores (anxiety scale) and ↑ working memory among black children, while DEs was associated with worse working memory scores. No association was observed with PON1 polymorphism	VH
McCanlies et al. [87]	CHARGE Case-control / California (USA)	2 and 5 yo Both / N=951	Oc; Pesticides Postnatal mother/father interviews	Mullen Scales of Early Learning / Vineland Adaptive Behavior Scales / Autism Diagnostic Observation Schedule / Autism Diagnostic Interview – Revised / Social Communication Questionnaire	No association between pesticides and autism / ASD	VH
Ostrea et al. [88]	Cohort / Bulacan (Philippines)	2 yo Both / N=754	B; CM (Propoxur) and PT / Prenatal maternal blood, hair and postnatal cord blood and children hair	Griffiths Test	Motor development were affected after Propoxur exposure among boys No association was observed between Propoxur exposure and social behavior	VH
Shelton et al. [90]	CHARGE Case-Control / California (USA)	Between 2 and 5 yo Both / N=970	B; OP, CM, PT and OC Prenatal household (3 mo before conception and during pregnancy)	Autism Diagnostic Observation Schedule / Social Communication Questionnaire / Mullen Scales of Early Learning / Vineland Adaptive Behavior Scales	↑ autism risk after prenatal OP pesticides (1st and 2nd trimester) and PT (3 mo before conception and 3rt trimester) / ASD	VH
Von Ehrenstein et al. [83]	Case-Control / California (USA)	1 yo / Both N=38,331	Env; Pesticides Prenatal (3 mo before conception and during pregnancy) and postnatal (first year of life) residential samples	Diagnostic and Statistical Manual of Mental Disorders – 4th edition, revised	↑ autism risk after prenatal exposure to pesticides such as Glyphosate, CPF, MAL, Diazinon, Avermectin and Permethrin / ASD	VH

Gw = gestational week. gm, mo = gestational/month old. yo = year old. Oc = occupational. Env = environmental. B = both. DAP = dialkyl phosphate. DM = dimethylphosphate. DE = diethyl phosphate. DMTP = dimethylthiophosphate. DDT = dichlorodiphenyltrichloroethylene. DEP = diethylphosphate. TCPy = 3,5,6-trichloro-2-pyridinol. DDE = dichlorodiphenyldichloroethylene. PON1 = paraxonase 1. DCCA = 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid. DBCA = cis-3-(2,2-dibromovinyl)-2,2-DCCA. 3-PBA = 3-phenoxybenzoic acid. PBO = piperonyl buroxide. HCH = hexachlorocyclohexane. HCB = hexachlorobenzene. Mn = manganese fungicides. IQ = intelligence quotient. FA = folic acid. CPF = Chlorpyrifos. MAL = Malathion. VH = Very high. H = High quality. MH = Medium-high quality. ML = Medium-low quality.

As previously stated, we evaluated studies of prenatal exposure that measure OCs metabolites in maternal blood during pregnancy, along with breast milk samples of children given postnatal exposure. The literature focuses on DDT and one of its principal breakdown products DDEs. Authors that studied the effects of this exposure also assessed other metabolites such as hexachlorocyclohexane (HCH) and hexachlorobenzene (HCB) [63–71]. Only two studies assessed other OC compounds (Mirex, [72] and Chlordecone, [73]).

Two studies evaluated children during the first year of life [63,64]. Although no effects were found on reflexes, neurological or psychomotor development after prenatal OC exposure at one month of age [63], a national birth cohort study (Finland) which evaluated children within the age range at risk of ASD (between 0 and 7 years of age) found a link between DDE exposure and an increased likelihood of developing autism [64].

Only one study assessed children's developmental problems at 18 months of age [73]. Prenatal Chlordecone exposure was measured from umbilical cord blood, whereas postnatal exposure was measured from breast milk collected at 3 months postpartum. Nevertheless, boys prenatally exposed to this OC compound showed poorer motor abilities, whilst no significant association was found between childhood exposure and personal-social, communication, problem solving, fine and gross motor development [73]. A further evaluation was conducted in children at 4 years of age [72]. While no effects were observed on perceptual performance, verbal, and motor areas, the INMA cohort showed deficits in cognition, particularly in working memory and quantitative areas (numerical memory or counting and sorting) [72].

Further, four studies evaluated the outcomes in children during the first five years of life, that is, from 13 to 24 months [65], 15 to 38 months [67], 42 to 60 months [71], and four to five years [66]. Even though analysis of maternal OC serum levels did not reveal any specific outcome [65], Jeddy and coworkers [67] reported that an increase of HCB levels was associated with vocabulary comprehension and production deficits in 15-month-old children, while a decrease in intelligibility scores were observed at 38 months of age. The same study found communication problems associated with maternal DDT levels in children aged 38-months-old, but no association was observed between β-HCH or DDE and communication scores in both exposure periods [67]. In addition, a study conducted in Morelos (Mexico) found that DDE exposure during the third trimester of pregnancy was associated with verbal comprehension, cognitive and memory problems [71], while the HOME cohort showed a link between *trans*-nonachlor and an increased risk of developing autism behaviors [66].

Two more studies evaluated children aged between three and 10 years [69] and from four to 9 years [70]. All of these works evaluated the EMA population based on a case-control study that identified biomarkers and their possible association with the risk of developing autism.

Lyall and coworkers [69] found no clear evidence that higher levels of DDE and *trans*-nonachlor in maternal serum analyzed during the second trimester of pregnancy increased the risk of the disorder, whilst Hamra et al. [70] found no association between OC exposure and the risk of developing autism.

Postnatal exposure to OC metabolites (measured from breast milk) was only assessed in one study [68] in children aged between 8 and 12 months, finding that exposure to DDT and *trans*-nonachlor were associated with socio-emotional, language, and cognitive deficits, along with motor problems.

3.4.3. Pyrethroid Compounds

With regard to PT, five out of 45 (11.1%) studies were included. Of these, four were classified as VH (80.0%, [74–77]) and only Watkins et al. [78] was classified as H-quality.All studies assessed prenatal exposure, except for Viel et al. [75,76] who evaluated prenatal and postnatal PT exposure.

The effects of PT exposure were generally assessed by measuring metabolites in maternal urine, cord blood or air samples, and/or child urine. All of these studies assessed PT differently, by 3-phenoxybenzoic acid (3-PBA) (a non-specific metabolite), Permethrin, Cypermethrin and Cyfluthrin (by their *cis* or *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (*cis* or *trans*-DCCA) metabolite), 4-Fluoro-3-PBA (4- F-3-PBA) (as a specific metabolite of cyfluthrin) and *cis*-3-(2,2-dibromovinyl)-2,2-DCCA (*cis*-DBCA) (as a deltamethrin specific metabolite) [74–76,78]. The only exception was the study by Horton et al. [77], which assessed Permethrin and Piperonyl butoxide (PBO) exposure.

One study evaluated children at two and three years of age [78], while Horton et al. [77] only evaluated children at three years of age. A study conducted in Mexico (the ELEMENT cohort) found lower mental development in 2-year-old children, with stronger effects in girls. The same study found no association between maternal urine 3-PBA levels and motor development at both ages of evaluation [78]. Moreover, studies carried out with a New York cohort (CCEH) also found mental development deficits with PBO air exposure during the third trimester of pregnancy (but not motor problems), whereas no association between Permethrin exposure and mental and motor development were found at two years of age [77]. Another New York cohort study (Mount Sinai Children's Environmental Health), which measured PT metabolites in maternal urine, assessed outcomes in children aged between one and nine years. The results revealed depression, somatization, behavioral and emotional deficits

after 3-PBA exposure, and while *cis*-DCCA was associated with externalizing problems and poorer behavioral and emotional regulation, the *trans*-DCCAisomer was not associated with any adverse effects [74].

Finally, whilst no effects were observed following prenatal PT exposure in the PELAGIE cohort, postnatal 3-PBA and *cis*-DBCA exposure assessed in urine in 6-year-old children was associated with deficits in verbal comprehension and working memory [75], whilst an increased risk of behavioral disorders was also observed following 3-PBA and *trans*-DCCA exposure [76].

3.4.4. Mixtures of Pesticides and Other Potential Neurotoxic Agents

The remaining articles, specifically 12 out of 45 (26.7%), studied the effects of exposure to a common mix of pesticides and other toxicants or several types of pesticides. All of these studies were classified as VH [46,80–85,87–91]. Eight out of 12 studies assessed only prenatal exposure, three out of 12 (25.0%, [46,83,88]) assessed both prenatal and postnatal exposure, whereas only one study [87] evaluated postnatal exposure.

Whilst previous studies evaluated a single exposure to pesticides in children, the most common situation in humans is that they are exposed to a wide variety of pesticides, something that is formally taken into account in recent studies related to the concept of exposome science [92]. In this regard, general exposure to pesticides was assessed in three out of 12 studies (25.0%, [82,83,87]). Moreover, six out of 12 studies assessed the effects of exposure to a mix of different pesticides (50.0%, [84,85,88–91]), whilst the remaining two assessed CM exposure (16.7%, [46,80]), and only Keil et al. [81] assessed NN exposure. With regard to CM, maternal urine, blood, hair, cord blood, or child urine samples were used as measures of prenatal and postnatal exposure to Propoxur, Carbofuranphenol, and Mancozeb [46,80,88]. In addition, prenatal exposure to NN was evaluated by measuring household levels of Imidacloprid [81]. The remainder of the included studies measured prenatal and/or postnatal exposure to OP, PT, or OC, as mentioned in the previous sections. Two studies evaluated children during the first two years of life [83,84]. A case-control study based on individuals with a primary diagnosis of autism disorder reported an increased risk of ASD following prenatal exposure to pesticides such as CPF, MAL, diazinon, avermectin, and Permethrin during the first year of life [83]. A study carried out in Limpopo (South Africa) found no adverse effects on neurodevelopment at one year of age, whilst DDT exposure was associated with motor problems, and two-year-old children showed communication and language deficits following DDE exposure. This same study assessed exposure to PT metabolite, measured by blood and urine. Socio-emotional problems

were observed during the first year of life, while *cis*-DBCA metabolite exposurewas linked to communication and language deficit in 1-year-old girls and 2-year-old children of both sexes [84].

Moreover, three studies evaluated outcomes in children aged from 2 to 5 years [87,89,90]. All of these studies evaluated a Californian population (CHARGE cohort), which showed an increased risk of autism in children following prenatal exposure to OP (during the second trimester) and PT (3 months before conception and during the third trimester of pregnancy) [90]. Similarly, Schmidt et al. [89] found the same increased risk of the disorder following OP, PT, and CM exposure, while folic acid (FA) intake decreased this risk. However, no association was found between autism and general postnatal exposure to pesticides [87].

Two more studies evaluated children from one to nine years of age [85] and six to 11 years [82]. One study assessed the Mount Sinai Children's Environmental Health cohort (New York) and found an association between prenatal urine DMP levels and internalizing problems and better scores in working memory in black children, and whilst DEP was associated with poorer working memory scores, no association was found with PON1 polymorphism [85]. A Denmark population, which evaluated general prenatal pesticide exposure, found an increase in brainstem evoked potential (BAEP) latency in both sexes, as well as impairments in neurobehavioral, language, motor speed, and short-term memory functions, but only in girls [82].

Gunier et al. [91] evaluated 7-year-old children using a number of tests. This study assessed the CHAMACOS cohort, which showed IQ and verbal comprehension deficits following OP, NN, and PT exposure. Furthermore, exposure to NN and PT was also linked to perceptual reasoning problems, while combined exposure to OP and CM was only reported to be associated with IQ deficits [91]. Further, three studies evaluated exposure to only CM at one year [80], two years [88], and three years of age [79]. During the first year of life, a decrease in cognitive abilities in girls, deficits in language and fine motor development in boys, and socio-emotional deficits in both sexes were observed following prenatal exposure to Mancozeb [80]. Moreover, exposure to Propoxur was also associated with motor development problems in boys, while no social behavior deficits were observed [88]. Finally, prenatal urine levels of Carbofuranphenol were linked to a decrease in social and adaptive behaviors, whereas postnatal exposure was associated with language and social behavior deficits [79].

Finally, only Keil et al. [81] assessed prenatal NN exposure using measurements of Imidacloprid in children aged between 3 and 4 years. This study, conducted with a

Californian population (CHARGE cohort), found an association between Imidacloprid and autism disorder [81].

4. Discussion

4.1. Preclinical Studies and ASD

A total of 16 preclinical studies were finally included in the present systematic review. All of them studied some effect on communication or social behavior after being prenatally or postnatally exposed to OP, PT, or GLA.

When dividing the analysis according to developmental stages and compounds, gestational exposure to CPF decreased sociability in mice exposed from GD12-15 to doses from 2 to 5 mg/kg in the best-qualified studies [37,38]. MH classified studies also revealed decreased USV rates and maternal behavior when exposure occurred later during gestation [31,32], but the opposite was also true for both vocalizations and social investigation in male BTBR mice [35] and social interaction rates in wild-type female mice [34]. However, this gestational exposure protocol from GD14 to 17 in BTBR ASD-like mice models was associated with deep alterations in secondary behavioral markers usually observed in ASD patients such as delayed neuromotor development [35], along with an increase in various biomarkers of oxidative stress that are typically associated with autism [36]. Interestingly, this phenomenon of enhanced social and communicative traits was also observed in another model of ASD-like genetic background by using heterozygotes *Reeler* mice and exposing them from GD13 to delivery, as found in Mullen et al. [39], a study categorized as ML quality. This study is of special relevance as the influences of CPO on social outcomes varied depending on sex, where $^{+/-}$ neonate female mice decreased USVs number whilst their male counterparts increased them, as well as exposed females generally enhanced their social interaction during adolescence. These results support the notion that genetic background and environmental agents interact giving different results in a sex-dimorphic manner. As CPO exposure altered females' behavior in both $^{+/-}$ and $^{+/-}$ conditions, one explanation could be because of the differences that exist between sexes regarding the development of the cholinergic system during early neurodevelopment [93].

Studies of postnatal exposure also yielded inconclusive results. The only H-quality study shows that pre-weaning exposure to CPF altered maternal care and aggressive behavior but enhanced maternal social investigation without affecting the performance of pups in the three-chambers test [30]. Interestingly, one MH-quality study found that pre-weaning exposure to doses as low as 1 mg/kg/day differentially affected the reaction to social novelty

in mice depending on the APOE genotype background, with decreased (APOE3) and enhanced (APOE4) rates, both regulated by re-exposure to CPF during adulthood [40]. The earlier data is relevant since isoform 3 is most widely expressed in humans [94]. Finally, the results concerning continuous exposure to CPF [33] or MAL [41] during the whole developmental period (gestational and postnatal ages) showed opposing results characterized by both general enhancement (except for reaction to social novelty in females) and a significant decrease in social traits, respectively. However, we must point out that the differences in quality between these studies (MH vs. ML), the chemical studied (CPF vs. MAL), and the age range of exposure (medium-late gestation to the end of the second postnatal week vs. early gestation to weaning) prevent us from reaching common conclusions. Finally -and in relation to exposure to non-OP compounds- all the three studies included were categorized as MH, and all of them induced an ASD-like phenotype in their mice, using different doses of both GLA [43,44] or CYP [45] during the whole developmental period. Once again, the lack of studies limits the generalizability of these interesting, but insufficient, empirical results.

Based on all this information, we believe that there is not enough empirical support at any developmental stage or exposure protocol to confidently conclude that exposure to OPs or other pesticides can be linked to the development of ASD-like (core) behaviors. However, exposure to CPF during medium gestational ages (around GD12) seems to be the protocol that shows more promise in this regard [37,38]. This hypothesis gains support when using other well-known chemicals that can elicit these behavioral alterations when exposed at this age, as in the case of VPA [95], a drug that has also been linked to the diagnosis of ASD in humans [13]. However, the results provided by Lan and collaborators [37,38] must be replicated in other laboratories and in other rodent species such as rats, which are probably more appropriate models with regard to social outcomes [96], whilst there is also a need to include models with well-known genetic vulnerabilities to study this complex gene/environmental relationship that is thought to underlie ASD.

With respect to this last point, it is noteworthy that only two studies included in the present systematic review used genetic models of ASD (BTBR and heterozygous *Reeler* mice) to study behavioral outcomes [35,39] or molecular biomarkers [36], the two former studies finding enhanced social rates following CPF and CPO exposure. If the current hypothesis- those pesticides are environmental factors that can unmask or worsen the ASD phenotype codified in genes of vulnerability- is valid, these preliminary results point toward another direction, at least in relation to CPF. However, we consider that findings from a sum total of only two studies, which differ markedly, are an insufficient basis upon which to draw firm conclusions. We should not overlook the results obtained by De Felice et al. [35] regarding neuromotor outcomes and later [36] for different molecular markers that are generally linked to ASD.

4.2. Clinical Studies and ASD

As reported above, a total of 45 clinical studies were finally included in the present systematic review, 17 of which assessed OP exposure by means of metabolite measures, with two of the 17 studies assessing TCPy as a measure of CPF exposure [46] as well as other OP metabolites [60]. Moreover, 11 out of the 45 studies evaluated OC through metabolite measures, whilst two studied Mirex [72] and Chlordecone exposure [73]. Further, five out of 45 assessed general PT exposure by means of metabolite measures. CM exposure was assessed in two out of 45 (Carbofuranphenol, Zhang et al. [79] and Mancozeb, [80], whereas only Imidacloprid exposure was employed as a measure of NN exposure [81]. Finally, nine out of 45 studies assessed the effects of exposure to a common mix of pesticides.

As reported above, analyses were separated according to pesticide class (OP, OC, PT, CM, NN, and mixtures), period of exposure, and assessment outcome. Considering the studies analyzed assessing OP exposure, 14 out of 17 found effects on cognitive and behavioral functions after either prenatal or postnatal exposure. In particular, studies that assessed the effects of prenatal exposure to OP metabolites at very early ages (first days after delivery) pointed to altered primary reflexes, tone, and behavioral regulation [49,62]. Similarly, VH-quality studies observed alterations in motor function at 5 months [56], whereas H-quality works observed these alterations at 9 months of age [60]. However, these studies lack an adequate follow-up to establish future alterations during childhood or associations with ASD that are more likely to be diagnosed around the age of 18 months and beyond [97]. In this sense, another study classified as VH-quality [48] found associations between prenatal exposure to OPs and alterations in behavioral domains that could influence social relations, along with impaired social scores in boys evaluated at two years of age. Accordingly, social deficits among boys (7–9 years) were also found by Furlong et al. [54] (H-quality), with stronger effects observed in black participants following prenatal exposure, while a study classified as VH [58], with the HOME cohort, did not find any significant associations between OP exposure and autism when their model was adjusted for maternal sociodemographic and perinatal factors (8 years), with the authors indicating that a larger sample size might have allowed them to detect certain associations related to PON1 polymorphisms. Some of the discrepancies in results can be due to differences in OP insecticide exposure in the studied population, given that HOME study enrollment followed the U.S. EPA moves to restrict the residential use of OPs, thus lowering the environmental concentrations in comparison with those to which participants in the Mount Sinai Environmental Health study were exposed [98]. Moreover, another VH-quality study included in this review did not find any association between prenatal exposure to OP and ASD or ADHD [61]. The characteristics of the population included inthis study, that is, high socioeconomic level and high levels of exposure mainly through diet (fresh fruit and vegetables) raise an important question regarding the lifestyle and social factors that could

increase population resilience to toxicant effects. Further, another study with a long follow-up assessment (from one to 12 years of age) showed childhood and adolescence autism-related traits to be associated with prenatal OP exposure [59]. In addition, an H-quality study [47] found an association between prenatal OP exposure and autism diagnosis at three years of age, but in this case, the effects were only observed in girls. This study is one of the few that uses clinical diagnoses of autism instead of behavioral traits associated with social or communication abilities. Even though the quality of the study is high, the use of a population that is at high risk for ASD may have had some influence on this result. More studies are thus needed to assess OP effects on populations at high risk for ASD.

Moreover, studies categorized as VH-quality also found cognitive and developmental delays in children prenatally exposed to OP at two years of age [53,57] as well as neuropsychological impairments at later ages [50,52].

Regarding early postnatal exposure, VH-quality studies found social impairments among boys at two years of age [57], with similar results being reported by Guo et al. [46] related to CPF exposure at three years of age, whilst Wang et al. [48] reported some changes in the adaptive domain. Moreover, an H-quality study reported neuropsychological impairments associated with postnatal exposures [55].

With regard to OC exposure, 7 out of 11 studies found some cognitive but not social adverse effects in children exposed prenatally or postnatally to this type of pesticide. Two VH-quality studies reported autistic behaviors associated with blood OC metabolite levels [64,66]. All of these use a prenatal exposure protocol, evaluated at early ages (between two and four months of age) [64] and from four to five years of age [66]. In addition, a study using prenatal exposure with a follow-up period between one and five years reported intelligibility, vocabulary comprehension, and production deficits [67], while verbal comprehension, memory, and cognitive problems were observed by Torres-Sánchez et al. [71].

Similarly, cognitive deficits, particularly in working memory and quantitative areas were observed at four years of age following Mirex exposure [72], while Chlordecone exposure was linked to poorer motor ability amongst boys [73]. The same motor and cognitive deficits were also observed in children aged between eight and twelve months following postnatal exposure, in a study categorized as H-quality [68].

Conversely, the residential uses of PT have been rapidly increasing over the years [99]. In this review, except for the study by Watkins et al. [78] (H-quality), all studies were categorized as VH-quality. Moreover, all the included studies showed behavioral or development deficits, being more evident in the two studies which evaluated postnatal exposure. Prenatal exposure

to PT was associated with mental but not motor development problems in children between two and three years of age [77,78]. Children aged up to three years showed behavioral and emotional deficits [74], while the PELAGIE cohort showed an increased risk of behavioral disorders [76], in addition to verbal comprehension and working memory deficits after postnatal exposures [75]. These results are in accordance with those reported by Oulhote and Bouchard [100], who also found behavioral problems in children. In any case, the literature on PTs is scarce, and more longitudinal investigations are needed to verify these findings.

Likewise, all studies that assessed exposure to mixed or different types of pesticides were classified as VH-quality. Moreover, only one study did not find any association with autism symptomology after postnatal mixed exposure. Regarding prenatal exposure to some pesticides mentioned above (CPF, MAL, Diazinon, Avermectin, and Permethrin) appears to be associated with autistic behaviors in children assessed during their first year of life [83]. The same results were observed in the CHARGE cohort exposed to OP and PT [90] in addition to CM and NN [81,89]. Interestingly, Schmidt and coworkers [89] reported a decreased risk of the disorder when mothers received FA supplements during pregnancy. It has previously been reported that FA intake prevents neurodevelopmental and behavioral outcomes such as hyperactivity or verbal deficits, amongst others [101,102]. Accordingly, in a case-control study conducted by the same authors, it was found that gestational FA supplements near the time of conception were associated with a reduction in ASD risk (around 40%) [103]. Moreover, socio-emotional deficits were found after Pt exposure at one year of age, as well as communication and language problems among girls, while at two years of age this deficit was observed in both sexes. Similar communicative deficits were observed at two years of age after OC metabolite exposure, followed by motor problems [84]. The same outcomes were observed in girls when they were evaluated between six and 11 years of age, whilst impairments in neurobehavioral and cognitive functions were also observed in this sex [82]. In addition, Furlong et al. [74] found cognitive problems among black children, as well as internalizing deficits after OC exposure. In a similar vein, Gunier et al. [91] found deficits in IQ and comprehension as well as perceptual reasoning problems following OP, PT, and NN exposure, whilst combined OP and CM exposure was only associated with IQ deficits [91]. Prenatal exposure to only carbamates (Mancozeb) at one year of age was associated with cognitive problems among girls, motor and language deficits in boys, and lower socio-emotional scores in both sexes [80]. Further, when studying the effects of exposure at two years of age (Propoxur), Ostrea et al. [88] only found motor problems in boys, whilst at three years of age prenatal and postnatal Carbofuranphenol exposure was found to be associated with social behavior deficits [79].

Taking together, all of this information suggests that there are certain discrepancies in the way in which autism is assessed, with some studies relying on clinical diagnoses of autism

whilst others use scale scores to obtain data related to the symptomatology of ASD, which are also shared with other multiple neurodevelopmental disorders such as depression and mood disorders [54]. Moreover, other discrepancies could be related to the substantial variability between studies when assessing the source, route, and period of exposure. Many of the studies assessed exposure through metabolite levels in urine or blood, while others assessed specific compounds such as CPF or MAL. The presence of metabolites in urine may not be exclusively a result of environmental exposure, and in fact, these compounds could be ingested through the diet by humans; therefore, metabolite measures reflect exposure by both environmental and dietary routes [104]. In a similar vein,metabolite evaluation makes it difficult to conclude, because some of these biomarkers are nonspecific (e.g., DAPs or 3-PBA) and instead they arise from a multiple range of compounds with varying levels of toxicity and potency [98]. Further, it is also worth noting that studies conducted in different countries use different compounds and levels of exposure, which could explain some of the discrepancies between results [49,62].

Another challenge is that humans are constantly exposed to a wide variety of pesticides (through diet, house fumigation, or agricultural exposure), but most of the authors reported the effects of exposure to a single pesticide since they studied only a limited number of metabolites. It must also be taken into account that diet could have a pronounced impact on the outcomes observed in children. Diet is a strong variable, and, along with its inherent importance as the source of nutrients and vitamins, it is also associated with socioeconomic status. It remains unclear as to whether the consumption of fresh fruit and vegetables could partially counteract the adverse effects produced by pesticides, or whether there are potential beneficial effects of a diet supplemented with FA [89,103]. Moreover, some studies reported a stronger association between ASD or their symptomatology and sex. Although boys are almost four times more likely than girls to be diagnosed with autism [105], the results did not suggest the existence of a stronger association among boys, since an equal number of studies reported effects among girls. This indicates that environmental factors could equally affect both sexes. Alternatively, other studies find autism-related traits in children of black and unmarried women, thus highlighting the influence of other factors such as diet, vitamin deficiencies, socioeconomic status, or genetics [24].

Another important issue is related to the age at which the outcomes are evaluated since studies assessing behavior at very early stages found more adverse effects than those using a longer follow-up period. It appears that early detection of autism (one or two-week evaluations) can be extremely valuable for detection, prevention, and establishing more effective treatments [106].

4.3. Relationship between Preclinical and Clinical Studies Concerning ASD

Comparisons between the preclinical and clinical results summarized in the present systematic review can be made only with regard to OP compound exposure as the remaining families of pesticides have not been systematically studied using animal models. However, the type of compound differs among these studies; since animal studies are more focused on CPF exposure, whilst human studies focus on non-specific OP metabolites. As previously described, preclinical studies have yielded inconsistent results, but exposure during specific gestational ages (around GD12) has the strongest empirical support. Thus, in clinical studies, it is difficult to conclude because of the considerable variability observed in terms of age or type of exposure. Nonetheless, it seems that exposure to OP during prenatal or early postnatal periods is associated with cognitive and social deficits, OC exposure is linked to adverse effects on cognition, and PT exposure is linked to behavioral problems. Therefore, we can conclude that studies conducted in animals included more stable and controlled parameters than those conducted in humans.

5. Conclusions

This review provides a comprehensive synthesis of the available evidence from various sources and evaluates the link between exposure to a wide range of pesticides and ASD and associated symptomatology. Our search, however, revealed that OCs have been the most widely studied type of pesticides to date. In particular, the review of preclinical studies highlights that:

- The relation between exposure to different pesticides and the ASD-like phenotype concerning the core symptomatology of autism is relatively under-explored in preclinical research. Even in the case of those compounds for which there is a significant amount of empirical research regarding sociability and/or communicative outcomes (e.g., CPF), the considerable differences between studies regarding exposure protocols (e.g., gestational vs. postnatal or early vs. medium vs. late gestational) make it impossible, in the end, for us to draw any solid conclusions.

- There is a significant gap in the literature as only one study included in the review used rats. Although the relevance of the use of mice is unquestionable, it is known that rat models are closer to humans in terms of genetic background and behavioral regulation, particularly with regard to social behaviors [96].

- Future preclinical research should focus on a more in-depth analysis of exposure to developmental CPF and other pesticides concerning the core (sociability and USVs) and

secondary (e.g., neuromotor development) clinical signs of ASD, with a special emphasis on the gestational period around GD12, whilst it will also be necessary to include rat models along with the work carried out with mice.

- The study on wild-type mice should be complemented with the systematic analyses of the interactions of this exposure with the various genetic backgrounds of vulnerability associated with the ASD-like phenotype.

In relation to clinical studies:

- It is difficult to draw solid conclusions as there are a wide variety of studies that differ in many aspects such as route, age, or source of exposure.

- The study of exposure to a single pesticide in humans lacks ecological validity, due to the fact that humans are constantly exposed to a wide range of pesticides through arange of routes such as diet, house fumigation, or agriculture. This wide variability of compounds and environmental exposure could contribute to the heterogeneity of results found in the literature.

- Pesticide exposure appears to co-exist with other factors that may be harmful or beneficial for the development of the nervous system. Examples of other factors that could explain the association between pesticides and ASD are lifestyle, socioeconomic or educational status as well as ethnicity or gender. Moreover, maternal age is an important factor to consider, as the concentration of pesticides in the body increases with age, and so higher maternal ages are more strongly associated with an increased risk of autism in their offspring [107].

- Pesticide exposure did not always show harmful effects when authors considered different covariates, suggesting the existence of certain genetic polymorphisms which could interact with environmental factors and amplify the adverse effects of pesticides in relation to ASD (gene-environment interaction).

- Further clinical research is needed to homogenize exposure in human studies, particularly in terms of exposure to specific pesticides, consideration of other risk factors, as well as the use of a more well-defined follow-up period and validated tools for measuring behavioral outcomes.

Author Contributions: Conceptualization- M.T.C., F.S.-S., P.C., and J.L.A.; methodology, investigation- J.B.-B., S.M., and C.P.-F.; writing-original draft preparation- J.B.-B., S.M., and C.P.-F.; writing-review and editing-J.B.-B., S.M., C.P.-F., B.C., H.P., N.M.C., P.C., J.L.A., and F.M.-S.; supervision- M.T.C. and F.S.-S.; funding acquisition- M.T.C., F.S.-S., P.C., and J.L.A. All authors have read and agreed to the published version of the manuscript.

Funding: The study was supported by grants from the Spanish Goverment (Ministerio de Economía y Competitividad, Ministerio de Ciencia, Innovación y Universidades and Instituto Mixto de Investigación-Escuela Nacional de Sanidad (IMIENS)) and the Fondo Europeo de Desarrollo Regional (MINECO-FEDER) [Grant numbers: PSI2017-86847-C2-1-R MINECO-FEDER, PSI2017-86847-C2-2-R, PSI2017-86396-P, PSI2017-90806 REDT, PSI2013-45924-P, PSI2017-83038-P, PSI2017-83893-R, MINECO PSI2017-86396-P and IMIENS: PIC-IMIENS-2018-003].

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest

References

1. Del Barrio, V. Diagnostic and statistical manual of mental disorders. In *The Curated Reference Collection in Neuroscience and Biobehavioral Psychology*; Elsevier: Amsterdam, The Netherlands, 2016; ISBN 9780128093245.
2. Whyatt, C.; Craig, C. Sensory-motor problems in Autism. *Front. Integr. Neurosci.* **2013**, *7*, 51. [CrossRef]
3. Keehn, B.; Westerfield, M.; Müller, R.-A.; Townsend, J. Autism, Attention, and Alpha Oscillations: An Electrophysiological Study of Attentional Capture. *Biol. Psychiatry Cogn. Neurosci. Neuroimaging* **2017**, *2*, 528–536. [CrossRef] [PubMed]
4. Habib, A.; Harris, L.; Pollick, F.; Melville, C. A meta-analysis of working memory in individuals with autism spectrum disorders. *PLoS ONE* **2019**, *14*, e0216198. [CrossRef]
5. Christ, S.E.; Holt, D.D.; White, D.A.; Green, L. Inhibitory Control in Children with Autism Spectrum Disorder. *J. Autism Dev. Disord.* **2007**, *37*, 1155–1165. [CrossRef] [PubMed]
6. Sandin, S.; Lichtenstein, P.; Kuja-Halkola, R.; Hultman, C.; Larsson, H.; Reichenberg, A. The Heritability of Autism Spectrum Disorder Analysis method. B. *JAMA* **2017**, *318*, 1182–1184. [CrossRef] [PubMed]
7. Lázaro, M.T.; Golshani, P. The utility of rodent models of autism spectrum disorders. *Curr. Opin. Neurol.* **2015**, *28*, 103–109. [CrossRef]
8. Grove, J.; Ripke, S.; Als, T.D.; Mattheisen, M.; Walters, R.K.; Børglum, A.D. Identification of common genetic risk variants forautism spectrum disorder HHS Public Access Author manuscript. *Nat. Genet.* **2019**, *51*, 431–444. [CrossRef]

9. Sinzig, J. Autism Spectrum. *Monatsschrift Kinderheilkd* **2015**, *163*, 673–680. [CrossRef]

10. Hansen, S.N.; Schendel, D.E.; Parner, E.T. Explaining the Increase in the Prevalence of Autism Spectrum Disorders. *JAMA Pediatr.* **2015**, *169*, 56. [CrossRef]

11. Karimi, P.; Kamali, E.; Mousavi, S.; Karahmadi, M. Environmental factors influencing the risk of autism. *J. Res. Med. Sci.* **2017**, *22*. [CrossRef] [PubMed]

12. Chaste, P.; Leboyer, M. Autism risk factors: Genes, environment, and gene-environment interactions. *Dialogues Clin. Neurosci.* **2012**, *14*, 281–292. [CrossRef]

13. Christensen, J.; Grønborg, T.K.; Sørensen, M.J.; Schendel, D.; Parner, E.T.; Pedersen, L.H.; Vestergaard, M. Prenatal Valproate Exposure and Risk of Autism Spectrum Disorders and Childhood Autism. *JAMA* **2013**, *309*, 1696. [CrossRef] [PubMed]

14. Pelch, K.E.; Bolden, A.L.; Kwiatkowski, C.F. Environmental Chemicals and Autism: A Scoping Review of the Human and Animal Research. *Environ. Health Perspect.* **2019**, *127*, 046001. [CrossRef]

15. Roberts, J.R.; Dawley, E.H.; Reigart, J.R. Children's low-level pesticide exposure and associations with autism and ADHD: A review. *Pediatr. Res.* **2019**, *85*, 234–241. [CrossRef] [PubMed]

16. Modabbernia, A.; Velthorst, E.; Reichenberg, A. Environmental risk factors for autism: An evidence-based review of systematic reviews and meta-analyses. *Mol. Autism* **2017**, *8*, 13. [CrossRef] [PubMed]

17. DeBord, D.G.; Carreón, T.; Lentz, T.J.; Middendorf, P.J.; Hoover, M.D.; Schulte, P.A. Use of the "exposome" in the Practice of Epidemiology: A Primer on Omic Technologies. *Am. J. Epidemiol.* **2016**, *184*, 302–314. [CrossRef]

18. Coats, J.R. Mechanisms of toxic action and structure-activity relationships for organochlorine and synthetic pyrethroid insecticides. *Environ. Health Perspect.* **1990**, *87*, 255–262. [CrossRef] [PubMed]

19. Jayaraj, R.; Megha, P.; Sreedev, P. Review Article. Organochlorine pesticides, their toxic effects on living organisms and their fatein the environment. *Interdiscip. Toxicol.* **2016**, *9*, 90–100. [CrossRef]

20. Suppiramaniam, V.; Abdel-Rahman, E.A.; Buabeid, M.A.; Parameshwaran, K. Ion Channels. In *Comprehensive Toxicology*; Elsevier: Amsterdam, The Netherlands, 2010; Volume 11, pp. 129–171.

21. Gupta, R. *Toxicology of Organophosphate & Carbamate Compounds*; Elsevier Inc.: Amsterdam, The Netherlands, 2006; ISBN 9780120885237.

22. Casida, J.E. Neonicotinoids and Other Insect Nicotinic Receptor Competitive Modulators: Progress and Prospects. *Annu. Rev. Entomol.* **2018**, *63*, 125–144. [CrossRef]

23. Eaton, D.L.; Daroff, R.B.; Autrup, H.; Bridges, J.; Buffler, P.; Costa, L.G.; Coyle, J.; McKhann, G.; Mobley, W.C.; Nadel, L.; et al. Review of the Toxicology of Chlorpyrifos With an Emphasis on Human Exposure and Neurodevelopment. *Crit. Rev. Toxicol.* **2008**, *38*, 1–125. [CrossRef] [PubMed]

24. Becerra, T.A.; Von Ehrenstein, O.S.; Heck, J.E.; Olsen, J.; Arah, O.A.; Jeste, S.S.; Rodriguez, M.; Ritz, B. Autism spectrum disorders and race, ethnicity, and nativity: A population-based study. *Pediatrics* **2014**, *134*, 63–71. [CrossRef]

25. Silva, M.H. Effects of low-dose chlorpyrifos on neurobehavior and potential mechanisms: A review of studies in rodents, zebrafish, and *Caenorhabditis elegans*. *Birth Defects Res.* **2020**, *112*, 445–479. [CrossRef]

26. Moher, D.; Shamseer, L.; Clarke, M.; Ghersi, D.; Liberati, A.; Petticrew, M.; Shekelle, P.; Stewart, L.A.; Estarli, M.; Barrera, E.S.A.; et al. Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. *Rev. Esp. Nutr.Humana Diet.* **2016**, *20*, 148–160. [CrossRef] [PubMed]

27. Hooijmans, C.R.; Rovers, M.M.; De Vries, R.B.M.; Leenaars, M.; Ritskes-Hoitinga, M.; Langendam, M.W. SYRCLE's risk of bias tool for animal studies. *BMC Med. Res. Methodol.* **2014**, *14*, 43. [CrossRef] [PubMed]

28. Higgins, J.P.T.; Altman, D.G.; Gøtzsche, P.C.; Jüni, P.; Moher, D.; Oxman, A.D.; Savović, J.; Schulz, K.F.; Weeks, L.; Sterne, J.A.C. The Cochrane Collaboration's tool for assessing risk of bias in randomised trials. *BMJ* **2011**, *343*, d5928. [CrossRef]

29. Wells, G.; Shea, B.; O'Connell, D.; Robertson, J.; Peterson, J.; Welch, V.; Losos, M.; Tugwell, P. NOS for assessing quality of nonrandomised studies. The Ottawa Hospital: Research Institude. Available online: http://www.ohri.ca/programs/clinical_ epidemiology/oxford.asp (accessed on 6 June 2020).

30. Venerosi, A.; Cutuli, D.; Colonnello, V.; Cardona, D.; Ricceri, L.; Calamandrei, G. Neonatal exposure to chlorpyrifos affects maternal responses and maternal aggression of female mice in adulthood. *Neurotoxicol. Teratol.* **2008**, *30*, 468–474. [CrossRef]

31. Venerosi, A.; Ricceri, L.; Scattoni, M.L.; Calamandrei, G. Prenatal chlorpyrifos exposure alters motor behavior and ultrasonic vocalization in cd-1 mouse pups. *Environ. Health A Glob. Access Sci. Source* **2009**, *8*, 12. [CrossRef] [PubMed]

32. Venerosi, A.; Ricceri, L.; Rungi, A.; Sanghez, V.; Calamandrei, G. Gestational exposure to the organophosphate chlorpyrifos alters social–emotional behaviour and impairs responsiveness to the serotonin transporter inhibitor fluvoxamine in mice. *Psychopharmacology* **2010**, *208*, 99–107. [CrossRef] [PubMed]

33. Venerosi, A.; Tait, S.; Stecca, L.; Chiarotti, F.; De Felice, A.; Cometa, M.F.; Volpe, M.T.; Calamandrei, G.; Ricceri, L. Effects of maternal chlorpyrifos diet on social investigation and brain neuroendocrine markers in the offspring- A mouse study. *Environ.Health* **2015**, *14*, 32. [CrossRef] [PubMed]

34. De Felice, A.; Venerosi, A.; Ricceri, L.; Sabbioni, M.; Scattoni, M.L.; Chiarotti, F.; Calamandrei, G. Sex-dimorphic effects of gestational exposure to the organophosphate insecticide chlorpyrifos on social investigation in mice. *Neurotoxicol. Teratol.* **2014**, *46*, 32–39. [CrossRef]

35. De Felice, A.; Scattoni, M.L.; Ricceri, L.; Calamandrei, G. Prenatal Exposure to a Common Organophosphate Insecticide Delays Motor Development in a Mouse Model of Idiopathic Autism. *PLoS ONE* **2015**, *10*, e0121663. [CrossRef] [PubMed]

36. De Felice, A.; Greco, A.; Calamandrei, G.; Minghetti, L. Prenatal exposure to the organophosphate insecticide chlorpyrifos enhances brain oxidative stress and prostaglandin E2 synthesis in a mouse model of idiopathic autism. *J. Neuroinflamm.* **2016**, *13*, 149. [CrossRef] [PubMed]

37. Lan, A.; Kalimian, M.; Amram, B.; Kofman, O. Prenatal chlorpyrifos leads to autism-like deficits in C57Bl6/J mice. *Environ. HealthA Glob. Access Sci. Source* **2017**, *16*. [CrossRef]

38. Lan, A.; Stein, D.; Portillo, M.; Toiber, D.; Kofman, O. Impaired innate and conditioned social behavior in adult C57Bl6/J mice prenatally exposed to chlorpyrifos. *Behav. Brain Funct.* **2019**, *15*, 1–9. [CrossRef] [PubMed]

39. Mullen, B.R.; Khialeeva, E.; Hoffman, D.B.; Ghiani, C.A.; Carpenter, E.M. Decreased Reelin Expression and Organophosphate Pesticide Exposure Alters Mouse Behaviour and Brain Morphology. *ASN Neuro* **2013**, *5*, e00106. [CrossRef] [PubMed]

40. Basaure, P.; Guardia-Escote, L.; Biosca-Brull, J.; Blanco, J.; Cabré, M.; Peris-Sampedro, F.; Sánchez-Santed, F.; Domingo, J.L.; Colomina, M.T. Exposure to chlorpyrifos at different ages triggers APOE genotype-specific responses in social behavior, body weight and hypothalamic gene expression. *Environ. Res.* **2019**, *178*, 108684. [CrossRef] [PubMed]

41. Ouardi, F.Z.; Anarghou, H.; Malqui, H.; Ouasmi, N.; Chigr, M.; Najimi, M.; Chigr, F. Gestational and Lactational Exposure to Malathion Affects Antioxidant Status and Neurobehavior in Mice Pups and

Offspring. *J. Mol. Neurosci.* **2019**, *69*, 17–27. [CrossRef] [PubMed]

42. Hazarika, R. Neurotoxic impact of organophosphate pesticide phosphomedon on the albino rat. *J. Environ. Biol.* **2014**, *35*, 427–430.

43. Laugeray, A.; Herzine, A.; Perche, O.; HÃ©bert, B.; Aguillon-Naury, M.; Richard, O.; Menuet, A.; Mazaud-Guittot, S.; Lesné, L.; Briault, S.; et al. Pre- and Postnatal Exposure to Low Dose Glufosinate Ammonium Induces Autism-Like Phenotypes in Mice. *Front. Behav. Neurosci.* **2014**, *8*, 390. [CrossRef]

44. Dong, T.; Guan, Q.; Hu, W.; Zhang, M.; Zhang, Y.; Chen, M.; Wang, X.; Xia, Y. Prenatal exposure to glufosinate ammonium disturbs gut microbiome and induces behavioral abnormalities in mice. *J. Hazard. Mater.* **2020**, *389*. [CrossRef]

45. Laugeray, A.; Herzine, A.; Perche, O.; Richard, O.; Montecot-Dubourg, C.; Menuet, A.; Mazaud-Guittot, S.; Lesné, L.; Jegou, B.; Mortaud, S. In utero and lactational exposure to low-doses of the pyrethroid insecticide cypermethrin leads to neurodevelopmental defects in male mice- An ethological and transcriptomic study. *PLoS ONE* **2017**, *12*, e0184475. [CrossRef]

46. Guo, J.; Zhang, J.; Wu, C.; Lv, S.; Lu, D.; Qi, X.; Jiang, S.; Feng, C.; Yu, H.; Liang, W.; et al. Associations of prenatal and childhood chlorpyrifos exposure with Neurodevelopment of 3-year-old children. *Environ. Pollut.* **2019**, *251*, 538–546. [CrossRef]

47. Philippat, C.; Barkoski, J.; Tancredi, D.J.; Elms, B.; Barr, D.B.; Ozonoff, S.; Bennett, D.H.; Hertz-Picciotto, I. Prenatal exposure to organophosphate pesticides and risk of autism spectrum disorders and other non-typical development at 3 years in a high-risk cohort. *Int. J. Hyg. Environ. Health* **2018**, *221*, 548–555. [CrossRef]

48. Wang, Y.; Zhang, Y.; Ji, L.; Hu, Y.; Zhang, J.; Wang, C.; Ding, G.; Chen, L.; Kamijima, M.; Ueyama, J.; et al. Prenatal and postnatal exposure to organophosphate pesticides and childhood neurodevelopment in Shandong, China. *Environ. Int.* **2017**, *108*, 119–126. [CrossRef] [PubMed]

49. Zhang, Y.; Han, S.; Liang, D.; Shi, X.; Wang, F.; Liu, W.; Zhang, L.; Chen, L.; Gu, Y.; Tian, Y. Prenatal exposure to organophosphate pesticides and neurobehavioral development of neonates: A birth cohort study in Shenyang, China. *PLoS ONE* **2014**, *9*, e88491.[CrossRef] [PubMed]

50. Bouchard, M.F.; Chevrier, J.; Harley, K.G.; Kogut, K.; Vedar, M.; Calderon, N.; Trujillo, C.; Johnson, C.; Bradman, A.; Barr, D.B.; et al. Prenatal Exposure to Organophosphate Pesticides and IQ in 7-Year-Old Children. *Environ. Health Perspect.* **2011**, *119*, 1189–1195. [CrossRef] [PubMed]

51. Donauer, S.; Altaye, M.; Xu, Y.; Sucharew, H.; Succop, P.; Calafat, A.M.; Khoury, J.C.; Lanphear, B.; Yolton, K. An Observational Study to Evaluate Associations between Low-Level Gestational Exposure to Organophosphate Pesticides and Cognition during Early Childhood. *Am. J. Epidemiol.* **2016**, *184*, 410–418. [CrossRef] [PubMed]

52. Engel, S.M.; Wetmur, J.; Chen, J.; Zhu, C.; Barr, D.B.; Canfield, R.L.; Wolff, M.S. Prenatal exposure to organophosphates, paraoxonase 1, and cognitive development in childhood. *Environ. Health Perspect.* **2011**, *119*, 1182–1188. [CrossRef]

53. Eskenazi, B.; Huen, K.; Marks, A.; Harley, K.G.; Bradman, A.; Barr, D.B.; Holland, N. PON1 and Neurodevelopment in Children from the CHAMACOS Study Exposed to Organophosphate Pesticides in Utero. *Environ. Health Perspect.* **2010**, *118*, 1775–1781. [CrossRef] [PubMed]

54. Furlong, M.A.; Engel, S.M.; Barr, D.B.; Wolff, M.S. Prenatal exposure to organophosphate pesticides and reciprocal social behaviorin childhood. *Environ. Int.* **2014**, *70*, 125–131. [CrossRef] [PubMed]

55. González-Alzaga, B.; Hernández, A.F.; Rodríguez-Barranco, M.; Gómez, I.; Aguilar-Garduño, C.; López-Flores, I.; Parrón, T.; Lacasaña, M. Pre- and postnatal exposures to pesticides and neurodevelopmental effects in children living in agricultural communities from South-Eastern Spain. *Environ. Int.* **2015**, *85*, 229–237. [CrossRef]

56. Kongtip, P.; Techasaensiri, B.; Nankongnab, N.; Adams, J.; Phamonphon, A.; Surach, A.; Sangprasert, S.; Thongsuksai, A.; Srikumpol, P.; Woskie, S. The impact of prenatal organophosphate pesticide exposures on thai infant neurodevelopment. *Int. J. Environ. Res. Public Health* **2017**, *14*, 570. [CrossRef] [PubMed]

57. Liu, P.; Wu, C.; Chang, X.; Qi, X.; Zheng, M.; Zhou, Z. Adverse associations of both prenatal and postnatal exposure to organophosphorous pesticides with infant neurodevelopment in an agricultural area of Jiangsu Province, China. *Environ. Health Perspect.* **2016**, *124*, 1637–1643. [CrossRef] [PubMed]

58. Millenson, M.E.; Braun, J.M.; Calafat, A.M.; Barr, D.B.; Huang, Y.-T.; Chen, A.; Lanphear, B.P.; Yolton, K. Urinary organophosphate insecticide metabolite concentrations during pregnancy and children's interpersonal, communication, repetitive, and stereotypic behaviors at 8 years of age: The home study. *Environ. Res.* **2017**, *157*, 9–16. [CrossRef] [PubMed]

59. Sagiv, S.K.; Harris, M.H.; Gunier, R.B.; Kogut, K.R.; Harley, K.G.; Deardorff, J.; Bradman, A.; Holland, N.; Eskenazi, B. Prenatal Organophosphate Pesticide Exposure and Traits Related to Autism Spectrum Disorders in a Population Living in Proximity to Agriculture. *Environ. Health Perspect.* **2018**, *126*, 047012. [CrossRef]

60. Silver, M.K.; Shao, J.; Zhu, B.; Chen, M.; Xia, Y.; Kaciroti, N.; Lozoff, B.; Meeker, J.D. Prenatal naled and chlorpyrifos exposure is associated with deficits in infant motor function in a cohort of Chinese infants. *Environ. Int.* **2017**, *106*, 248–256. [CrossRef][PubMed]

61. van den Dries, M.A.; Guxens, M.; Pronk, A.; Spaan, S.; El Marroun, H.; Jusko, T.A.; Longnecker, M.P.; Ferguson, K.K.; Tiemeier, H. Organophosphate pesticide metabolite concentrations in urine during pregnancy and offspring attention-deficit hyperactivity disorder and autistic traits. *Environ. Int.* **2019**, *131*, 105002. [CrossRef]

62. Woskie, S.; Kongtip, P.; Thanasanpaiboon, W.; Kiatdamrong, N.; Charoonrungsirikul, N.; Nankongnab, N.; Surach, A.; Phamon- phon, A. A pilot study of maternal exposure to organophosphate pesticides and newborn neurodevelopment in Thailand. *Int. J. Occup. Environ. Health* **2017**, *23*, 193–201. [CrossRef] [PubMed]

63. Bahena-Medina, L.A.; Torres-Sánchez, L.; Schnaas, L.; Cebrián, M.E.; Chávez, C.H.; Osorio-Valencia, E.; Hernández, R.M.G.; López-Carrillo, L. Neonatal neurodevelopment and prenatal exposure to dichlorodiphenyldichloroethylene (DDE): A cohort study in Mexico. *J. Expo. Sci. Environ. Epidemiol.* **2011**, *21*, 609–614. [CrossRef]

64. Brown, A.S.; Cheslack-Postava, K.; Rantakokko, P.; Kiviranta, H.; Hinkka-Yli-Salomäki, S.; McKeague, I.W.; Surcel, H.-M.; Sourander, A. Association of Maternal Insecticide Levels With Autism in Offspring From a National Birth Cohort. *Am. J. Psychiatry* **2018**, *175*, 1094–1101. [CrossRef]

65. Kim, S.; Eom, S.; Kim, H.J.; Lee, J.J.; Choi, G.; Choi, S.; Kim, S.; Kim, S.Y.; Cho, G.; Kim, Y.D.; et al. Association between maternal exposure to major phthalates, heavy metals, and persistent organic pollutants, and the neurodevelopmental performances of their children at 1 to 2 years of age-CHECK cohort study. *Sci. Total Environ.* **2018**, *624*, 377–384. [CrossRef]

66. Braun, J.M.; Kalkbrenner, A.E.; Just, A.C.; Yolton, K.; Calafat, A.M.; Sjödin, A.; Hauser, R.; Webster, G.M.; Chen, A.; Lanphear, B.P. Gestational exposure to endocrine-disrupting chemicals and reciprocal social, repetitive, and stereotypic behaviors in 4- and 5-year-old children: The HOME study. *Environ. Health Perspect.* **2014**, *122*, 513–520. [CrossRef] [PubMed]

67. Jeddy, Z.; Kordas, K.; Allen, K.; Taylor, E.V.; Northstone, K.; Dana Flanders, W.; Namulanda, G.; Sjodin, A.; Hartman, T.J. Prenatal exposure to organochlorine pesticides and early childhood communication development in British girls. *Neurotoxicology* **2018**, *69*, 121–129. [CrossRef]

68. Kao, C.-C.; Que, D.E.; Bongo, S.J.; Tayo, L.L.; Lin, Y.-H.; Lin, C.-W.; Lin, S.-L.; Gou, Y.-Y.; Hsu, W.-

L.; Shy, C.-G.; et al. Residue Levels of Organochlorine Pesticides in Breast Milk and Its Associations with Cord Blood Thyroid Hormones and the Offspring's Neurodevelopment. *Int. J. Environ. Res. Public Health* **2019**, *16*, 1438. [CrossRef] [PubMed]

69. Lyall, K.; Croen, L.A.; Sjödin, A.; Yoshida, C.K.; Zerbo, O.; Kharrazi, M.; Windham, G.C. Polychlorinated Biphenyl and Organochlorine Pesticide Concentrations in Maternal Mid-Pregnancy Serum Samples: Association with Autism Spectrum Disorder and Intellectual Disability. *Environ. Health Perspect.* **2017**, *125*, 474–480. [CrossRef]

70. Hamra, G.B.; Lyall, K.; Windham, G.C.; Calafat, A.M.; Sjödin, A.; Volk, H.; Croen, L.A. Prenatal Exposure to Endocrine-disrupting Chemicals in Relation to Autism Spectrum Disorder and Intellectual Disability. *Epidemiology* **2019**, *30*, 418–426. [CrossRef] [PubMed]

71. Torres-Sánchez, L.; Schnaas, L.; Rothenberg, S.J.; Cebrián, M.E.; Osorio-Valencia, E.; Hernández, M.d.C.; García-Hernández, R.M.; López-Carrillo, L. Prenatal p,p'-DDE Exposure and Neurodevelopment among Children 3.5–5 Years of Age. *Environ. Health Perspect.* **2013**, *121*, 263–268. [CrossRef]

72. Puertas, R.; Lopez-Espinosa, M.J.; Cruz, F.; Ramos, R.; Freire, C.; Pérez-García, M.; Abril, A.; Julvez, J.; Salvatierra, M.; Campoy, C.; et al. Prenatal exposure to mirex impairs neurodevelopment at age of 4 years. *Neurotoxicology* **2010**, *31*, 154–160. [CrossRef] [PubMed]

73. Boucher, O.; Simard, M.-N.; Muckle, G.; Rouget, F.; Kadhel, P.; Bataille, H.; Chajès, V.; Dallaire, R.; Monfort, C.; Thomé, J.-P.; et al. Exposure to an organochlorine pesticide (chlordecone) and development of 18-month-old infants. *Neurotoxicology* **2013**, *35*, 162–168. [CrossRef]

74. Furlong, M.A.; Barr, D.B.; Wolff, M.S.; Engel, S.M. Prenatal exposure to pyrethroid pesticides and childhood behavior and executive functioning. *Neurotoxicology* **2017**, *62*, 231–238. [CrossRef]

75. Viel, J.-F.; Warembourg, C.; Le Maner-Idrissi, G.; Lacroix, A.; Limon, G.; Rouget, F.; Monfort, C.; Durand, G.; Cordier, S.; Chevrier, C. Pyrethroid insecticide exposure and cognitive developmental disabilities in children: The PELAGIE mother–child cohort. *Environ. Int.* **2015**, *82*, 69–75. [CrossRef]

76. Viel, J.-F.; Rouget, F.; Warembourg, C.; Monfort, C.; Limon, G.; Cordier, S.; Chevrier, C. Behavioural disorders in 6-year-old children and pyrethroid insecticide exposure: The PELAGIE mother–child cohort. *Occup. Environ. Med.* **2017**, *74*, 275–281. [CrossRef] [PubMed]

77. Horton, M.K.; Rundle, A.; Camann, D.E.; Boyd Barr, D.; Rauh, V.A.; Whyatt, R.M. Impact of Prenatal Exposure to Piperonyl Butoxide and Permethrin on 36-Month Neurodevelopment. *Pediatrics* **2011**, *127*, e699–e706. [CrossRef]

78. Watkins, D.J.; Fortenberry, G.Z.; Sánchez, B.N.; Barr, D.B.; Panuwet, P.; Schnaas, L.; Osorio-Valencia, E.; Solano-González, M.; Ettinger, A.S.; Hernández-Ávila, M.; et al. Urinary 3-phenoxybenzoic acid (3-PBA) levels among pregnant women in Mexico City: Distribution and relationships with child neurodevelopment. *Environ. Res.* **2016**, *147*, 307–313. [CrossRef]

79. Zhang, J.; Guo, J.; Wu, C.; Qi, X.; Jiang, S.; Lu, D.; Feng, C.; Liang, W.; Chang, X.; Zhang, Y.; et al. Exposure to carbamate and neurodevelopment in children: Evidence from the SMBCS cohort in China. *Environ. Res.* **2019**, *177*, 108590. [CrossRef] [PubMed]

80. Mora, A.M.; Córdoba, L.; Cano, J.C.; Hernandez-Bonilla, D.; Pardo, L.; Schnaas, L.; Smith, D.R.; Menezes-Filho, J.A.; Mergler, D.; Lindh, C.H.; et al. Prenatal Mancozeb Exposure, Excess Manganese, and Neurodevelopment at 1 Year of Age in the Infants' Environmental Health (ISA) Study. *Environ. Health Perspect.* **2018**, *126*, 057007. [CrossRef]

81. Keil, A.P.; Daniels, J.L.; Hertz-Picciotto, I. Autism spectrum disorder, flea and tick medication, and adjustments for exposure misclassification: The CHARGE (Childhood Autism Risks from Genetics and Environment) case-control study. *Environ. Heal. A Glob. Access Sci. Source* **2014**, *13*, 1–10.

[CrossRef] [PubMed]

82. Andersen, H.R.; Debes, F.; Wohlfahrt-Veje, C.; Murata, K.; Grandjean, P. Occupational pesticide exposure in early pregnancy associated with sex-specific neurobehavioral deficits in the children at school age. *Neurotoxicol. Teratol.* **2015**, *47*, 1–9. [CrossRef]

83. von Ehrenstein, O.S.; Ling, C.; Cui, X.; Cockburn, M.; Park, A.S.; Yu, F.; Wu, J.; Ritz, B. Prenatal and infant exposure to ambient pesticides and autism spectrum disorder in children: Population based case-control study. *BMJ* **2019**, *364*, l962. [CrossRef] [PubMed]

84. Eskenazi, B.; An, S.; Rauch, S.A.; Coker, E.S.; Maphula, A.; Obida, M.; Crause, M.; Kogut, K.R.; Bornman, R.; Chevrier, J. Prenatal Exposure to DDT and Pyrethroids for Malaria Control and Child Neurodevelopment: The VHEMBE Cohort, South Africa. *Environ. Health Perspect.* **2018**, *126*, 047004. [CrossRef] [PubMed]

85. Furlong, M.A.; Herring, A.; Buckley, J.P.; Goldman, B.D.; Daniels, J.L.; Engel, L.S.; Wolff, M.S.; Chen, J.; Wetmur, J.; Barr, D.B.; et al. Prenatal exposure to organophosphorus pesticides and childhood neurodevelopmental phenotypes. *Environ. Res.* **2017**, *158*, 737–747. [CrossRef] [PubMed]

86. Gunier, R.B.; Bradman, A.; Castorina, R.; Holland, N.T.; Avery, D.; Harley, K.G.; Eskenazi, B. Residential proximity to agricultural fumigant use and IQ, attention and hyperactivity in 7-year old children. *Environ. Res.* **2017**, *158*, 358–365. [CrossRef] [PubMed]

87. McCanlies, E.C.; Ma, C.C.; Gu, J.K.; Fekedulegn, D.; Sanderson, W.T.; Ludeña-Rodriguez, Y.J.; Hertz-Picciotto, I. The CHARGE study: An assessment of parental occupational exposures and autism spectrum disorder. *Occup. Environ. Med.* **2019**, *76*, 644–651. [CrossRef]

88. Ostrea Jr, E.M.; Reyes, A.; Villanueva-Uy, E.; Pacifico, R.; Benitez, B.; Ramos, E.; Bernardo, R.C.; Bielawski, D.M.; Delaney-Black, V.; Chiodo, L.; et al. Fetal exposure to propoxur and abnormal child neurodevelopment at 2 years of age. *Neurotoxicology* **2012**, *33*, 669–675. [CrossRef] [PubMed]

89. Schmidt, R.J.; Kogan, V.; Shelton, J.F.; Delwiche, L.; Hansen, R.L.; Ozonoff, S.; Ma, C.C.; McCanlies, E.C.; Bennett, D.H.; Hertz- Picciotto, I.; et al. Combined Prenatal Pesticide Exposure and Folic Acid Intake in Relation to Autism Spectrum Disorder. *Environ. Health Perspect.* **2017**, *125*, 097007. [CrossRef] [PubMed]

90. Shelton, J.F.; Geraghty, E.M.; Tancredi, D.J.; Delwiche, L.D.; Schmidt, R.J.; Ritz, B.; Hansen, R.L.; Hertz-Picciotto, I. Neurodevelopmental Disorders and Prenatal Residential Proximity to Agricultural Pesticides: The CHARGE Study. *Environ. Health Perspect.* **2014**, *122*, 1103–1109. [CrossRef]

91. Gunier, R.B.; Bradman, A.; Harley, K.G.; Kogut, K.; Eskenazi, B. Prenatal Residential Proximity to Agricultural Pesticide Use and IQ in 7-Year-Old Children. *Environ. Health Perspect.* **2017**, *125*, 057002. [CrossRef]

92. Hu, H.; Zheng, Y.; Wen, X.; Smith, S.S.; Nizomov, J.; Fishe, J.; Hogan, W.R.; Shenkman, E.A.; Bian, J. An external exposome-wide association study of COVID-19 mortality in the United States. *Sci. Total Environ.* **2021**, *768*, 144832. [CrossRef] [PubMed]

93. Berger-Sweeney, J. The cholinergic basal forebrain system during development and its influence on cognitive processes: Important questions and potential answers. *Neurosci. Biobehav. Rev.* **2003**, *27*, 401–411. [CrossRef]

94. Williams, T.; Borchelt, D.R.; Chakrabarty, P. Therapeutic approaches targeting Apolipoprotein E function in Alzheimer's disease. *Mol. Neurodegener.* **2020**, *15*, 8. [CrossRef]

95. Nicolini, C.; Fahnestock, M. The valproic acid-induced rodent model of autism. *Exp. Neurol.* **2018**, *299*, 217–227. [CrossRef]

96. Ellenbroek, B.; Youn, J. Rodent models in neuroscience research: Is it a rat race? *Dis. Model. Mech.* **2016**, *9*, 1079–1087. [CrossRef]

97. Thabtah, F.; Peebles, D. Early autism screening: A comprehensive review. *Int. J. Environ. Res. Public Health* **2019**, *16*, 3502. [CrossRef]

98. Engel, S.M.; Bradman, A.; Wolff, M.S.; Rauh, V.A.; Harley, K.G.; Yang, J.H.; Hoepner, L.A.; Barr, D.B.; Yolton, K.; Vedar, M.G.; et al. Prenatal Organophosphorus Pesticide Exposure and Child Neurodevelopment at 24 Months: An Analysis of Four Birth Cohorts. *Environ. Health Perspect.* **2016**, *124*, 822–830. [CrossRef] [PubMed]

99. Quirós-Alcalá, L.; Mehta, S.; Eskenazi, B. Pyrethroid Pesticide Exposure and Parental Report of Learning Disability and Attention Deficit/Hyperactivity Disorder in U.S. Children: NHANES 1999–2002. *Environ. Health Perspect.* **2014**, *122*, 1336–1342. [CrossRef]

100. Oulhote, Y.; Bouchard, M.F. Urinary Metabolites of Organophosphate and Pyrethroid Pesticides and Behavioral Problems in Canadian Children. *Environ. Health Perspect.* **2013**, *121*, 1378–1384. [CrossRef]

101. Julvez, J.; Fortuny, J.; Mendez, M.; Torrent, M.; Ribas-Fitó, N.; Sunyer, J. Maternal use of folic acid supplements during pregnancy and four-year-old neurodevelopment in a population-based birth cohort. *Paediatr. Perinat. Epidemiol.* **2009**, *23*, 199–206. [CrossRef][PubMed]

102. Roza, S.J.; van Batenburg-Eddes, T.; Steegers, E.A.P.; Jaddoe, V.W.V.; Mackenbach, J.P.; Hofman, A.; Verhulst, F.C.; Tiemeier, H. Maternal folic acid supplement use in early pregnancy and child behavioural problems: The Generation R Study. *Br. J. Nutr.* **2010**, *103*, 445–452. [CrossRef] [PubMed]

103. Schmidt, R.J.; Hansen, R.L.; Hartiala, J.; Allayee, H.; Schmidt, L.C.; Tancredi, D.J.; Tassone, F.; Hertz-Picciotto, I. Prenatal Vitamins, One-carbon Metabolism Gene Variants, and Risk for Autism. *Epidemiology* **2011**, *22*, 476–485. [CrossRef]

104. Lu, C.; Bravo, R.; Caltabiano, L.M.; Irish, R.M.; Weerasekera, G.; Barr, D.B. The Presence of Dialkylphosphates in Fresh Fruit Juices: Implication For Organophosphorus Pesticide Exposure and Risk Assessments. *J. Toxicol. Environ. Health Part A* **2005**, *68*, 209–227. [CrossRef] [PubMed]

105. Baio, J.; Wiggins, L.; Christensen, D.L.; Maenner, M.J.; Daniels, J.; Warren, Z.; Kurzius-Spencer, M.; Zahorodny, W.; Rosenberg, R.C.; White, T.; et al. Prevalence of Autism Spectrum Disorder Among Children Aged 8 Years- Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2014. *MMWR Surveill. Summ.* **2018**, *67*, 1–23. [CrossRef] [PubMed]

106. Dawson, G. Early behavioral intervention, brain plasticity, and the prevention of autism spectrum disorder. *Dev. Psychopathol.* **2008**, *20*, 775–803. [CrossRef] [PubMed]

107. Shelton, J.F.; Tancredi, D.J.; Hertz-Picciotto, I. Independent and dependent contributions of advanced maternal and paternal ages to autism risk. *Autism Res.* **2010**, *3*, 30–39. [CrossRef] [PubMed]

PUBLICATION II

4.2. Publication II

Influence of gestational chlorpyrifos exposure and *APOE* polymorphism on autistic-like behaviors: differences with the valproic acid animal model

Judit Biosca-Brull, Pia Basaure, Laia Guardia-Escote, Maria Cabré, Jordi Blanco, Miguel Morales-Navas, Fernando Sánchez-Santed, José L. Domingo, Maria Teresa Colomina

Study II overview

What do we already know?	ASD is characterized by social and communication impairments, as well as repetitive patterns of behavior. People with autism often have co-occurring condition such as anxiety. Although its etiology is still unknown, there are some genetic and environmental factors that make a child more likely to develop the disorder. The OP pesticide CPF, as well as hundreds of genes, have been strongly associated with the disorder.
What does the study add?	This study evaluates the effects of prenatal exposure to CPF on communication skills, early development and long-lasting anxiety behaviors, and compares them to a pharmacological model of autism. At the same time, the different characteristics conferred by the *APOE* genotype are also studied, in search of a plausible cause that can explain the etiology of autism.
Highlights	Prenatal exposure to CPF and VPA were observed to have opposite effects, on communication skills. Basal differences between the *APOE* genotype were observed in young and adolescent transgenic mice. VPA-treated mice showed autism-like behaviors in all parameters evaluated.

Influence of gestational chlorpyrifos exposure and *APOE* polymorphism on autistic-like behaviors: differences with the valproic acid animal model

Judit Biosca-Brull [abc], Pia Basaure [a], Laia Guardia-Escote [ab], Maria Cabré [ad], Jordi Blanco [ace], Miguel Morales-Navas [f], Fernando Sánchez-Santed [f], José L. Domingo [c], Maria Teresa Colomina [abc*]

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

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

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

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

[e] Universitat Rovira i Virgili, Department of Basic Medical Sciences, Reus, Spain

[f] Department of Psychology, Health Research Center (CEINSA), Almeria University, 04120 Almeria, Spain

Abstract: Autism spectrum disorder (ASD) encompasses several neurodevelopmental conditions characterized by communication and social impairment, and repetitive patterns of behavior. It can co-occur with other mental conditions such as anxiety. The massive use of chlorpyrifos (CPF) has been linked to the increase the prevalence of the disorder, especially when the exposure occurs during development. ASD has also been closely linked to a wide variety of genetic factors. The aim of the present investigation is to study how gestational CPF exposure and *APOE* polymorphism affects communication skills, early development and mid-term anxiety-like behaviors. C57BL/6J and humanized apoE3 and apoE4 homozygous mice were exposed to 0 or 1 mg/kg/day of CPF through the diet, from gestational day (GD) 12 to 18. In addition, a group of C57BL/6J females were injected subcutaneously with 300 mg/kg/day of valproic acid (VPA) on GD 12 and 13. This group was used as a positive control for studying some core and associated autism-like behaviors. Dams were evaluated on maternal care and nest quality, whereas pups communication skills by means of ultrasonic vocalizations and physical/motor development were assessed during the preweaning period. Moreover, locomotor activity and anxiety-like behaviors were evaluated during adolescence. Our results showed that C57BL/6J mice prenatally exposed to CPF or VPA showed a decrease in body weight and a delay in eye opening, while communication and anxiety behaviors were affected differently depending on treatment. In addition, none of the parameters evaluated in apoE transgenic mice exposed to CPF were affected, but there were differences between genotypes. Therefore, we suggest that prenatal CPF exposure does not influence communication and anxiety behaviors as VPA exposure does, while the *APOE* genetic background confers resistance to prenatal CPF exposure and influences the maturation rate by conditioning the behavioral response.

Keywords: Development, Autism, Chlorpyrifos, Anxiety, Ultrasonic vocalizations, *APOE*

1. Introduction

Organophosphate (OP) pesticides have been used worldwide to control insect pests in agricultural and residential areas. In 2003, the European Commission reported that 59.1 % of the total used pesticies were OP, which amounts to 4,645 tons of this pesticide. Of all the OP, chlorpyrifos (CPF) was the most used with 15.6 % (1,226 tones) (European Commission, 2007). It was also highly used in the United States (US) (28,500 tons annually). After a considerable number of scientific reports on the health effects associated with CPF, a number of regulatory measures were introduced in both US and Europe. In brief, in 2001, the US Environmental Protection Agency (EPA) prohibited its residential use (EPA, 2002), and in the last few years, the US and the European Union have completely banned CPF, even though it is still used in developing countries (EPA, 2021; EFSA, 2019).

Although CPF is intended to have detrimental effects on pest species, some studies have reported effects on non-target organisms including humans (Eaton et al., 2008; Maggio et al., 2021; Nandi et al., 2022). Once CPF has been absorbed, cytochrome P450 metabolizes it to undergo either oxidative desulfuration which gives its active metabolite (CPF-oxon), or diarylation, which gives 3,5,6-trichloro-2-pyridinol (TCPy) and diethyl-thiophosphate. CPF-oxon can be hydrolyzed by A- or B-esterases to TCPy and diethyl-phosphate (Chanda, 1997; Kamataki et al., 1976; Pond et al., 1998). CPF and, in particular, CPF-oxon inhibits the activity of acetylcholinesterase (AChE) by binding to its active site. This inhibition led to an accumulation of acetylcholine in the synaptic cleft and a subsequent overstimulation of the postsynaptic cholinergic receptors (Pope and Liu, 1997; Sultatos, 1994). In rodents, AChE inhibition has been described at a threshold of 1 mg/kg/day of CPF (Silva, 2020), although doses below this threshold have been described without cholinergic signs of toxicity but with alterations in locomotor activity (Lee et al., 2015; Silva et al., 2017), cognition (Gómez-Giménez et al., 2017; Jett et al., 2001) or anxiety-like behaviors (Carr et al., 2017; Silva et al., 2017), since CPF has other targets such as the cannabinoid or endocrine system (Abreu-Villaça and Levin, 2017; Casida and Quistad, 2004; Otênio et al., 2022). In addition, several studies have demonstrated that young animals and humans are more sensitive to CPF toxicity than adults (Moser et al., 1998; Pope and Liu, 1997; Timchalk et al., 2006).

Recent investigations suggest that CPF exposure contributes to the etiology of neurodevelopmental disorders such as autism spectrum disorder (ASD) (Berg et al., 2020; Biosca-Brull et al., 2021; Perez-Fernandez et al., 2022). Autism is a heterogeneous disorder characterized by difficulties in social interaction and communication, as well as repetitive patterns of behavior (WHO, 2022). Apart from these three core symptoms, ASD also presents significant impairments in the motor domain and can co-occur with other mental health conditions (i.e., anxiety) that may precede social and adaptative functioning deficits in children (Bhat, 2020; Kerns et al., 2015; Leary and Hill, 1996). Genetics was first associated with this disorder in the 1980s, and today hundreds of genes have

been suggested as risk candidates for autism (Rylaarsdam and Guemez-Gamboa, 2019). In our laboratory, we have investigated the interactions between CPF exposure and different polymorphisms of the apolipoprotein E (*APOE*) gene. In humans, there are three major isoforms – apoE2, apoE3 and apoE4 – of which apoE3 is the most abundant in the population (Marais, 2021). The apoE protein is involved in lipid transport and metabolism and competes with reelin (RELN) to bind to very low-density lipoprotein and apoE receptor-2 (D'Arcangelo et al., 1999; Mahley, 1988). The *RELN* gene has been closely related to ASD, making the *APOE* a possible candidate (Scala et al., 2022). However, results on the association between the *APOE* polymorphism and ASD are scarce and contradictory. Although Raiford et al. (2004) and Ashley-Koch et al. (2007) observed that the different polymorphisms of the *APOE* gene are not a risk for autism, other studies have associated the hypermethylation of this gene and the apoE2 isoform with increased risk (Hu et al., 2018; Persico et al., 2004).

Mice predominantly communicate using ultrasonic vocalizations (USVs). USVs emitted by pups while they are separated from their mother and littermates are important in terms of social motivation (Ehret, 2005). The genetic background of the animals and exposure to environmental factors make a considerable contribution to USV modifications. Moreover, altered USVs during the early stages of development may be an early indicator of long-term behavioral alterations. For example, changes in the total number of calls can be linked to anxiety-like behaviors (Budylin et al., 2019). Along these lines, Morales-Navas et al. (2020) observed that prenatal CPF exposure (1 mg/kg/day) reduces the number of vocalizations and increases the latency to emit the first call on postnatal day (PND) 7 in rats. These results were similar to those observed for rats treated with valproic acid (VPA), a well-established pharmacological autism model (Mabunga et al., 2015). Likewise, CD-1 mice exposed to 6 mg/kg/day of CPF during gestation also showed few vocalizations, a high latency and a tendency to hyporeflexia (Venerosi et al., 2009). On the other hand, in a study with a validated mouse model of idiopathic autism (BTBR mice) exposed to 6 mg/kg/day of CPF, De Felice et al. (2015) observed an increase in the number of USVs and a reduction in the motor activity. In terms of the *APOE* genotype, there is no evidence of prenatal exposure to CPF, but the evaluation of postnatal CPF exposure (1 mg/kg/day) by Basaure et al. (2018) showed differences between the *APOE* genotype in terms of body weight and eye opening, and a general effect of CPF.

As reported above, anxiety-like behaviors affect 40 % of children diagnosed with autism (Kent and Simonoff, 2017). Studies with rodents observed that prenatal and postnatal exposure to CPF can increase or trigger anxiety-like behavior (Braquenier et al., 2010; Silva et al., 2017). Accordingly, Sánchez-Amate et al. (2001) suggested that CPF has an anxiogenic effect on rats exposed during adulthood and Robertson et al. (2005) demonstrated that apoE isoforms have differential effects on anxiety measures, with *APOE* ε4 carriers being the most affected individuals. Anxiety also co-occurs

with Alzheimer's disease and, in this case, the apoE4 isoform is the most prevalent genetic risk factor (Safieh et al., 2019).

The aim of this investigation is to study the contribution of prenatal CPF exposure and the *APOE* genotype to the etiology of autism. To this end, communication, physical and motor development, as well as anxiety-like behaviors were first studied in a pharmacological model of autism and then compared with C57BL/6J mice exposed to CPF. Subsequently, these variables were also evaluated in humanized apoE3 and apoE4 homozygous mice prenatally exposed to CPF. To the best of our knowledge, this is the first study that assesses communication, early development and anxiety behavior in apoE transgenic mice prenatally exposed to CPF and their relation to autism.

2. Material and Methods

2.1. Experimental animals

Adult C57BL/6J mice from Charles Rivers Laboratories (Barcelona, Spain) and humanized apoE3- and apoE4 target replacement (TR) homozygous mice from Taconic Europe (Lille Skensved, Denmark) were used in this study. The apoE-TR mice were generated by replacing the murine *ApoE* gene with one of the two human *APOE* alleles (ε3 or ε4) without altering any endogenous regulatory sequence (Sullivan et al., 1997). After one week of quarantine, one male and two females of the same strain were mated for 3 h. The presence of a vaginal plug was assigned as gestational day (GD) 0. All the mice were housed in plastic cages containing between two and five animals until GD 12, when pregnant females were housed individually. Pregnant C57BL/6J mice were randomly assigned to one of the three treatments (control [CNT], CPF or VPA), whereas human apoE-TR were randomly selected to receive one of the two treatments (CNT or CPF). The day of delivery was assigned as postnatal day (PND) 0, and the number of litters and live pups were recorded. Only litters with at least four live pups were used in this study. Animals were maintained in a 12-hour light/dark automatic cycle (light ON between 8 a.m. and 8 p.m.) with controlled temperature (22 ± 2 ºC) and humidity (50 ± 10%). Food (SAFE® A04 diet, Panlab, Barcelona, Spain) and tap water were administered *ad libitum*. The present study was given an authorization code (number 10735) by the Government of Catalonia and conducted following the ARRIVE Guidelines (Percie du Sert et al., 2020). It was approved by the Animal Care and Use Committee of the Rovira i Virgili University (Catalonia, Spain) and in compliance with Spanish Royal Decree 53/2013 on the protection of animals used in experiments and the European Communities Council Directive (86/609/EEC).

2.2. Chemicals, treatment, and experimental design

Two parallel experiments were conducted in this study (Figure 1). In the first experiment, we exposed C57BL/6J mice to either 0 or 1 mg/kg/day of CPF (0,0-diethyl O-(3,5,6-trichloropyridin-2-yl) phosphorothioate) (Sigma-Aldrich, Madrid, Spain) through a supplemented diet from GD 12 to 18, and we included a group treated with a subcutaneous injection of 300 mg/kg/day of VPA (2-propypentanoic acid sodium) (Sigma-Aldrich, Madrid, Spain), administered on two consecutive days (GD 12 and 13). The group treated with VPA was used as a pharmacological animal model for autism (Sakai et al., 2018). In the second experiment, apoE-TR mice were exposed to 0 or 1 mg/kg/day of CPF in the diet from GD 12 to 18. In both experiments, standard food was supplemented with 15 mg CPF/kg chow (Panlab, Barcelona, Spain). The body weight and food intake of dams were monitored daily to adjust the amount of food and achieve a daily dose of 1 mg/kg of CPF. All animals were provided with regular chow *ad libitum*.

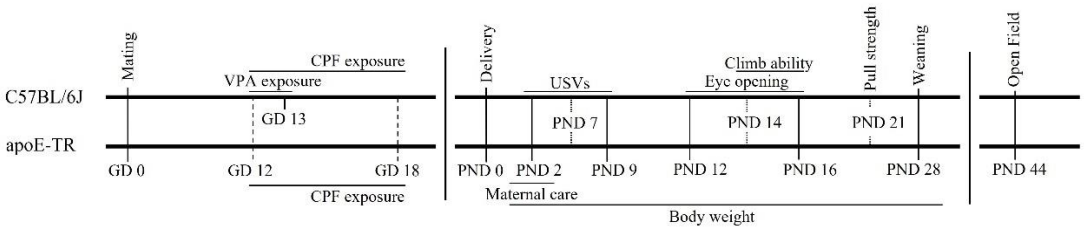


Figure 1. Experimental schema of C57BL/6J and apoE-TR mice. A group of C57BL/6J mice were exposed to 300 mg/kg/day of VPA by a subcutaneous injection on GD 12 and 13. Pregnant females of both strains were orally exposed to CPF (1 mg/kg/day) between GD 12 and 18. USVs were assessed on PND 2, 7 and 9. Physical and motor development were assessed from PND 2 to 28, whereas locomotor activity and anxiety-like behavior were evaluated during adolescence (PND 44).

During the preweaning period, communication skills were evaluated, in both strains, on PND 2, 7 and 9. After USV analysis on PND 2, maternal care and nest quality were evaluated, and until weaning on PND 28, the physical and motor development of the litter were evalauted. At weaning, mice were assigned to groups of two to five animals of the same sex and treatment per cage. The unit of analysis was the litter. Measures obtained from individuals of the same litter and sex were subject to statistical analysis. All the animals and litters used in this study are summarized in Table 1.

Table 1. Number of litters used in this study.

	C57BL/6J			apoE3		apoE4	
	CNT	CPF	VPA	CNT	CPF	CNT	CPF
Ultrasonic vocalizations							
Litters	15	14	18	12	13	16	13
Males	15	12	14	12	12	15	12
Females	12	12	15	12	12	15	12
Maternal care							
Litters	15	15	15	15	13	15	15
Physical and motor development							
Litters	10	10	8	8	10	10	10
Open field							
Litters	18	13	21	12	16	19	15
Males	14	10	16	12	12	15	11
Females	15	13	10	10	12	17	12

CNT-Control; CPF-Chlorpyrifos; VPA-Valproic acid

2.3. Pre-weaning assessment

2.3.1. Litter characteristics and maternal care

On the day of delivery (PND 0), we recorded the number of living and dead pups. The mortality between PND 0 and 2 was recorded to calculate the viability index (live pups PND 2/litter size PND 0) (Basaure et al., 2018).

Nest quality and maternal care were assessed on PND 2, after pup communication had been analyzed. First, we rated the quality of the nest as described in Table 2. Afterwards, the dam was removed from the cage while the pups were weighed and placed in the corner opposite the nest. The dam was then returned to the home cage and the time that she took to collect the first pup (latency) and all the pups were recorded for a maximum of three minutes. In addition, we calculated a new parameters named efficiency (time to collect all the pups - latency)/(number of pups in the litter) (Reverte et al., 2014a).

2.3.2. Evaluation of the litter's physical and motor development

On the basis of previous studies conducted in our laboratory (Basaure et al., 2018; Reverte et al., 2014a), the developmental timeline of mice was monitored between PND 2 and 28 to evaluate physical and motor development at different timepoints (see Table 2). Physical development was assessed by body weight and eye opening, whereas motor development was evaluated by the climbing ability and pull-strength force. To assess climbing ability, we placed a metal grid (24 x 24 cm) at an angle of 45º in a plastic cage with the pups at the bottom, and to measure force we gently pulled the pup backwards by the tail three times and recorded the highest value, with a grip strength meter (Ugo Basile, Gemonio, Italy). The value for each pup within the litter was used to calculate the mean value of the litter.

Table 2. Assessment of maternal care and physical and motor development.

Test	Day of evaluation (PND)	Measure/score
Body weight	2, 7, 9, 14, 16, 21, 23, 28	Weight (g)
Maternal care	2	Latency (s) to collect the first pup and time (s) taken to collect all pups
Nest quality	2	0 = no nest, 1 = not all the pups are in the nest, 2 = the nest is well-defined
Eye opening	12-16	0 = both eyes are closed, 1 = one eye open and one closed, 2 = both eyes are open
Climb ability	14-16	0 = no movement, 1 = less than half of the grid, 2 = more than half of the grid
Pull strength	21	Force (g)

2.3.3. Communication assessment: Ultrasonic Vocalizations

Communication was assessed by recording mice USVs, with SeaWave software (Gianni Pavan©, Pavia, Italy) and a UltraMic 250 (Dodotronic, Italy) in a sound-attenuating chamber.

The USV call rate increases during the first 5-6 days, peaks are around PND 6-7 and then starts to decrease until it completely disappears (Elwood and Keeling, 1982). In order to determine the best days to carry out the test, we recorded mice communication for 3 minutes on PND 2, 4, 6, 8 and 10. Our data, together with that reported by Branchi et al. (2001), led us to select PND 2, 7 and 9 as the best days on which to observe the complete profile of vocalizations.

USVs were recorded in one male and one female from each litter. Animals were moved to the testing room in their home cage five minutes before the start of the test. Then, we placed a

random pup in the sound-attenuated chamber for five minutes. The distance between the microphone and the pup was set at 10 cm (Scattoni et al., 2008). Subsequently, the chamber was cleaned with ethanol 70 % to prevent olfactory clues and a pup from the same litter but opposite sex was randomly selected and placed into the chamber to follow the same protocol. This protocol was performed on every day of the evaluation. Changes over the five-minute period, total number of calls, and the average of duration, frequency and intensity, as well as the latency to emit the first call were assessed.

2.4. Behavioral assessment in adolescent mice: open field test

Locomotor and anxiety-like behaviors were assessed at PND 44 in both strains using an open field test. Briefly, the apparatus consists of an open wooden box measuring 60 x 60 cm and with a 50 cm high wall. The field was divided into two zones: the center zone covering an area of 30 x 30 cm in the middle of the field and the periphery which covers the rest of the space. Five minutes before starting the test, the animals were transported to the testing room, placed in the center of the arena and allowed to explore freely for 30 minutes. After the test had finished, the open box was cleaned with ethanol 70 % to prevent olfactory clues. Locomotor activity was evaluated by recording the total distance travelled in periods of five minutes and anxiety-like behaviors by measuring the time that the animals spent in the center zone in comparison to the periphery, the velocity at which they moved and the distance they covered. To this end, we used a video camera (Sony CCD-IRIS) and a video-tracking program (Etho-Vision XT 11.5, Noldus Information Technologies, Wageningen, The Netherlands) (Kraeuter et al., 2019).

2.5. Sacrifice, sampling and determination of brain AChE activity

Biological samples were obtained on PND 2. Four animals from different litters, sex and experimental group were sacrificed by decapitation. Brains were flash-frozen and stored at -80 ºC until AChE activity was evaluated.

Brain samples were weighed and homogenized in cold PBS 0.1 M at pH 8 with 1 % Triron X-100. Then, homogenates were centrifuged at 2,000 g for 10 minutes at 4 ºC and the supernatant removed for analysis. AChE activity was measured in duplicates and determined spectrophotometrically using a semiautomatic COBAS MIRA analyzer (Hoffman-La Roche & Co., Basel, Switzerland) and an updated version of the Ellman method (Ellman et al., 1961; Peris-Sampedro et al., 2015). The enzyme activity was calculated relative to the protein concentration,

which was assessed by the Lowry method (Lowry et al., 1951) described in our previous study (Biosca-Brull et al., 2022). Brain AChE activity was represented as U/mg proteins.

2.7. Statistical analysis

Data were analyzed using the SPSS 27.0 software (IBM Corp. Chicago, IL, USA). A three-way analysis of variance (ANOVA) was done to analyze the general effects of sex, treatment and genotype, as well as their interactions. In those cases in which a variable was assessed over time, effects were evaluated by using repeated measures ANOVA (RMANOVA). The number of pups in each litter was used as a co-variable to analyze maternal care latency and all the developmental landmarks evaluated, while in the study of litter characteristics (litter size and viability index) the co-variable was the age of the dams. The homogeneity of variance was assessed by the Levene test. Then, parametric data was further analyzed by one-way ANOVA followed by a post-hoc Tukey or a two-sample t-test, while non-parametric data was analyzed by the Kruskal-Wallis or Mann-Whitney U test to assess differences between groups when necessary. The results are represented as mean values ± S.E.M, with the statistical significance set at $p<0.05$.

3. Results

3.1. AChE activity was unaffected by prenatal exposure and APOE genotype

The activity of AChE was assessed on PND 2 in the brains of pups from both strains. No signs of AChE inhibition or differences between groups were observed in either C57BL/6J or apoE-TR groups (data not shown). Therefore, prenatal exposure to low doses of CPF (1 mg/kg/day) during the late gestation of mice did not affect the activity of the AChE enzyme in pup brains, which indicates that the cholinergic system was functioning correctly.

3.2. Prenatal treatment with CPF and APOE genetic background do not alter the litter characteristics or maternal care

The analysis of litter size and viability index using a one-way (treatment) or two-way (treatment and genotype) ANOVA depending on the strain used showed that neither the treatment nor the genotype were of any statistical significance (data not shown).

As far as maternal behavior is concerned, all the dams evaluated prepared a well-defined nest with all the pups inside (data not shown). The latency to collect the first pup and the total time corrected by the latency and the number of pups in each litter were analyzed using a one-way (treatment) ANOVA in C57BL/6J mice and a two-way (treatment and genotype) ANOVA in apoE-TR mice, but no differences between treatment or genotype were observed (Figure 2).

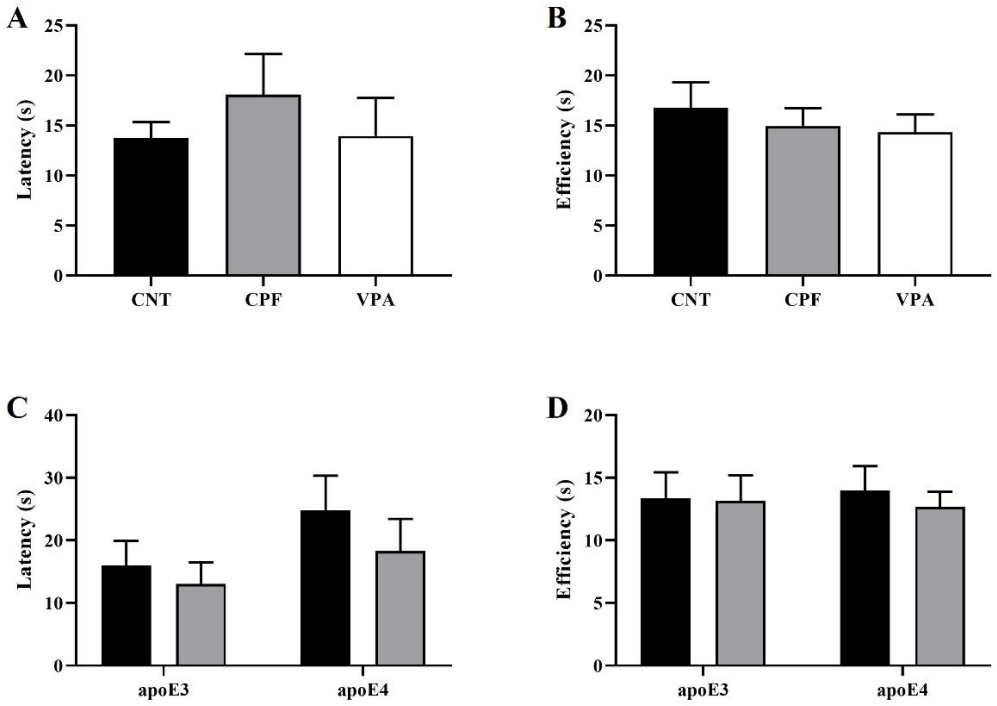


Figure 2. Maternal care on PND 2. Latency and efficiency in C57BL/6 (**A** and **B**) and apoE-TR mice (**C** and **D**).

3.3. Assessment of litter development

The influence of the treatment, sex and genotype on the early development of mice was determined by analyzing physical and motor endpoints evaluated between PND 2 and PND 28 (Figure 3 and 4).

3.3.1. Prenatal CPF and VPA exposure reduces body weight and delays eye opening in C57BL/6J mice

The analysis of body weight with a two-way RMANOVA (sex and treatment), showed an increase in weight with time [$F_{7,70}$=609.595, p<0.001] and an interaction between PND and treatment [$F_{14,142}$=2.602, p=0.002] (Figure 3A). Subsequent analysis with one-way ANOVA or the Kruskal-Wallis test (PND 14 and PND 21) revealed differences between CNT and CPF (PND2 (p=0.031),

PND 7 (p=0.013), PND 14 (p=0.004), PND 16 (p=0.001), PND 21 (p<0.001), PND 23 (p<0.001), PND 28 (p<0.001)) and also between CNT and VPA (PND2 (p<0.001), PND 14 (p=0.001), PND 16 (p=0.005), PND 21 (p=0.001), PND 23 (p<0.001) and PND 28 (p<0.001)), with CNT being the heaviest group (Figure 3A).

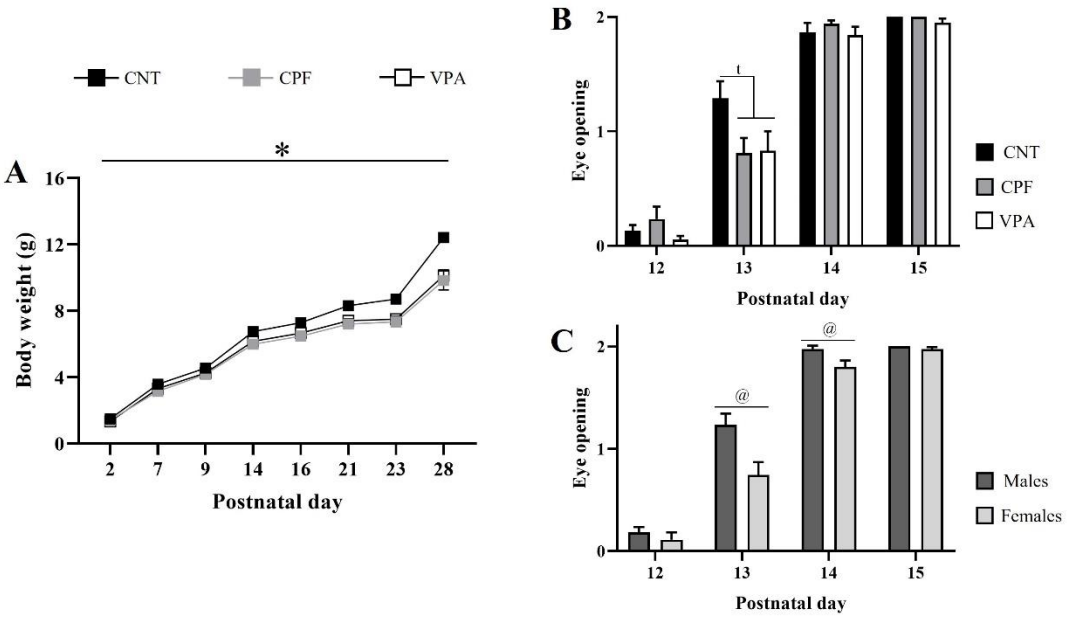


Figure 3. Physical development of C57BL/6J mice. Body weight from PND 2 to 28 (**A**) and eye opening from PND 12 to 15 according to treatment (**B**) and sex (**C**). Symbols indicate significant differences between treatments (*) and sex (@) at p<0.05, while t indicates a tendency.

Eye opening scores were analyzed from PND 12 to 16 using a two-way RMANOVA (sex and treatment). Maturation of this parameter was observed over time [$F_{4,48}$=409.877, p<0.001], and by PND 15 both eyes were completely open in all groups. We also found an interaction between PND and treatment [$F_{8,98}$=2.891, p=0.006] and PND and sex [$F_{4,48}$=2.769, p=0.038] (Figure 3B and 3C). Analysis with the Kruskal-Wallis test showed a downward trend on PND 13 (p=0.057), when the lowest eye opening score was for CPF- and VPA-treated groups (Figure 3B). Differences between males and females were assessed by the Mann-Whitney U test (PND 13 and PND 15) and a two-sample t-test. We observed that males opened their eyes earlier than females, and this was significant on PND 13 (p=0.005) and PND 14 (p=0.017) (Figure 3C).

No effects of sex or treatment were observed on climbing ability or pull strength, but a RMANOVA analysis showed progressive motor improvement from PND 14 to 16 [$F_{2,48}$=110.260, p<0.001] (data not shown).

3.3.2. Physical and motor development of apoE-TR mice was not affected by prenatal exposure to CPF, but differences between genotypes were observed

Body weight was analyzed with a three-way RMANOVA (sex, treatment and genotype). It showed a progressive increase in weight over time [$F_{7,83}$=857.260, p<0.001], and an interaction between PND and genotype [$F_{7,83}$=5.034, p<0.001], and PND and sex [$F_{7,83}$=2.423, p=0.026] (Figure 4A). A two-sample t-test with the genotype as independent variable indicates that apoE4 mice had lower body weight over the time (PND 2 [t_{95}=2.957, p=0.004], PND 7 [t_{95}=3.095, p=0.003], PND 9 [t_{95}=3.841, p<0.001], PND 14 [t_{95}=3.841, p<0.001], PND 16 [t_{95}=3.936, p<0.001], PND 21 [t_{95}=5.527, p<0.001], PND 23 [t_{95}=5.255, p<0.001] and PND 28 [t_{95}=5.542, p<0.001]) (Figure 4A).

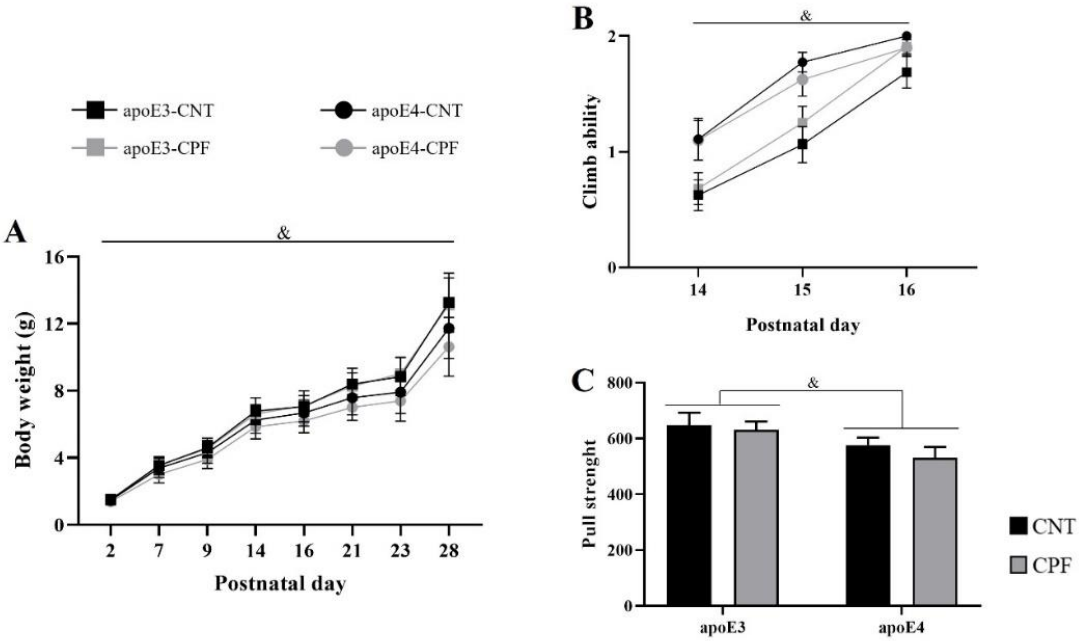


Figure 4. Physical and motor development in apoE-TR mice. Body weight from PND 2 to 28 (**A**), climbing ability from PND 14 to 16 (**B**) and pull strength on PND 21 (**C**). The symbol & indicates significant differences between genotypes at p<0.05.

Treatment, sex and genotype were observed to have no effect on eye opening, but there was an overall effect of time [$F_{4,65}$=483.972, p<0.001], indicating that all mice opened their eyes during the period studied (data not shown).

In terms of motor development, a three-way RMANOVA (sex, treatment and genotype) revealed a progressive improvement in climbing ability [$F_{2,69}$=74.524, p<0.001] and an interaction between PND and genotype [$F_{2,69}$=5.045, p=0.009]. Further analysis with a two-sample t-test or

the Mann-Whitney U test (PND 16) revealed that apoE4 mice achieved top scores earlier than apoE3 mice, which indicates different rhythms of motor maturation (PND 14 [t_{73}=-2.869, p=0.055], PND 15 [t_{76}=-3.951, p<0.001] and PND 16 (p=0.040)) (Figure 4B). In addition, a three-way ANOVA (sex, treatment and genotype) [$F_{1,72}$=5.497, p=0.022] showed an overall effect of genotype on the pull strength, the highest values being for the *APOE3* genotype (Figure 4C).

3.4. Assessment of USVs

Mouse USVs were analyzed to study changes over the five-minutes period evaluated in periods of one minute (data not shown) and the total number of vocalizations and the average changes on duration, frequency and intensity, as well as the latency to the first vocalization (Figure 5 and 6).

3.4.1. CPF and VPA produced opposite effects on communication skills in C57BL/6J mice

As reported above, USVs were recorded on three different days. On PND 2, a two-way (sex and treatment) ANOVA showed a general effect of treatment on the total number of calls [$F_{2,79}$=8.657, p<0.001], duration [$F_{2,79}$=4.830, p=0.011], frequency [$F_{2,79}$=3.449, p=0.037] and intensity [$F_{2,79}$=5.359, p=0.007] of vocalizations (Figure 5A to 5E). The subsequent analysis with a Kruskal-Wallis test showed that VPA-treated animals emitted fewer vocalizations (CNT vs VPA (p<0.001), CPF vs VPA (p<0.001)) with less duration (CPF vs VPA (p=0.011)) and intensity (CPF vs VPA (p=0.014)), especially in comparison with the animals treated with CPF (Figure 5A, 5C and 5E).

However, these differences disappeared on PND 7 (data not shown), but on PND 9 treatment was observed to have an overall effect on the total number of vocalizations [$F_{2,78}$=11.032, p<0.001], latency [$F_{2,78}$=4.590, p=0.013], duration [$F_{2,78}$=4.709, p=0.012] and intensity [$F_{2,78}$=3.942, p=0.024] of calls (Figure 5A to 5E). The analysis with the Kruskal-Wallis test showed differences in the total number of vocalizations between CNT and VPA (p=0.036), CPF and VPA (p<0.001) and CNT and CPF (p=0.046) (Figure 5A). In addition, a significant difference was observed between CPF and VPA (p=0.004) in the latency to the first vocalization, while differences in call intensity were observed between CNT and CPF and between CPF and VPA at p=0.001 (Figure 5B and 5E). The duration of USVs was also analyzed by one-way ANOVA (treatment) [$F_{2,78}$=4.449, p=0.015]. Post-hoc analysis showed significant differences between the CNT and VPA-treated group (p=0.017) (Figure 5C). These results indicate that the treatment with VPA reduces the number, duration and intensity of USVs and increases the time to emit the first call, which results in inefficient communication, while CPF does the opposite.

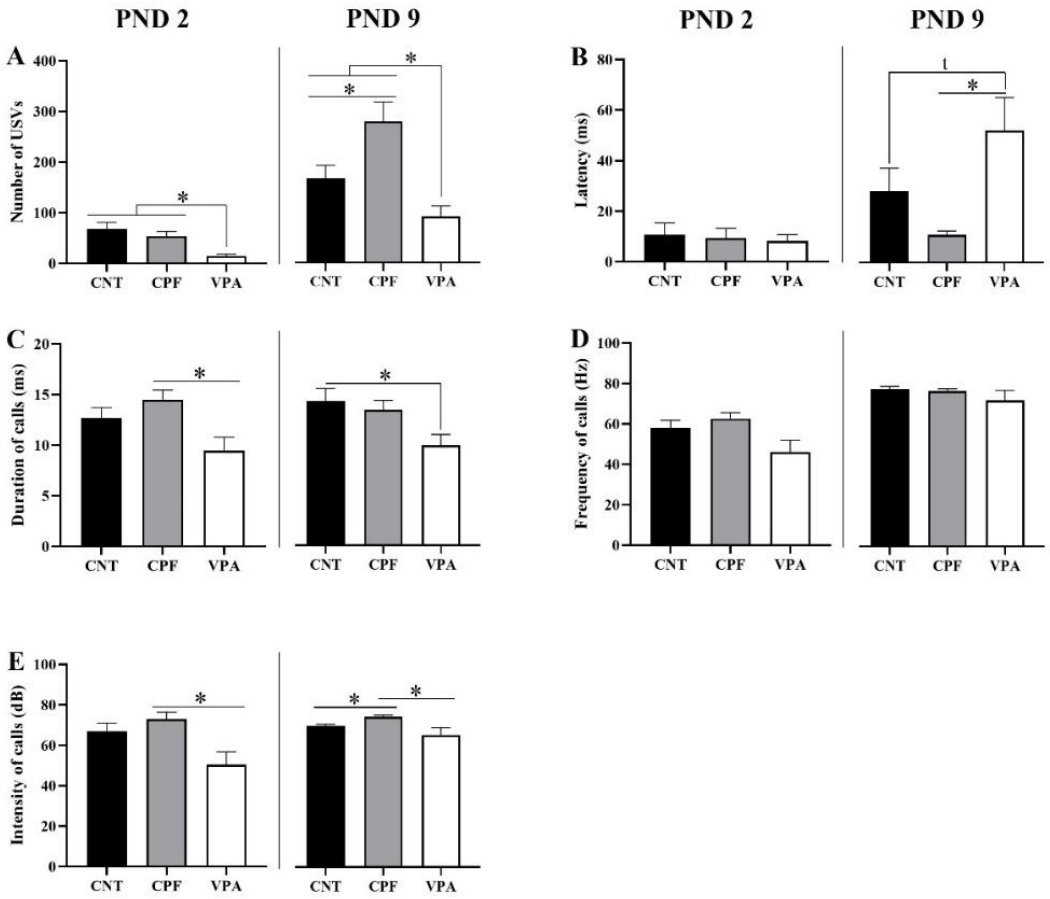


Figure 5. Communication assessment by measuring USVs on PND 2 and 9 in C57BL/6J mice. Average of total number of calls (**A**), latency (**B**), duration (**C**), frequency (**D**) and intensity (**E**). An asterisk indicates significant differences between treatments at $p<0.05$, whereas a t indicates a tendency.

3.4.2. Prenatal exposure to CPF does not affect communication skills in apoE-TR mice, but genotype does

Although, on PND 7 we observed significant differences in some USV parameters between genotypes, these effects were not observed on PND 2 (data not shown) and disappeared by PND 9 (Figure 6). On PND 7, a three-way ANOVA (sex, treatment and genotype) showed an overall effect of genotype on all parameters, except for the total number of vocalizations and latency (duration [$F_{1,101}=7.771$, $p=0.006$], frequency [$F_{1,101}=19.877$, $p<0.001$] and intensity [$F_{1,101}=5.157$, $p=0.025$]). The *APOE4* genotype shows an increase in the duration and intensity of calls, whereas frequency decreased (Figure 6C to 6E).

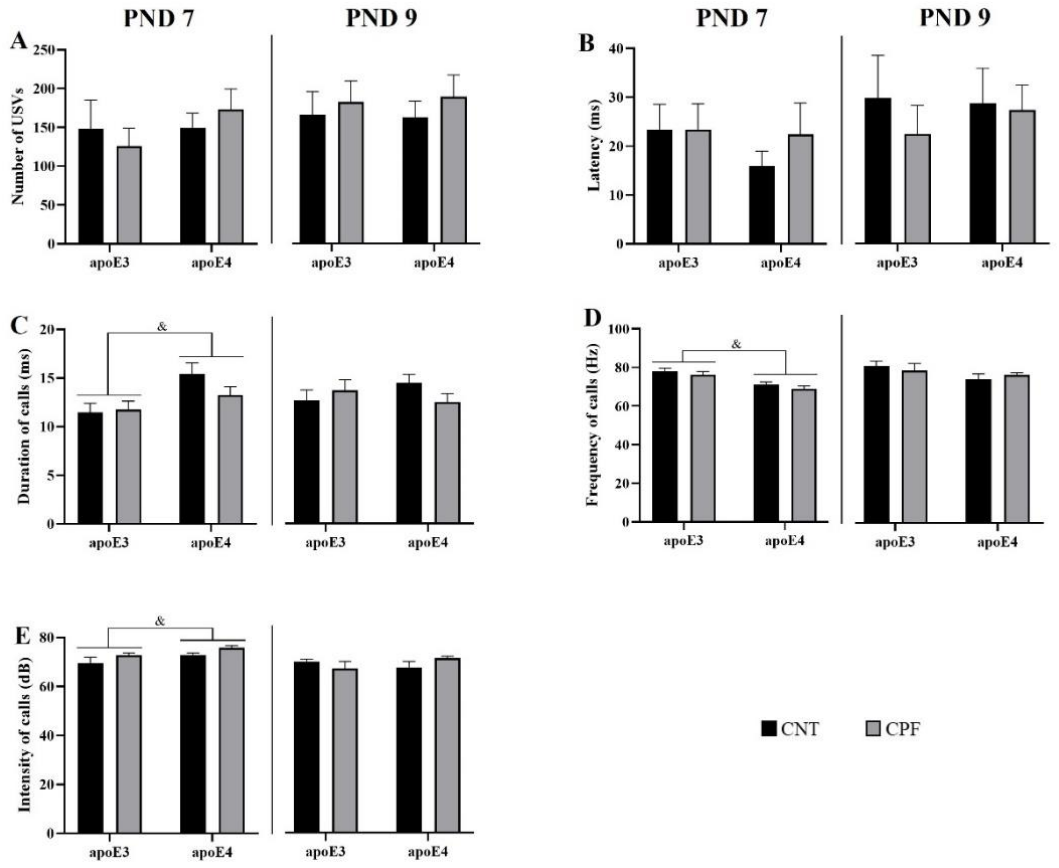


Figure 6. Communication assessment by measuring of USVs on PND 7 and 9 in apoE-TR mice. Average of total number of calls (**A**), latency (**B**), duration (**C**), frequency (**D**) and intensity (**E**). The symbol & indicates significant differences between genotypes at $p<0.05$.

3.5. Anxiety-like behavior in an open field test

3.5.1. C57BL/6J mice treated with VPA showed anxiety-like behavior and decreased locomotor activity

General activity in the open field test was analyzed by a two-way RMANOVA (sex and treatment). The total time (30 min) was divided into six periods of five-minutes. Locomotor activity decreased during the testing period in all groups, except for the VPA-treated group in which it increased at two different evaluation points, suggesting impaired habituation. However, time was observed to have an overall effect [$F_{5,45}=5.753$, $p<0.001$] (Figure 7A). In addition, the total average of distance traveled and the distance and velocity in the central area were also assessed (Figure 7B to 7E). Although no significant differences were observed in the distance treveled in the center, a two-way ANOVA (sex and treatment) showed an overall effect of treatment on the total average of distance [$F_{2,54}=3.953$, $p=0.026$] and velocity [$F_{2,54}=4.975$, $p=0.011$] (Figure 7B and 7E). Subsequent analysis by a one-way ANOVA (treatment) revealed

significant differences between CPF and VPA (total average distance traveled (p=0.011) and velocity in the center (p=0.005)) (Figure 7B and 7E), with the group treated with CPF traveling longer distances at greater velocity.

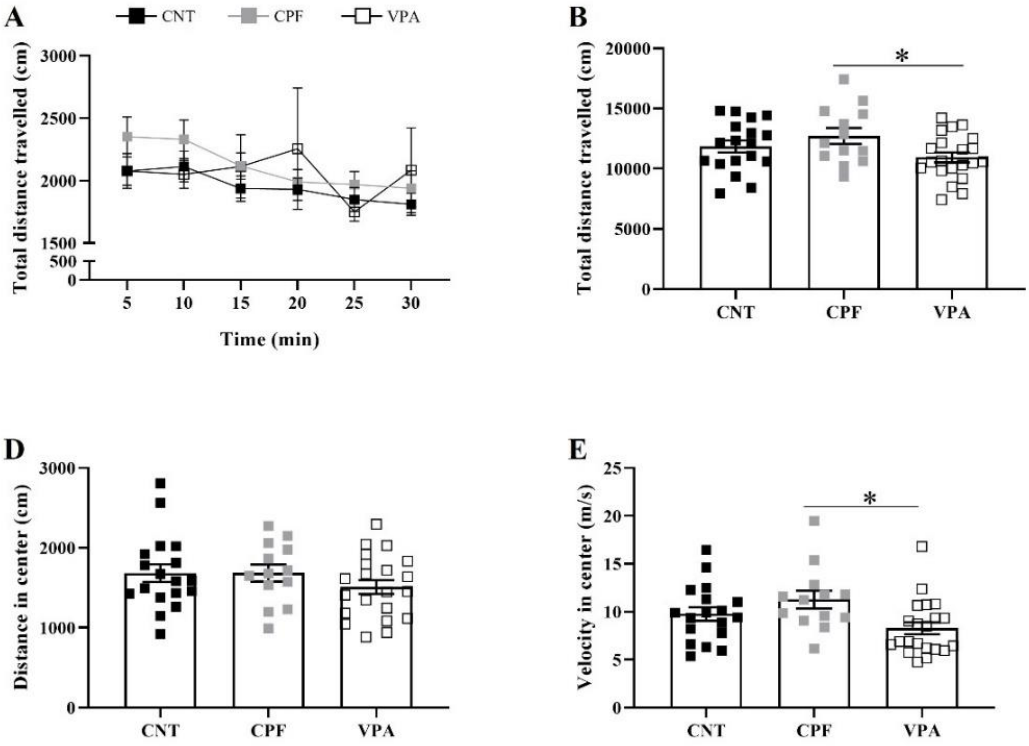


Figure 7. Evaluation of locomotor activity and anxiety behavior in adolescent C57BL/6J mice. Total distance travelled during the 30 minutes of the test in six five-minute periods (**A**). Total average distance traveled (**B**) and distance (**C**) and velocity (**D**) in the central area of the open field. An asterisk indicates significant differences between treatments at p<0.05.

3.5.2. APOE4 genotype showed more anxiety-like behavior than APOE3

As reported above, locomotor activity was assessed by three-way RMANOVA (sex, treatment and genotype). Both apoE3 and apoE4 mice habituated to the novel space regardless of the treatment [$F_{5,51}$=20.358, p<0.001]. However, interaction between time and treatment [$F_{5,51}$=2.443, p=0.046] was observed. This, indicates that the CNT group presented greater activity than the treated groups and that the differences were significantly different at 10 [t_{61}=2.453, p=0.017] and 25 min [t_{61}=2.298, p=0.025] of the test (Figure 8A). The analysis of the total average distance traveled and the distance and velocity in the central zone using a three-way ANOVA (sex, treatment and genotype) revealed an overall effect of the genotype (distance in the center [$F_{1,62}$=5.423, p=0.024] and velocity in the center [$F_{1,62}$=5.918, p=0.018]), and that

the apoE4 mice traveled the shortest distances more slowly in the center (Figure 8B to 8D). This may indicate a more anxious phenotype in those mice carrying the *APOE* ε4 allele.

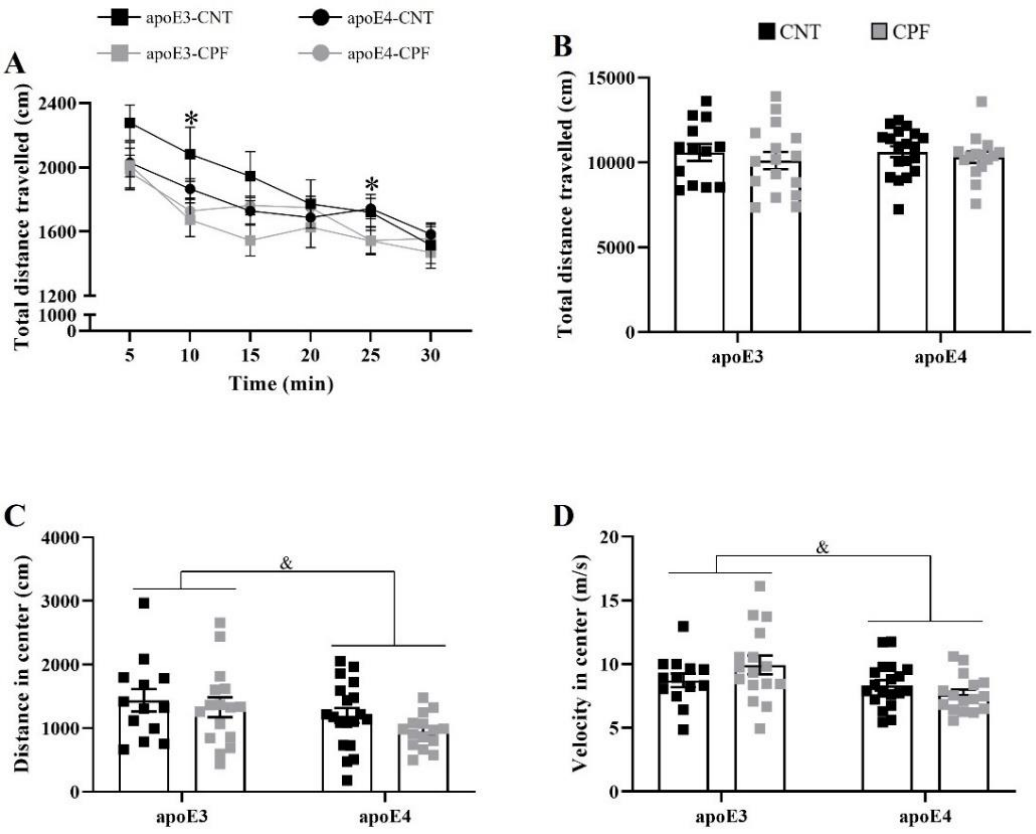


Figure 8. Evaluation of locomotor activity and anxiety behavior in adolescent apoE-TR mice. Total distance travelled during the 30 minutes of the test in six five-minute periods (**A**). Total average distance travelled (**B**) and distance (**C**) and velocity (**D**) in the central area of the open field. Symbols indicte significant differences between genotypes (&) and treatment (*) at $p<0.05$.

4. Discussion

In the current study, we evaluate the potential risk associated with gestational exposure to CPF of developing ASD-like symptomatology in various animal models. We studied the genetic vulnerability and interactions with the pesticide in males and females in the VPA pharmacological autism model, and a transgenic mouse model carrying the human *APOE* alleles ε3 and ε4. In previous studies carried out in our laboratory, we observed that CPF exposure during late gestation affects social behaviors in adolescence in a sex dependent manner (Biosca-Brull et al., 2022). Moreover, Basaure et al. (2019) observed that social deficits in adult mice also depended on their *APOE* genetic background.

Thus, we studied how prenatal CPF exposure and *APOE* genotype affect early communication and developmental landmarks, which are core and associated symptoms of ASD. To this end, we designed two experiments that exposed C57BL/6J to VPA and CPF and apoE-TR mice to CPF during gestation. Communication and physical/motor development was evaluated in the preweaning period, and locomotor activity and anxiety behavior were assessed in adolescence (PND 44). In C57BL/6J we observed that both CPF and VPA decreased body weight and delayed eye opening. Mice treated with VPA showed communication deficits after birth and anxiety-like behavior in adolescence, while prenatal CPF affected both communication and anxiety but in the opposite way. Prenatal CPF exposure did not alter any of the parameters evaluated in the *APOE* genetic background but significant differences were observed between *APOE* genotypes, with a greater effect in *APOE* ε4 carriers. This lack of any gestational toxic effects suggests a more resistant phenotype in mice carrying the human *APOE* gene, and deserves more investigations.

Since no effect was observed on AChE activity, we can confirm that the dose used in this study had no toxicity or clinical effects, as Silva (2020) reported in her review. However, it must be taken into account that the time elapsed between maternal exposure and AChE determination is long enough for any effects to have disappeared (Basaure et al., 2018; Carr, 2001; Morales-Navas et al., 2020; Perez-Fernandez et al., 2020b, 2020a).

As mentioned above, mice communicate by emitting USVs as distress calls when they are isolated from the nest so that they can be retrieved by their mother (Portfors, 2007), although retrieval is affected as much by the dams emotional and motivational state as by the quality and quantity of USVs. Indeed, mothers involved in maternal behaviors such as lactation or actively caring for pups showed a faster response than the non-lactating mothers (Ehret and Haack, 1984). Nevertheless, our results were unable to detect any maternal behavior that could explain differences between pups in terms of physical development or behavior later in life. In fact, body weight from PND 2 to 28 was lower in C57BL/6J exposed to both CPF and VPA. In addition, these treated animals showed a delay in eye opening, but no motor alterations. Although the literature about the influence of prenatal CPF exposure on fetal growth is scarce, a study on women living in New York associated the levels of CPF in umbilical cord with lower body weigh and length at birth (Whyatt et al., 2004). On the other hand, VPA exposure is associated with body weight gain in adolescents (Biton et al., 2003; Wirrell, 2003) and adults (Mattson et al., 1992), but Espinosa et al. (2008) did not observe any increase in the body mass index of prepubertal children. Likewise, apoE3 mice showed higher body weight but poorer climbing ability, even though motor deficits were reversed as the animals grew (PND 21), since the mice carrying the *APOE* ε3 allele were stronger than *APOE* ε4 carriers. Published data indicate that the *APOE3* genotype is the most vulnerable to metabolic alterations such as obesity (Arbones-Mainar et al., 2008; Johnson et al., 2017; Tejedor et al., 2014) so this could explain the differences observed in body weight.

Communication skills are affected by such variables as the strain, age, sex and environmental factors (Caruso et al., 2022; Sasaki et al., 2020; Scattoni et al., 2009). Rodent USVs increase during the first PNDs which is when pups strictly depend on their mothers for survival, but progressively decrease as they develop physically and in terms of motor function (Caruso et al., 2020). In CD-1 mice exposed to 6 mg/kg/day of CPF during late gestation, Venerosi et al. (2009) found that the number and duration of calls decreased but the latency to the first vocalization on PND 10 increased. Along these lines, Morales-Navas et al. (2020) showed similar results in rats on PND 7, and these alterations were associated with those observed in VPA-treated rats. On the other hand, our results showed opposite effects for CPF and VPA in C57BL/6J; CPF significantly increases the number of calls on PND 9 in comparison to both control and VPA mice. These discrepancies between the two experiments may be related to differences in the species and strain used. On the other hand, increased USV rates have also been reported for ASD animal models. In fact, several studies with idiopathic and knockout mouse models of autism showed an increase in vocalizations (De Felice et al., 2015; Picker et al., 2006; Scattoni et al., 2008; Tsai et al., 2012). However, we did not observe any significant effect of prenatal exposure to CPF on apoE-TR mice, but rather a genotype effect. This is the first study to assesse communication skills in apoE-TR mice, and we should point out the need to investigate the USV profile in this genetic background to determine whether some apoE isoforms follow an idiopathic communication profile or whether there are different rhythms of maturation.

Likewise, several studies have used developmental USVs to predict later-life anxious behaviors (Budylin et al., 2019; Lukas and Wöhr, 2015; Yamauchi et al., 2022). Although the literature on USVs and late anxiety-like behaviors has discussed CPF and the *APOE* genotype very little, it is well known that treatment with VPA increases anxiety levels in rodents (Olexová et al., 2016; Schneider et al., 2008, 2006). In agreement with this, our VPA-treated mice showed a reduction in center velocity, indicating that they showed inactive behaviors in the inner zone as a sign of anxiety. Furthermore, several studies observed that CPF exposure during late gestation or the first postnatal week can induce long-term alterations in terms of anxious behaviors in rodents (Braquenier et al., 2010; Ribeiro-Carvalho et al., 2020; Venerosi et al., 2010). However, the results obtained in this study show no differences between CNT and CPF-exposed mice. Our results on the *APOE* genotype are in agreement with those of Reverte et al. (2014b), which show that the *APOE* genetic background, and in particular the *APOE4* genotype, confers different anxiety behaviors. However, it was not affected by CPF treatment.

In summary, pregnancy is probably the most important stage in the development of a human or animal. Exposure to environmental toxics during this period can have short-, mid- and long-term adverse effects related to neurodevelopmental disorders, and these effects may be influenced by the genetic background. Our results showed that communication and anxiety behaviors in animals after prenatal exposure to CPF differ from those observed in animals after exposure to VPA, whereas

both treatments reduced body weight and delayed eye opening in C57BL/6J mice. On the other hand, we observed basal differences between *APOE* genotypes in all the variables evaluated, but they were not affected by treatment with CPF. For this reason, we suggest that different human apoE isoforms confer some protection against prenatal CPF exposure. Finally, we should point out that small differences in the maturation profile between species or produced by treatment could be masking some effects, and further research is needed to evaluate the possible long-lasting effects of CPF in adults.

Acknowledgments: The authors would like to thank Dr. Celeste de Paolo, Esperanza Chernichero and Juan Valencia for their skillful technical support with the care of animals.

Funding: This research was supported by the Martí Franquès Grant Program – Doctoral modality (Reference: 2019PMF-PIPF-28) and the Spanish Government (Ministry of the Economy and Competitiveness (MINECO, Spain)) (Reference: PSI2017-86847-C2-R).

References

Abreu-Villaça, Y., Levin, E.D., 2017. Developmental neurotoxicity of succeeding generations of insecticides. Environ. Int. 99, 55–77. https://doi.org/10.1016/j.envint.2016.11.019

Arbones-Mainar, J.M., Johnson, L.A., Altenburg, M.K., Maeda, N., 2008. Differential modulation of diet-induced obesity and adipocyte functionality by human apolipoprotein E3 and E4 in mice. Int. J. Obes. 32, 1595–1605. https://doi.org/10.1038/ijo.2008.143

Ashley-Koch, A.E., Jaworski, J., Ma, D.Q., Mei, H., Ritchie, M.D., Skaar, D.A., Robert Delong, G., Worley, G., Abramson, R.K., Wright, H.H., Cuccaro, M.L., Gilbert, J.R., Martin, E.R., Pericak-Vance, M.A., 2007. Investigation of potential gene–gene interactions between apoe and reln contributing to autism risk. Psychiatr. Genet. 17, 221–226. https://doi.org/10.1097/YPG.0b013e32809c2f75

Basaure, P., Guardia-Escote, L., Biosca-Brull, J., Blanco, J., Cabré, M., Peris-Sampedro, F., Sánchez-Santed, F., Domingo, J.L., Colomina, M.T., 2019. Exposure to chlorpyrifos at different ages triggers APOE genotype-specific responses in social behavior, body weight and hypothalamic gene expression. Environ. Res. 178, 108684. https://doi.org/10.1016/j.envres.2019.108684

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

Berg, E.L., Ching, T.M., Bruun, D.A., Rivera, J.K., Careaga, M., Ellegood, J., Lerch, J.P., Wöhr, M., Lein, P.J., Silverman, J.L., 2020. Translational outcomes relevant to neurodevelopmental disorders following early life exposure of rats to chlorpyrifos. J. Neurodev. Disord. 12, 40. https://doi.org/10.1186/s11689-020-09342-1

Bhat, A.N., 2020. Is Motor Impairment in Autism Spectrum Disorder Distinct From Developmental Coordination Disorder? A Report From the SPARK Study. Phys. Ther. 100, 633–644. https://doi.org/10.1093/ptj/pzz190

Biosca-Brull, J., Guardia-Escote, L., Blanco, J., Basaure, P., Cabré, M., Sánchez-Santed, F., Domingo, J.L., Colomina, M.T., 2022. Prenatal, but not postnatal exposure to chlorpyrifos affects social behavior of mice and the excitatory-inhibitory balance in a sex-dependent manner. Food Chem. Toxicol. 169, 113423. https://doi.org/10.1016/j.fct.2022.113423

Biosca-Brull, J., Pérez-Fernández, C., Mora, S., Carrillo, B., Pinos, H., Conejo, N.M., Collado, P., Arias, J.L., Martín-Sánchez, F., Sánchez-Santed, F., Colomina, M.T., 2021. Relationship between Autism Spectrum Disorder and Pesticides: A Systematic Review of Human and Preclinical Models. Int. J. Environ. Res. Public Health 18, 5190. https://doi.org/10.3390/ijerph18105190

Biton, V., Levisohn, P., Hoyler, S., Vuong, A., Hammer, A.E., 2003. Lamotrigine Versus Valproate Monotherapy—Associated Weight Change in Adolescents With Epilepsy: Results From a Post Hoc Analysis of a Randomized, Double-Blind Clinical Trial. J. Child Neurol. 18, 133–139. https://doi.org/10.1177/08830738030180021701

Branchi, I., Santucci, D., Alleva, E., 2001. Ultrasonic vocalisation emitted by infant rodents: a tool for assessment of neurobehavioural development. Behav. Brain Res. 125, 49–56. https://doi.org/10.1016/S0166-4328(01)00277-7

Braquenier, J.B., Quertemont, E., Tirelli, E., Plumier, J.C., 2010. Anxiety in adult female mice following perinatal exposure to chlorpyrifos. Neurotoxicol. Teratol. 32, 234–239. https://doi.org/10.1016/j.ntt.2009.08.008

Budylin, T., Guariglia, S.R., Duran, L.I., Behring, B.M., Shaikh, Z., Neuwirth, L.S., Banerjee, P., 2019. Ultrasonic vocalization sex differences in 5-HT-R deficient mouse pups: Predictive phenotypes associated with later-life anxiety-like behaviors. Behav. Brain Res. 373, 112062. https://doi.org/10.1016/j.bbr.2019.112062

Carr, R.L., 2001. Effects of Repeated Oral Postnatal Exposure to Chlorpyrifos on Open-Field Behavior in Juvenile Rats. Toxicol. Sci. 59, 260–267. https://doi.org/10.1093/toxsci/59.2.260

Carr, R.L., Armstrong, N.H., Buchanan, A.T., Eells, J.B., Mohammed, A.N., Ross, M.K., Nail, C.A., 2017. Decreased anxiety in juvenile rats following exposure to low levels of chlorpyrifos during development. Neurotoxicology 59, 183–190. https://doi.org/10.1016/j.neuro.2015.11.016

Caruso, A., Marconi, M.A., Scattoni, M.L., Ricceri, L., 2022. Ultrasonic vocalizations in laboratory mice: strain, age, and sex differences. Genes, Brain Behav. 21, 1–16. https://doi.org/10.1111/gbb.12815

Caruso, A., Ricceri, L., Scattoni, M.L., 2020. Ultrasonic vocalizations as a fundamental tool for early and adult behavioral phenotyping of Autism Spectrum Disorder rodent models. Neurosci. Biobehav. Rev. 116, 31–43. https://doi.org/10.1016/j.neubiorev.2020.06.011

Casida, J.E., Quistad, G.B., 2004. Organophosphate Toxicology: Safety Aspects of Nonacetylcholinesterase Secondary Targets. Chem. Res. Toxicol. 17, 983–998. https://doi.org/10.1021/tx0499259

Chanda, S., 1997. Tissue-Specific Effects of Chlorpyrifos on Carboxylesterase and Cholinesterase Activity in Adult Rats: Anin Vitroandin VivoComparison. Fundam. Appl. Toxicol. 38, 148–157. https://doi.org/10.1006/faat.1997.2329

D'Arcangelo, G., Homayouni, R., Keshvara, L., Rice, D.S., Sheldon, M., Curran, T., 1999. Reelin Is a Ligand for Lipoprotein Receptors. Neuron 24, 471–479. https://doi.org/10.1016/S0896-6273(00)80860-0

De Felice, A., Scattoni, M.L., Ricceri, L., Calamandrei, G., 2015. Prenatal Exposure to a Common Organophosphate Insecticide Delays Motor Development in a Mouse Model of Idiopathic Autism. PLoS One 10, e0121663. https://doi.org/10.1371/journal.pone.0121663

Eaton, D.L., Daroff, R.B., Autrup, H., Bridges, J., Buffler, P., Costa, L.G., Coyle, J., McKhann, G., Mobley, W.C., Nadel, L., Neubert, D., Schulte-Hermann, R., Spencer, P.S., 2008. Review of the Toxicology of Chlorpyrifos With an

Emphasis on Human Exposure and Neurodevelopment. Crit. Rev. Toxicol. 38, 1–125. https://doi.org/10.1080/10408440802272158

EFSA, 2019. Statement on the available outcomes of the human health assessment in the context of the pesticides peer review of the active substance chlorpyrifos. EFSA J. 17. https://doi.org/10.2903/j.efsa.2019.5809

Ehret, G., 2005. Infant Rodent Ultrasounds - A Gate to the Understanding of Sound Communication. Behav. Genet. 35, 19–29. https://doi.org/10.1007/s10519-004-0853-8

Ehret, G., Haack, B., 1984. Motivation and Arousal Influence Sound-induced Maternal Pup-retrieving Behavior in Lactating House Mice. Z. Tierpsychol. 65, 25–39. https://doi.org/10.1111/j.1439-0310.1984.tb00370.x

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95. https://doi.org/10.1016/0006-2952(61)90145-9

Elwood, R.W., Keeling, F., 1982. Temporal organization of ultrasonic vocalizations in infant mice. Dev. Psychobiol. 15, 221–227. https://doi.org/10.1002/dev.420150306

EPA, 2021. Chlorpyrifos; Tolerance Revocations. Fed. Regist. / Vol. 86, No. 165 / Monday, August 30, 2021 / Rules Regul. 86, 21–42.

EPA, 2002. Interim Reregistration Eligibility Decision (IRED) for Chlorpyrifos. EPA 1–259.

Espinosa, P.S., Salazar, J.C., Yu, L., Mendiondo, M.S., Robertson, W.C., Baumann, R.J., 2008. Lack of Valproic Acid-Associated Weight Gain in Prepubertal Children. Pediatr. Neurol. 39, 177–180. https://doi.org/10.1016/j.pediatrneurol.2008.05.006

European Comission, 2007. The use of plant protection products in the European Union. Statisticalbooks.

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

Hu, Z., Yang, Y., Zhao, Y., Yu, H., Ying, X., Zhou, D., Zhong, J., Zheng, Z., Liu, J., Pan, R., Zhang, W., Cheng, F., Duan, S., 2018. APOE hypermethylation is associated with autism spectrum disorder in a Chinese population. Exp. Ther. Med. 15, 4749–4754. https://doi.org/10.3892/etm.2018.6069

Jett, D.A., Navoa, R. V., Beckles, R.A., McLemore, G.L., 2001. Cognitive Function and Cholinergic Neurochemistry in Weanling Rats Exposed to Chlorpyrifos. Toxicol. Appl. Pharmacol. 174, 89–98. https://doi.org/10.1006/taap.2001.9198

Johnson, L.A., Torres, E.R.S., Impey, S., Stevens, J.F., Raber, J., 2017. Apolipoprotein E4 and Insulin Resistance Interact to Impair Cognition and Alter the Epigenome and Metabolome. Sci. Rep. 7, 43701. https://doi.org/10.1038/srep43701

Kamataki, T., Lee Lin, M.C.M., Belcher, D.H., Neal, R.A., 1976. Studies of the metabolism of parathion with an apparently homogeneous preparation of rabbit liver cytochrome P 450. Drug Metab. Dispos. 4, 180–189.

Kent, R., Simonoff, E., 2017. Prevalence of Anxiety in Autism Spectrum Disorders, in: Anxiety in Children and Adolescents with Autism Spectrum Disorder. Elsevier, pp. 5–32. https://doi.org/10.1016/B978-0-12-805122-1.00002-8

Kerns, C.M., Kendall, P.C., Zickgraf, H., Franklin, M.E., Miller, J., Herrington, J., 2015. Not to Be Overshadowed or Overlooked: Functional Impairments Associated With Comorbid Anxiety Disorders in Youth With ASD. Behav. Ther. 46, 29–39. https://doi.org/10.1016/j.beth.2014.03.005

Kraeuter, A.K., Guest, P.C., Sarnyai, Z., 2019. The Open Field Test for Measuring Locomotor Activity and Anxiety-Like Behavior, in: Methods in Molecular Biology. pp. 99–103. https://doi.org/10.1007/978-1-4939-8994-2_9

Leary, M.R., Hill, D.A., 1996. Moving on: Autism and movement disturbance. Ment. Retard. 34, 39–53.

Lee, I., Eriksson, P., Fredriksson, A., Buratovic, S., Viberg, H., 2015. Developmental neurotoxic effects of two pesticides: Behavior and biomolecular studies on chlorpyrifos and carbaryl. Toxicol. Appl. Pharmacol. 288, 429–438. https://doi.org/10.1016/j.taap.2015.08.014

Lowry, O., Rosebrough, N., Farr, A.L., Randall, R., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275. https://doi.org/10.1016/S0021-9258(19)52451-6

Lukas, M., Wöhr, M., 2015. Endogenous vasopressin, innate anxiety, and the emission of pro-social 50-kHz ultrasonic vocalizations during social play behavior in juvenile rats. Psychoneuroendocrinology 56, 35–44. https://doi.org/10.1016/j.psyneuen.2015.03.005

Mabunga, D.F.N., Gonzales, E.L.T., Kim, J., Kim, K.C., Shin, C.Y., 2015. Exploring the Validity of Valproic Acid Animal Model of Autism. Exp. Neurobiol. 24, 285–300. https://doi.org/10.5607/en.2015.24.4.285

Maggio, S.A., Janney, P.K., Jenkins, J.J., 2021. Neurotoxicity of chlorpyrifos and chlorpyrifos-oxon to Daphnia magna. Chemosphere 276, 130120. https://doi.org/10.1016/j.chemosphere.2021.130120

Mahley, R.W., 1988. Apolipoprotein E: Cholesterol Transport Protein with Expanding Role in Cell Biology. Science. 240, 622–630. https://doi.org/10.1126/science.3283935

Marais, A.D., 2021. Apolipoprotein E and Atherosclerosis. Curr. Atheroscler. Rep. 23, 34. https://doi.org/10.1007/s11883-021-00933-4

Mattson, R.H., Cramer, J.A., Collins, J.F., 1992. A Comparison of Valproate with Carbamazepine for the Treatment of Complex Partial Seizures and Secondarily Generalized Tonic–Clonic Seizures in Adults. N. Engl. J. Med. 327, 765–771. https://doi.org/10.1056/NEJM199209103271104

Morales-Navas, M., Castaño-Castaño, S., Pérez-Fernández, C., Sánchez-Gil, A., Teresa Colomina, M., Leinekugel, X., Sánchez-Santed, F., 2020. Similarities between the Effects of Prenatal Chlorpyrifos and Valproic Acid on Ultrasonic Vocalization in Infant Wistar Rats. Int. J. Environ. Res. Public Health 17, 6376. https://doi.org/10.3390/ijerph17176376

Moser, V.C., Padilla, S., Hunter, D.L., Marshall, R.S., McDaniel, K.L., Phillips, P.M., 1998. Age- and Gender-Related Differences in the Time Course of Behavioral and Biochemical Effects Produced by Oral Chlorpyrifos in Rats. Toxicol. Appl. Pharmacol. 149, 107–119. https://doi.org/10.1006/taap.1997.8354

Nandi, N.K., Vyas, A., Akhtar, M.J., Kumar, B., 2022. The growing concern of chlorpyrifos exposures on human and environmental health. Pestic. Biochem. Physiol. 185, 105138. https://doi.org/10.1016/j.pestbp.2022.105138

Olexová, L., Štefánik, P., Kršková, L., 2016. Increased anxiety-like behaviour and altered GABAergic system in the amygdala and cerebellum of VPA rats — An animal model of autism. Neurosci. Lett. 629, 9–14. https://doi.org/10.1016/j.neulet.2016.06.035

Otênio, J.K., Souza, K.D., Alberton, O., Alberton, L.R., Moreno, K.G.T., Gasparotto Junior, A., Palozi, R.A.C., Lourenço, E.L.B., Jacomassi, E., 2022. Thyroid-disrupting effects of chlorpyrifos in female Wistar rats. Drug Chem. Toxicol. 45, 387–392. https://doi.org/10.1080/01480545.2019.1701487

Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M.T., Baker, M., Browne, W.J., Clark, A., Cuthill, I.C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S.T., Howells, D.W., Karp, N.A., Lazic, S.E., Lidster, K., MacCallum, C.J., Macleod, M., Pearl, E.J., Petersen, O.H., Rawle, F., Reynolds, P., Rooney, K., Sena, E.S., Silberberg, S.D., Steckler, T., Würbel, H., 2020. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLOS Biol. 18, e3000410. https://doi.org/10.1371/journal.pbio.3000410

Perez-Fernandez, C., Matamala Montoya, M., Morales-Navas, M., Guardia-Escote, L., Cabré, M., Colomina, M.T., Giménez, E., Sánchez-Santed, F., 2022. Influence of Gestational Chlorpyrifos Exposure on ASD-like Behaviors in an fmr1-KO Rat Model. Mol. Neurobiol. 59, 5835–5855. https://doi.org/10.1007/s12035-022-02933-0

Perez-Fernandez, C., Morales-Navas, M., Aguilera-Sáez, L.M., Abreu, A.C., Guardia-Escote, L., Fernández, I., Garrido-Cárdenas, J.A., Colomina, M.T., Giménez, E., Sánchez-Santed, F., 2020a. Medium and long-term effects of low doses of Chlorpyrifos during the postnatal, preweaning developmental stage on sociability, dominance, gut microbiota and plasma metabolites. Environ. Res. 184, 109341. https://doi.org/10.1016/j.envres.2020.109341

Perez-Fernandez, C., Morales-Navas, M., Guardia-Escote, L., Garrido-Cárdenas, J.A., Colomina, M.T., Giménez, E., Sánchez-Santed, F., 2020b. Long-term effects of low doses of Chlorpyrifos exposure at the preweaning developmental stage: A locomotor, pharmacological, brain gene expression and gut microbiome analysis. Food Chem. Toxicol. 135, 110865. https://doi.org/10.1016/j.fct.2019.110865

Peris-Sampedro, F., Cabré, M., Basaure, P., Reverte, I., Domingo, J.L., Teresa Colomina, M., 2015. Adulthood dietary exposure to a common pesticide leads to an obese-like phenotype and a diabetic profile in apoE3 mice. Environ. Res. 142, 169–176. https://doi.org/10.1016/j.envres.2015.06.036

Persico, A.M., D'Agruma, L., Zelante, L., Militerni, R., Bravaccio, C., Schneider, C., Melmed, R., Trillo, S., Montecchi, F., Elia, M., Palermo, M., Rabinowitz, D., Pascucci, T., Puglisi-Allegra, S., Reichelt, K.-L., Muscarella, L., Guarnieri, V., Melgari, J.-M., Conciatori, M., Keller, F., 2004. Enhanced APOE2 transmission rates in families with autistic probands. Psychiatr. Genet. 14, 73–82. https://doi.org/10.1097/01.ypg.0000128768.37838.17

Picker, J.D., Yang, R., Ricceri, L., Berger-Sweeney, J., 2006. An altered neonatal behavioral phenotype in Mecp2 mutant mice. Neuroreport 17, 541–544. https://doi.org/10.1097/01.wnr.0000208995.38695.2f

Pond, A.L., Chambers, H.W., Coyne, C.P., Chambers, J.E., 1998. Purification of two rat hepatic proteins with a-esterase activity toward chlorpyrifos-oxon and paraoxon. J. Pharmacol. Exp. Ther. 286, 1404–1411.

Pope, C.N., Liu, J., 1997. Age-related differences in sensitivity to organophosphorus pesticides. Environ. Toxicol. Pharmacol. 4, 309–314. https://doi.org/10.1016/S1382-6689(97)10029-1

Portfors, C. V., 2007. Types and functions of ultrasonic vocalizations in laboratory rats and mice. J. Am. Assoc. Lab. Anim. Sci. 46, 28–34.

Raiford, K.L., Shao, Y., Allen, I.C., Martin, E.R., Menold, M.M., Wright, H.H., Abramson, R.K., Worley, G., DeLong, G.R., Vance, J.M., Cuccaro, M.L., Gilbert, J.R., Pericak-Vance, M.A., 2004. No association between theAPOE gene and autism. Am. J. Med. Genet. 125B, 57–60. https://doi.org/10.1002/ajmg.b.20104

Reverte, I., Domingo, J.L., Colomina, M.T., 2014a. Neurodevelopmental effects of decabromodiphenyl ether (BDE-209) in APOE transgenic mice. Neurotoxicol. Teratol. 46, 10–17. https://doi.org/10.1016/j.ntt.2014.08.003

Reverte, I., Pujol, A., Domingo, J.L., Colomina, M.T., 2014b. Thyroid hormones and fear learning but not anxiety are affected in adult apoE transgenic mice exposed postnatally to decabromodiphenyl ether (BDE-209). Physiol. Behav. 133, 81–91. https://doi.org/10.1016/j.physbeh.2014.05.013

Ribeiro-Carvalho, A., Lima, C.S., Dutra-Tavares, A.C., Nunes, F., Nunes-Freitas, A.L., Filgueiras, C.C., Manhães, A.C., Meyer, A., Abreu-Villaça, Y., 2020. Mood-related behavioral and neurochemical alterations in mice exposed to low chlorpyrifos levels during the brain growth spurt. PLoS One 15, e0239017. https://doi.org/10.1371/journal.pone.0239017

Robertson, J., Curley, J., Kaye, J., Quinn, J., Pfankuch, T., Raber, J., 2005. apoE isoforms and measures of anxiety in probable AD patients and Apoe−/− mice. Neurobiol. Aging 26, 637–643. https://doi.org/10.1016/j.neurobiolaging.2004.06.003

Rylaarsdam, L., Guemez-Gamboa, A., 2019. Genetic Causes and Modifiers of Autism Spectrum Disorder. Front. Cell. Neurosci. 13, 1–15. https://doi.org/10.3389/fncel.2019.00385

Safieh, M., Korczyn, A.D., Michaelson, D.M., 2019. ApoE4: an emerging therapeutic target for Alzheimer's disease. BMC Med. 17, 64. https://doi.org/10.1186/s12916-019-1299-4

Sakai, A., Matsuda, T., Doi, H., Nagaishi, Y., Kato, K., Nakashima, K., 2018. Ectopic neurogenesis induced by prenatal antiepileptic drug exposure augments seizure susceptibility in adult mice. Proc. Natl. Acad. Sci. 115, 4270–4275. https://doi.org/10.1073/pnas.1716479115

Sánchez-Amate, M.C., Flores, P., Sánchez-Santed, F., 2001. Effects of chlorpyrifos in the plus-maze model of anxiety. Behav. Pharmacol. 12, 285–292. https://doi.org/10.1097/00008877-200107000-00007

Sasaki, E., Tomita, Y., Kanno, K., 2020. Sex differences in vocalizations to familiar or unfamiliar females in mice. R. Soc. Open Sci. 7, 201529. https://doi.org/10.1098/rsos.201529

Scala, M., Grasso, E.A., Di Cara, G., Riva, A., Striano, P., Verrotti, A., 2022. The Pathophysiological Link Between Reelin and Autism: Overview and New Insights. Front. Genet. 13. https://doi.org/10.3389/fgene.2022.869002

Scattoni, M.L., Crawley, J., Ricceri, L., 2009. Ultrasonic vocalizations: A tool for behavioural phenotyping of mouse models of neurodevelopmental disorders. Neurosci. Biobehav. Rev. 33, 508–515. https://doi.org/10.1016/j.neubiorev.2008.08.003

Scattoni, M.L., Gandhy, S.U., Ricceri, L., Crawley, J.N., 2008. Unusual Repertoire of Vocalizations in the BTBR T+tf/J Mouse Model of Autism. PLoS One 3, e3067. https://doi.org/10.1371/journal.pone.0003067

Schneider, T., Roman, A., Basta-Kaim, A., Kubera, M., Budziszewska, B., Schneider, K., Przewłocki, R., 2008. Gender-specific behavioral and immunological alterations in an animal model of autism induced by prenatal exposure to valproic acid. Psychoneuroendocrinology 33, 728–740. https://doi.org/10.1016/j.psyneuen.2008.02.011

Schneider, T., Turczak, J., Przewłocki, R., 2006. Environmental Enrichment Reverses Behavioral Alterations in Rats Prenatally Exposed to Valproic Acid: Issues for a Therapeutic Approach in Autism. Neuropsychopharmacology 31, 36–46. https://doi.org/10.1038/sj.npp.1300767

Silva, J.G., Boareto, A.C., Schreiber, A.K., Redivo, D.D.B., Gambeta, E., Vergara, F., Morais, H., Zanoveli, J.M., Dalsenter, P.R., 2017. Chlorpyrifos induces anxiety-like behavior in offspring rats exposed during pregnancy. Neurosci. Lett. 641, 94–100. https://doi.org/10.1016/j.neulet.2017.01.053

Silva, M.H., 2020. Effects of low-dose chlorpyrifos on neurobehavior and potential mechanisms: A review of studies in rodents, zebrafish, and <scp> Caenorhabditis elegans </scp>. Birth Defects Res. 112, 445–479. https://doi.org/10.1002/bdr2.1661

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

Sultatos, L.G., 1994. Mammalian toxicology of organophosphorus pesticides. J. Toxicol. Environ. Health 43, 271–289. https://doi.org/10.1080/15287399409531921

Tejedor, M.T., Garcia-Sobreviela, M.P., Ledesma, M., Arbones-Mainar, J.M., 2014. The Apolipoprotein E Polymorphism rs7412 Associates with Body Fatness Independently of Plasma Lipids in Middle Aged Men. PLoS One 9, e108605. https://doi.org/10.1371/journal.pone.0108605

Timchalk, C., Poet, T.S., Kousba, A.A., 2006. Age-dependent pharmacokinetic and pharmacodynamic response in preweanling rats following oral exposure to the organophosphorus insecticide chlorpyrifos. Toxicology 220, 13–25. https://doi.org/10.1016/j.tox.2005.11.011

Tsai, P.T., Hull, C., Chu, Y., Greene-Colozzi, E., Sadowski, A.R., Leech, J.M., Steinberg, J., Crawley, J.N., Regehr, W.G., Sahin, M., 2012. Autistic-like behaviour and cerebellar dysfunction in Purkinje cell Tsc1 mutant mice. Nature 488, 647–651. https://doi.org/10.1038/nature11310

Venerosi, A., Ricceri, L., Rungi, A., Sanghez, V., Calamandrei, G., 2010. Gestational exposure to the organophosphate chlorpyrifos alters social–emotional behaviour and impairs responsiveness to the serotonin transporter inhibitor fluvoxamine in mice. Psychopharmacology (Berl). 208, 99–107. https://doi.org/10.1007/s00213-009-1713-2

Venerosi, A., Ricceri, L., Scattoni, M.L., Calamandrei, G., 2009. Prenatal chlorpyrifos exposure alters motor behavior and ultrasonic vocalization in cd-1 mouse pups. Environ. Heal. 8, 12. https://doi.org/10.1186/1476-069X-8-12

WHO, 2022. Autism. URL https://www.who.int/news-room/fact-sheets/detail/autism-spectrum-disorders (accessed 8.19.22).

Whyatt, R.M., Rauh, V., Barr, D.B., Camann, D.E., Andrews, H.F., Garfinkel, R., Hoepner, L.A., Diaz, D., Dietrich, J., Reyes, A., Tang, D., Kinney, P.L., Perera, F.P., 2004. Prenatal Insecticide Exposures and Birth Weight and Length among an Urban Minority Cohort. Environ. Health Perspect. 112, 1125–1132. https://doi.org/10.1289/ehp.6641

Wirrell, E.C., 2003. Valproic acid-associated weight gain in older children and teens with epilepsy. Pediatr. Neurol. 28, 126–129. https://doi.org/10.1016/S0887-8994(02)00505-2

Yamauchi, T., Yoshioka, T., Yamada, D., Hamano, T., Ohashi, M., Matsumoto, M., Iio, K., Ikeda, M., Kamei, M., Otsuki, T., Sato, Y., Nii, K., Suzuki, M., Ichikawa, H., Nagase, H., Iriyama, S., Yoshizawa, K., Nishino, S., Miyazaki, S., Saitoh, A., 2022. Cold-Restraint Stress-Induced Ultrasonic Vocalization as a Novel Tool to Measure Anxiety in Mice. Biol. Pharm. Bull. 45, b21-00776. https://doi.org/10.1248/bpb.b21-00776

PUBLICATION III

4.3. Publication III

Prenatal, but not postnatal exposure to chlorpyrifos affects social behavior of mice and the excitatory-inhibitory balance in a sex-dependent manner

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

Food and Chemical Toxicology 2022; 169; 113423
https://doi.org/10.1016/j.fct.2022.113423

Study III overview

What do we already know?	CPF is a widely used OP in agriculture even though its uncontrolled used has had negative effects on human health. Specifically, neurodevelopmental disorders such as ASD have been associated with the massive use of CPF, as well as a consequent imbalance between excitatory and inhibitory neurotransmitters that could lead to inappropriate brain development.
What does the study add?	This study focuses on evaluating the effects of prenatal CPF exposure on social behavior at two developmental stages. In addition, it studies the glutamatergic and GABAergic systems in order to confirm the imbalance observed in ASD population.
Highlights	Social novelty preference was impaired in male mice exposed prenatally to CPF or VPA. Prenatal, but not postnatal exposure to CPF alters social behaviors. Male mice prenatally exposed to CPF or VPA showed altered gene expression in both the GABAergic and glutamatergic systems. The toxic effects of prenatal CPF exposure involve different pathways depending on sex.

* No edited version of the online article

Prenatal, but not postnatal exposure to chlorpyrifos affects behavior of mice and the excitatory-inhibitory balance in a sex-dependent manner

Judit Biosca-Brull [a,b,c], Laia Guardia-Escote [a,b], Jordi Blanco [a,c,d], Pia Basaure [a], Maria Cabré [a,e], Fernando Sánchez-Santed [f], José L. Domingo [c], Maria Teresa Colomina [a,b,c,*]

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

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

[c] Universitat Rovira i Virgili, Laboratory of Toxicology and Environmental Health (TECNATOX), Reus, Spain

[d] Universitat Rovira i Virgili, Department of Basic Medical Sciences, Reus, Spain

[e] Universitat Rovira I Virgili, Department of Biochemistry, Tarragona, Spain

[f] Department of Psychology, Health Research Center (CEINSA), Almeria University, 04120, Almeria, Spain

Abstract: The balance between excitatory and inhibitory neurotransmitters is essential for proper brain development. An imbalance between these two systems has been associated with neurodevelopmental disorders. On the other hand, literature also associates the massive use of pesticides with the increase of these disorders, with a particular focus on chlorpyrifos (CPF) a world-wide used organophosphate pesticide. This study was aimed at assessing social autistic-like behaviors on mice pre or postnatally exposed to CPF (0 or 1 mg/kg/day), in both sexes. In prenatal exposure, C57BL/6J pregnant mice were exposed to CPF through the diet, between gestational days (GD) 12 and 18, while a positive control group for some autistic behaviors was exposed to valproic acid (VPA) on GD 12 and 13. To assess postnatal exposure, C57BL/6J mice were orally exposed to the vehicle (corn oil) or CPF, from postnatal days (PND) 10–15. Social behavior and gene expression analysis were assessed on PND 45. Results showed social alterations only in males prenatally treated. GABA system was upregulated in CPF-treated females, whereas an increase in both systems was observed in both treated males. These findings suggest that males are more sensitive to prenatal CPF exposure, favoring the sex bias observed in ASD.

Keywords: Chlorpyrifos, Autism, GABA, Glutamate, Social behavior, Excitatory/inhibitory balance

1. Introduction

Pesticides have been used extensively in developed countries for both agriculture and residential uses, with consequential effects on non-target organisms such as humans (Karalliedde et al., 2003; Suratman et al., 2015). Exposure to pesticides can occur through different routes: ingestion (e.g., food contamination), inhalation (e.g., environmental and household pollution) and dermal absorption (e.g., farmers) (Kim et al., 2017).

Organophosphate (OP) pesticides, and specially chlorpyrifos (CPF), are widely used in agriculture to protect crops from insect attacks (Jokanovíc, 2001). However, there is a great deal of evidence demonstrating adverse health effects on humans leading to regulations in the United State, where agricultural and food uses were banned in 2022 (EPA, 2021). In turn, in 2020, the European Union did not continue renewing the authorization to use CPF (EFSA, 2019). However, several countries still use this pesticide, which will lead to negative effects on human health for years to come.

Bioactivation of CPF is produced by its oxidation to CPF-oxon (active metabolite) in the liver by cytochrome P450. Subsequently, CPF-oxon is hydrolyzed to diethylphosphate and 3,5,6-trichloro-2-pyridinol (TCPy) (Chambers and Chambers, 1989; Sams et al., 2004). The toxic effects of OP are related to the irreversible inhibition of the enzyme cholinesterase (ChE), which causes an overstimulation of the cholinergic system (Casida and Quistad, 2004; Flaskos, 2012). However, other targets have also been identified (Casida, 2017).

There is a growing body of literature that points towards the relationship between CPF exposure and the increase in neurodevelopmental disorders such as autism spectrum disorder (ASD), which is characterized by difficulties with social interaction and communication, as well as a high prevalence of repetitive patterns of behavior or stereotypes (Baird, 2003). The increased prevalence in ASD observed in the last few years has triggered many authors to hypothesize a gene-environment interaction involved in autism etiology (Havdahl et al., 2021; Waye and Cheng, 2018). Although existing data are not always consistent regarding CPF exposure and its relation to autism symptomatology, a systematic review recently conducted by our group (Biosca-Brull et al., 2021) informed consistent positive associations between OP exposure and neurodevelopmental disorders, including autism-related traits, in clinical studies. Moreover, in preclinical studies, exposure to low or high doses of CPF, during gestation or the first developmental stages, produce social deficits in the adulthood in a sex-dependent manner (Venerosi et al., 2006, 2015), while effects in earlier stages such as adolescence, are more heterogeneous (Ricceri et al., 2003; Venerosi et al., 2008). Besides, studies that evaluated both prenatal and postnatal exposure also reported short- and long-term impairments in motor and novelty-related response (Laporte et al., 2018). Nonetheless, Lan et al. (2019) reported that autism is a sexually dimorphic disorder, with a male bias inasmuch as for every four males diagnosed, only one female is affected.

The Three-Chamber test described by Crawley (2004) has been largely used to study social behavior and social recognition in rodents (Silverman et al., 2010). Brain regions such as amygdala, anterior cingulate cortex, medial prefrontal cortex, and hippocampus are critical for social memory formation and consolidation (Tanimizu et al., 2017). A considerable amount of literature has highlighted the CA2 hippocampal region as the principal area involved in social memory regulation (Garrido-Zinn et al., 2016; Hitti and Siegelbaum, 2014; Montagrin et al., 2018). Furthermore, it is suggested that an imbalance between excitatory and inhibitory neurotransmitters (glutamate and gamma-aminobutyric acid (GABA), respectively) is present in some neurodevelopmental disorders (Lopatina et al., 2018). GABA is synthesized from glutamate by glutamic acid decarboxylase (GAD). Then, vesicular GABA or glutamate transporters release their neurotransmitters to the synapses through exocytosis, leading GABA or glutamate to join their receptors (Rowley et al., 2012). Alterations in neurotransmitter balance, together with neurodevelopmental delays, were observed in the offspring of epileptic mothers treated with valproic acid (VPA) (Godhe-Puranik et al., 2013). Exposure to VPA during the first trimester, when organogenesis occurs, is associated with a high prevalence of ASD in offspring (Arndt et al., 2005). In mice and rats, organogenesis is developing between gestational day (GD) 8 and GD 15 (Ergaz et al., 2016). Prenatal VPA exposure (GD 11.5) showed deficits in GAD levels and a downregulation of the GABAergic system in rats (Win-Shwe et al., 2018), while glutamate expression levels were upregulated (Markram et al., 2008). On the other hand, post-mortem studies of autism showed a reduction in hippocampal $GABA_A$ receptor subunits and GAD (Bozzi et al., 2018). In the same line, developmental exposure to low and high doses of CPF was associated with an increase in the glutamate N-methyl-D-aspartate (NMDA) receptor, particularly, in the GluN2A and GluN2B receptor subunits) (Gómez-Giménez et al., 2018; Gultekin et al., 2007). In contrast, less is known about the effects of CPF in the GABA system. However, Perez-Fernandez et al. (2020a) found an upregulation of GABA-A α2 subunits in rats that were postnatally exposed to low doses of CPF, but no effects were observed in GAD1, GAD2 and GABA-A α1 subunit. Taking together all these results, it could be suggested that both CPF and VPA exposure impair GABAergic neurotransmission and enhance glutamatergic neurotransmission, leading to an altered excitatory/inhibitory (E/I) balance.

Some evidence points towards the contribution of CPF on neurodevelopmental disorders being the developmental period of exposure critical in producing different deficits. For this reason, the aims of this study were to evaluate the effects of prenatal and early postnatal exposure to CPF on social behavior, as well as to determine whether low pesticide exposure alters GABAergic and glutamatergic hippocampal signaling, while looking for a plausible association between CPF exposure and neurodevelopmental disorders.

2. Material and methods

2.1. Animals

Adult male and female C57BL/6J mice were obtained from Charles River Laboratories (Barcelona, Spain). One male and two females were mated for 3 h. When a vaginal plug was detected, this day was designated as GD 0. Animals were housed in plastic cages containing between three and five mice until GD 12, when pregnant females were housed individually. The delivery day was designated as postnatal day (PND) 0. Only litters with at least four live pups were used in this study. Pregnant females were randomly assigned to one of the three treatments (control (CNT), chlorpyrifos (CPF_1) or VPA) for the prenatal exposure experiment or to one of the two treatment groups (vehicle or CPF_2) for the postnatal exposure experiment. Animals were maintained in a 12-h light/dark automatic cycle (light ON between 8 a.m. and 8 p.m.) with controlled temperature (22 ± 2 ºC) and humidity (50 ± 10%). Food and water were administered *ad libitum*. The present study was assigned an authorization code (number 10735) by the Government of Catalonia. It was conducted in compliance with Spanish Royal Decree 53/2013 on the protection of animals used in experiments and the European Communities Council Directive (86/609/EEC) and was approved by the Animal Care and Use Committee of the Universitat Rovira i Virgili (Catalonia, Spain).

2.2. Treatment and experimental design

Two experiments were designed in order to assess the effects of low doses of CPF at two different developmental periods: late prenatal and early postnatal (Fig. 1). For the prenatal exposure experiment, pregnant mice were exposed through the diet to a 0 or 1 mg/kg/day dose of CPF (0,0-diethyl O-(3,5,6-trichloropyridin-2-yl) phosphorothioate) 99.5% purity) (Sigma-Aldrich, Madrid, Spain) between GD 12 and GD 18. The positive control group was based on a model of autism-like behaviors taken from previous literature (Sakai et al., 2018). For the autism model mice, pregnant females were treated with a dose of 300 mg/kg/day of VPA (2-propypentanoic acid sodium, purity 98%) (Sigma-Aldrich, Madrid, Spain) administered by subcutaneous injection for two consecutive days (GD 12 and GD 13). For the postnatal exposure experiment, CPF was dissolved in corn oil (vehicle) and adjusted to administer an oral dose of 1 mg/kg in 1 µL/g of body weight, using a micropipette. Treatment was administered to pups from PND 10 to PND 15. The control group received the vehicle during the same period.

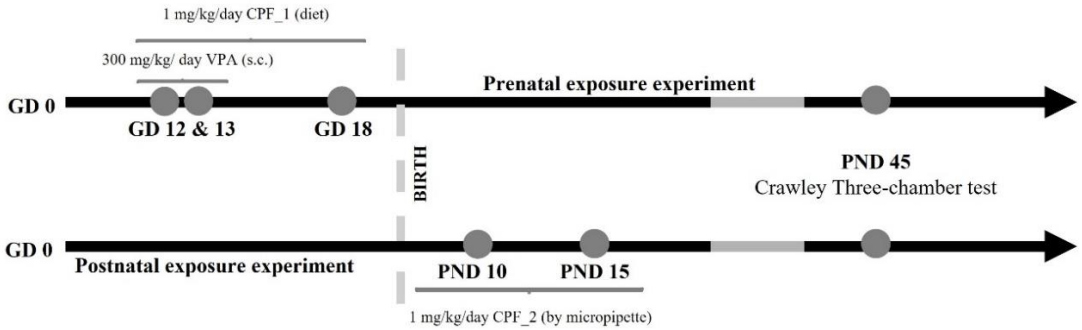


Fig. 1. Experimental design of both exposure periods. In the prenatal exposure experiment, pregnant females were exposed to CPF_1 (1 mg/kg/day) from GD 12 to GD 18, whereas in the positive control of autism, mice were exposed to VPA (300 mg/kg/day) on GD 12 and GD 13. In the postnatal exposure experiment, mice were exposed to the vehicle (corn oil) or CPF_2 (1 mg/kg/day) from PND 10 to PND 15. A social behavioral test was performed on PND 45.

In both cases, on PND 28 (weaning day), mice were separated in order to assess social behavior. Animals were housed in groups of two to four animals of the same sex per cage until behavioral test day (PND 45). On PND 46, animals were euthanized by exsanguination under isoflurane anesthesia, where brains were removed and stored at - 80 ºC until analysis. The number of mice used for each analysis is given in Table 1.

Table 1
Number of animals used in the study.

	Prenatal exposure			Postnatal exposure	
	CNT	CPF	VPA	CNT	CPF
Social behavior					
Males	15	10	15	10	10
Females	15	13	13	10	10
Gene expression					
Males	5	6	6	-	-
Females	6	6	5	-	-
Protein expression					
Males	4	5	6	-	-
Females	5	5	6	-	-

CNT-Control; CPF-Chlorpyrifos; VPA-Valproic acid

2.3. Behavioral assessment: three-chamber test

Sociability and preference for social novelty were evaluated in adolescent males and females on PND 45, using a nonautomated three-chamber Plexiglas box based on the Three-Chamber test described by Crawley (2004). Briefly, the apparatus consisted of a rectangular box (60 × 30 × 30 cm) with three interconnected chambers (20 × 30 × 30 cm). The two middle walls of the box had doorways allowing the mice to move between chambers. There were also two empty wire cups (7 × 7 cm) at both ends of the box. Before starting the test, the mice were transported to the testing room. Then, the animals were introduced into the space and allowed to explore freely for 10 min. Next, we assessed sociability by placing an inanimate object (i.e., red plastic frog, 2.5 × 2.5 cm) in one of the two cups (non-social chamber) and an unfamiliar mouse (same sex and age) in the other wire cup (social chamber). Finally, we evaluated the preference for social novelty by replacing the inanimate object with a novel unfamiliar mouse of same sex and age (novel chamber), while the first mouse was kept inside the same wire cup as the familiar mouse (non-novel chamber). In both, the social and novel phases, mice were also allowed to freely explore the three compartments for 6 min. Upon test completion, ethanol 70% was used to clean and eliminate olfactory cues. The time that the subject mouse spent in each of the three compartments was recorded using a video camera (Sony CCD-IRIS), and then computerized using a video-tracking program (Etho-Vision©, Noldus Information Technologies, Wageningen, The Netherlands) to evaluate the innate preference for the novel stimulus (i.e., the preference to explore a new mouse over a familiar one or an inanimate object) (Crawley, 2007). We also analyzed two social variables, which were represented by sociability ratio ((time spent in the social chamber-time in the non-social chamber)/total time exploring), and novelty ratio ((time spent in the novel chamber-time in the non-novel chamber)/total time exploring). "Total time exploring" refers to the sum of time the mice spent in the two lateral compartments.

2.4. Gene expression analysis

Hippocampal RNA was extracted using the SPEEDTOOLS Total RNA Extraction Kit (Biotools, Madrid, Spain). RNA concentration and purity were measured with a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was synthetized from 0.21 µg of RNA samples using a Maxima First Strand cDNA Kit for RT-qPCR (ThermoFisher Scientific, Waltham, MA, USA). Duplicates of each RNA sample were included in the quantitative real-time polymerase chain reaction (qPCR), which was performed with a Maxima SYBR Green/ROX qPCR Master Mix (2X) kit (ThermoFisher Scientifics, Waltham, MA, USA) and the Rotor-Gene Q Real-time Q cycler (Qiagen Inc., Hilden, Germany). A quantitative PCR analysis was performed to study the expression of glutamate- and GABA-related genes such as glutamate

decarboxylase 1 and 2 (*Gad1* and *Gad2*), vesicular GABA transporter (*Slc32a1*), glutamate ionotropic receptor NMDA type, subunit 2A (*Grin2a*) and 2B (*Grin2b*), $GABA_A$ receptor subunit alpha 1 (*Gabra1*), alpha 2 (*Gabra2*), alpha 5 (*Gabra5*) and beta 3 (*Gabrb3*), solute carrier family 12-member 5 (*Slc12a5*) and 2 (*Slc12a2*), parvalbumin (*Pvalb*) and matrix metallopeptidase 9 (*Mmp9*). Primer sequences are described in detail in Table 2. To calculate the cycle threshold (Ct) we used Rotor-Gene Q Real-Time PCR 2.0 software (Qiagen Inc., Hilden, Germany). Each sample was normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ΔCt) and then standardized to the average of each male control group (ΔΔCt) to assess the relative gene expression levels in accordance with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 2
Sequences of primers used for the qPCR analysis.

***Mus musculus* gene**	**Article Symbology**	**Forward primer**	**Reverse primers**	**Source**
Gad1	GAD1	ATGATACTTGGTGTGGCGTAG	GACTCTTCTCTTCCAGGCTATTG	Lee et al., 2017
Gad2	GAD2	CTCCGGCTTTTGGTCCTTCG	ATGCCGCCCGTGAACTTTTG	Lee et al., 2017
Slc32a1	VGAT	TCATCGAGCTGGTGATGACG	CTTGGACACGGCCTTGAGAT	Oka et al., 2015
Grin2a	GluN2A	CCATCAGCAGAGGTATCTAC	CAGTCTGAATGCGTGAAGCT	Chen et al., 2016
Grin2b	GluN2B	TCCAGGAGTAATGGCACTGTTTC	CGAACATCATCACCCAGACAG	Tsang et al., 2015
Gabra1	GABA-A α1	CACCATGAGGTTGACCGTGA	CTACAACCACTGAACGGGCT	Mitchell et al., 2018
Gabra2	GABA-A α2	TTACAGTCCAAGCCGAATGTCCC	ACTTCTGAGGTTGTGTAAGCGTAGC	Tan et al., 2011
Gabra5	GABA-A α5	CCCTCCTTGTCTTCTGTATTTCC	TGATGTTGTCATTGGTCTCGTCT	Tan et al., 2011
Gabrb3	GABA-A β3	GAGGTCTTCACAAGCTCAAAATC	AGGCAGGGTAATATTTCACTCAG	Provenzano et al., 2020
Slc12a5	KCC2	CTCAACAACCTGACGGACTG	GCACAACACCATTGGTT GCG	Aguado et al., 2003
Slc12a2	NKCC1	AACCGCTTCGTGGTTACATC	TTGCAAGTGATGCATGGAAT	Liu et al., 2014
Pvalb	PVALB	TGTCGATGACAGACGTGCTC	TTCTTCAACCCCAATCTTGC	Huo et al., 2018
Mmp9	MMP-9	ACCAAGGGTACAGCCTGTTCCT	GGTAGCTATACAGCGGGTACATGA	Kizaki et al., 2006

2.5. Western blot analysis

PVALB protein levels were analyzed by western blotting. Hippocampal samples containing 25 µg of protein/sample were mixed with RIPA buffer (Merck, Darmstadt, Germany). Samples were shaken for 40 min and then centrifuged for 15 min (4 ºC, 20.000 g). The proteins of the supernatant were separated by electrophoresis on 15% acrylamide gels. Then, proteins were transferred to Immobilon-P PVDF sheets (Millipore Corp., Bedford, MA, USA) using a transblot apparatus (BioRad, Madrid, Spain). Membranes were blocked for 1 h with 5% non-fat milk

dissolved in TBS-T buffer (50 mM Tris, 1.5% NaCl, 0.05% Tween 20 at pH 7.5). Primary monoclonal antibody (Cell Signaling, Danvers, MA, USA) against PVALB (molecular weight [MW]: 12 kDa) and GAPDH ([MW]: 37 KDa) was incubated overnight. Blots were washed thoroughly in TBS-T buffer and then incubated with peroxidase-conjugated IgG antibody for 1 h. An ImmunStar Chemiluminescence Kit (BioRad, Madrid, Spain) was used to visualize immunoreactive proteins. Digital images were obtained using the VersaDoc system (BioRad, Madrid, Spain) to perform semi-quantification of the band intensity (Image Lab, Bio-Rad, Madrid, Spain). The protein load was periodically monitored via the immune detection of GAPDH.

2.6. Statistical analysis

The data were analyzed using SPSS 27.0 software (IBM Corp. Chicago, IL, USA). General univariate and multivariate (body weight, sociability/novelty ratio and gene/protein expression) analyses of variance (ANOVA) were performed to screen general differences of sex, treatment or sex x treatment interaction. A one-way ANOVA followed by the *post-hoc* DMC test was used to analyze the differences between treatments or sex x treatment interactions when it was appropriate. Both social behaviors were evaluated with a paired samples *t*-test in order to find differences in the time spent in social/novel versus non-social/non-novel chambers. The one sample *t*-test was performed to analyze the sociability and novelty ratio. A principal component analysis (PCA) of ΔCt was carried out in gene expression as a general screening. Levene test was performed to assess the homogeneity of variance. Kruskal-Wallis or Mann-Whitney *U* test were performed when variances were not homogeneous. All data are presented as the mean values ± S.E.M. Statistical significance was set at a threshold of $p < 0.05$.

3. Results

3.1. Body weight

Body weight was measured in all animals during developmental period, from PND 2 to PND 28. Differences of treatment were observed in both exposure periods (prenatal exposure [$F_{7,70}$ = 2.602, $p \leq 0.002$] and postnatal exposure [$F_{9,16}$ = 3.061, p = 0.025]), being in the prenatal exposure the CNT group the ones that had higher body weight, whereas in postnatal exposure higher weight was observed in the CPF-treated group (data not shown). During development treated groups reach the value of controls and by PND 45, both pre or postnatal exposure had no effect on body weight. We observed that sex had a general effect on both periods of exposure (prenatal exposure

[$F_{1,66}$ = 73.991, p < 0.001] and postnatal exposure [$F_{1,30}$ = 90.799, p < 0.001]), indicating that females were leaner than males, as expected (data not shown).

3.2. Social behavior: sociability and preference for social novelty in the three-chamber test

Sociability and the preference for social novelty are shown as follows: **i.** The time that the animal spent in each compartment (social or novel vs non-social or non-novel) and **ii.** Sociability and novelty ratio, which was compared to the fixed value basal equal exploration (i.e., 0), indicating a social or novel preference when this ratio is positive and a non-social or non-novel preference when this ratio is negative (Figs. 2 and 3).

3.2.1. Prenatal exposure to CPF and VPA disrupts the novelty preference

Male mice's social memory was affected, particularly in that's treated with CPF or VPA. No adverse effects were observed during the sociability preference phase, with all groups displaying a significant preference for the social stimulus. A paired sample *t*-test analysis showed a significant preference for the social stimulus versus the inanimate object (CNT: [t_{29} = 6.642, p < 0.001], CPF_1: [t_{22} = 6.722, p < 0.001] and VPA: [t_{32} = 7.024, p < 0.001]) (Fig. 2A). Similar results were observed when we evaluated the sociability ratio by conducting one sample *t*-test analysis, which also showed significant differences compared to the basal exploration in all groups (CNT: [t_{29} = 6.662, p < 0.001], CPF_1: [t_{22} = 6.784, p < 0.001] and VPA: [t_{32} = 7.016, p < 0.001]), indicating a general preference for the social stimulus (Fig. 2B).

In contrast, adverse effects on the preference for the novel stimulus were observed in the novelty phase in some treated groups. Although we did not observe general significant differences related to sex [$F_{1,65}$ = 1.939, p = 0.151], a paired sample *t*-test analysis showed that males had a significant preference for the unfamiliar mouse, but only in the CNT groups [t_{14} = 2.230, p = 0.043], while females showed a significant preference for the novel stimulus in both the CNT [t_{14} = 3.599, p = 0.003] and CPF-treated group [t_{12} = 2.210, p = 0.047]. A tendency was observed in VPA-treated females [t_{14} = 2.006, p = 0.058] (Fig. 2C). Regarding the novelty ratio, no differences related to sex were observed [$F_{1,65}$ = 2.874, p = 0.094], even though the one sample *t*-test analysis indicated that males in the CNT group [t_{14} = 2.200, p = 0.045] showed a novel preference, whereas CPF_1 [t_{9} = 0.157, p = 0.879] and VPA-treated males [t_{17} = 0.129, p = 0.899] did not show a preference for the novel stimulus. Moreover, CNT females [t_{14} = 3.710, p = 0.002] also showed a preference for the unfamiliar mice, while in CPF_1 [t_{12} = 2.083, p = 0.059] and

VPA-treated females [t_{14} = 2.096, p = 0.055], the level of statistical significance was not reached (Fig. 2D).

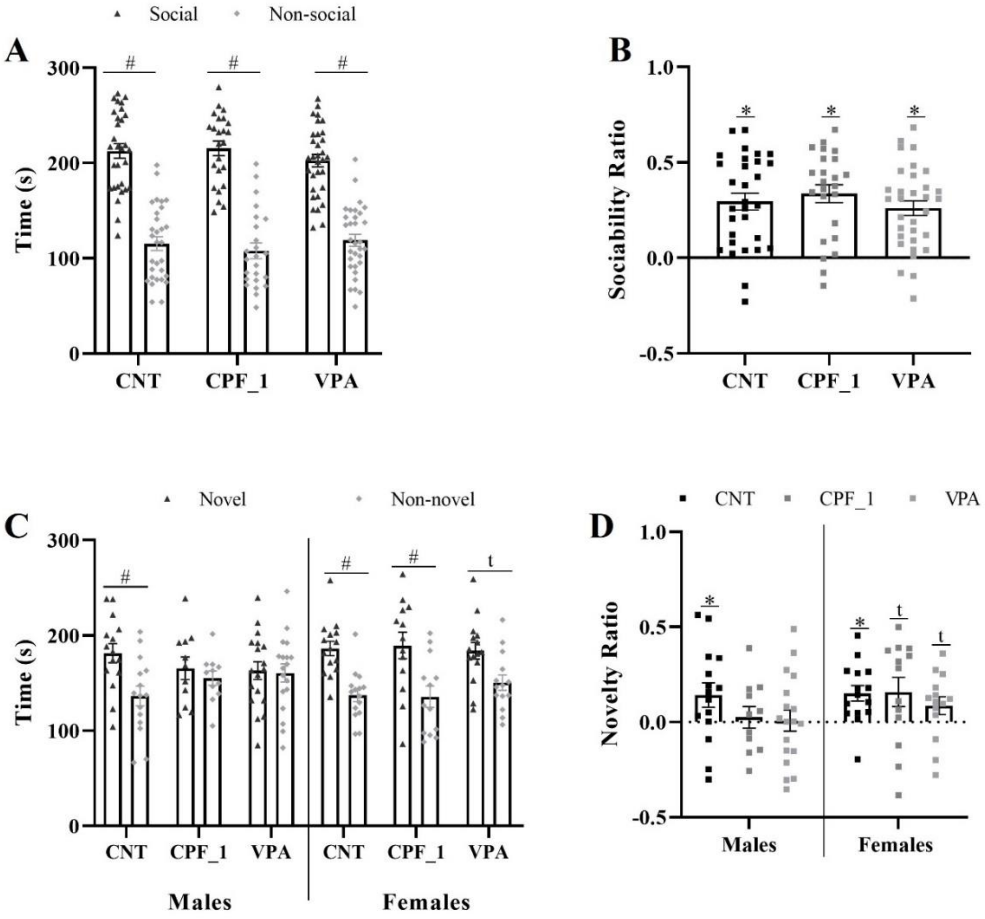


Fig. 2. Social behavioral assessment in prenatal exposure experiment. The time that the animal spends in the social or novel chamber versus the non-social or non-novel chamber (**A and C**). Sociability or novelty ratio calculated as (time spent in social or novel chamber-time spent in non-social or non-novel chamber)/total time exploring (**B and D**). The symbol # indicates the differences between the social or novel and non-social or non-novel chamber at $p < 0.05$. Differences with the chance level (i.e., 0) are represented by an asterisk, while trends are indicated with a t.

3.2.2. Postnatal exposure to CPF did not affect social behavior

Similar to prenatal exposure, all groups showed a preference for the social stimulus, but no significant effects were observed in the social novelty preference phase. A paired sample *t*-test analysis showed significant preference for the social chamber in both vehicle [t_{19} = 6.294, $p <$ 0.001] and CPF-treated groups [t_{19} = 6.631, $p < 0.001$] when the time that the animals spent in the social versus non-social chamber was compared (Fig. 3A). Moreover, the analysis of

sociability ratio by one sample *t*-test also showed significant preference for the social compartment in both the vehicle [$t_{19} = 6.442$, $p < 0.001$] and CPF-treated groups [$t_{19} = 6.640$, $p < 0.001$]) (Fig. 3B), confirming that the treatment had no effect on social preference.

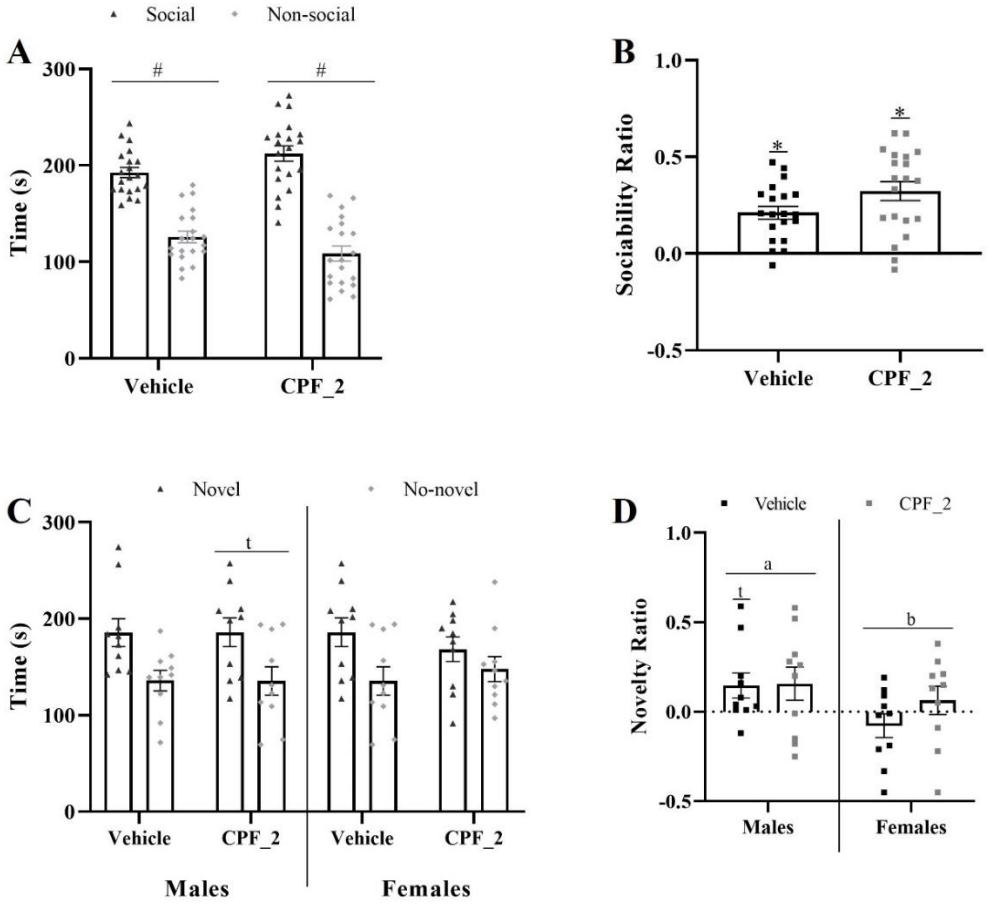


Fig. 3. Social behavioral assessment in postnatal exposure experiment. The time that the animal spends in the social or novel chamber versus the non-social or non-novel chamber (**A and C**). Sociability or novelty ratio calculated as (time spent in social or novel chamber-time spent in non-social or non-novel chamber)/total time exploring (**B and D**). The symbol # indicates the differences between the social or novel and non-social or non-novel chamber, while different letters indicate differences between sexes at $p < 0.05$. Differences with the chance level (i.e., 0) are represented by an asterisk, while trends are indicated with a t.

Regarding the novelty phase, a non-significant effect of sex was observed [$F_{2,35} = 3.139$, $p = 0.056$]. When we analyzed the time that the animal spent in the novel versus the non-novel compartment using a paired sample *t*-test, we saw a tendency for the unfamiliar mouse in CPF-treated males [$t_9 = 1.711$, $p = 0.061$], whereas others groups did not show any preference (vehicle-treated males [$t_7 = 1.458$, $p = 0.094$], vehicle-treated females [$t_8 = -0.662$, $p = 0.263$] and

CPF-treated females [t_9 = 0.811, p = 0.219]) (Fig. 3C). In the same regard, when we analyzed the novelty ratio, we observed a significant effect of sex [$F_{1,39}$ = 4.185, p = 0.048] indicating that male mice had a greater preference for the novel stimulus in comparison to female. A one sample *t*-test showed a tendency in vehicle-treated males [t_9 = 2.106, p = 0.064], while other groups did not present any preference for the novel stimulus (vehicle-treated females [t_9 = - 1.179, p = 0.268], CPF-treated males [t_9 = 1.706, p = 0.122] and CPF-treated females [t_9 = 0.823, p = 0.432]) (Fig. 3D).

3.3. Hippocampal gene expression

Prenatal, but not postnatal exposure to CPF, disrupted social behavior, especially in treated male mice. Therefore, we assessed hippocampal gene expression only in the prenatal exposure experiment.

First, we performed a screening of all evaluated genes with a PCA (Fig. 4). The results showed three main components. Principal component (PC) 1 accounted for about 49.05% of the variance and included genes related to the GABAergic system such as GAD1 (r = 0.866), GAD2 (r = 0.655), VGAT (r = 0.804), NKCC1 (r = 0.792), KCC2 (r = 0.811), GABA-A α1 (r = 0.905) and PVALB (r = 0.565). PC 2 accounted for about 22.82% of the variance, where GABA-A α2 (r = 0.536), GABA-A α5 (r = 0.788), GABA-A β3 (r = 0.552), GluN2A (r = 0.536) and GluN2B (r = 0.560) were strongly correlated. Finally, PC 3 accounted for about 11.64% of the variance, with MMP9 (r = - 0.882) being the only gene in this component. It is important to highlight that gene clustering in the same PC shares a similar gene expression pattern.

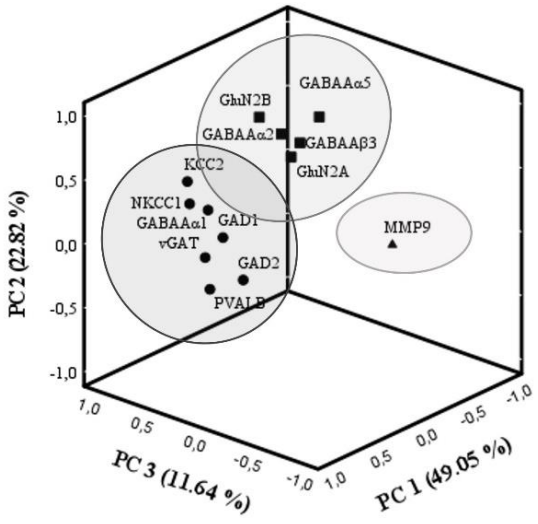


Fig. 4. Principal component analysis (PCA) of hippocampal GABA- and glutamate-related genes in adolescent mice prenatally exposed to CPF and VPA.

3.3.1. Genes clustered in PC1

Prenatal exposure to CPF modulates RNA expression in treated females (Fig. 5). All GABAergic-related genes clustered in PC 1 (GAD1, GAD2, VGAT, NKCC1, KCC2, GABA-A α1 and PVALB) are part of the GABA components in synapses, which leads to a phasic response. A Kruskal-Wallis test indicated that CPF-treated females showed an increase in GAD1 expression in comparison to CNT males (p = 0.034), CPF-treated males (p = 0.037) and VPA-treated females (p = 0.011), whereas a non-clear increase was observed with respect to CNT females (p = 0.072) (Fig. 5A). On the other hand, a multivariate analysis of all the genes clustered in PC 1 only showed an interaction between sex and treatment [$F_{2,33}$ = 3.832, p = 0.034] in GABA-A α1 subunit. *Post-hoc* analysis indicated a non-significant increase in females, between CNT and CPF (p = 0.092), as well as a tendency between both treated groups (p = 0.056) (Fig. 5F).

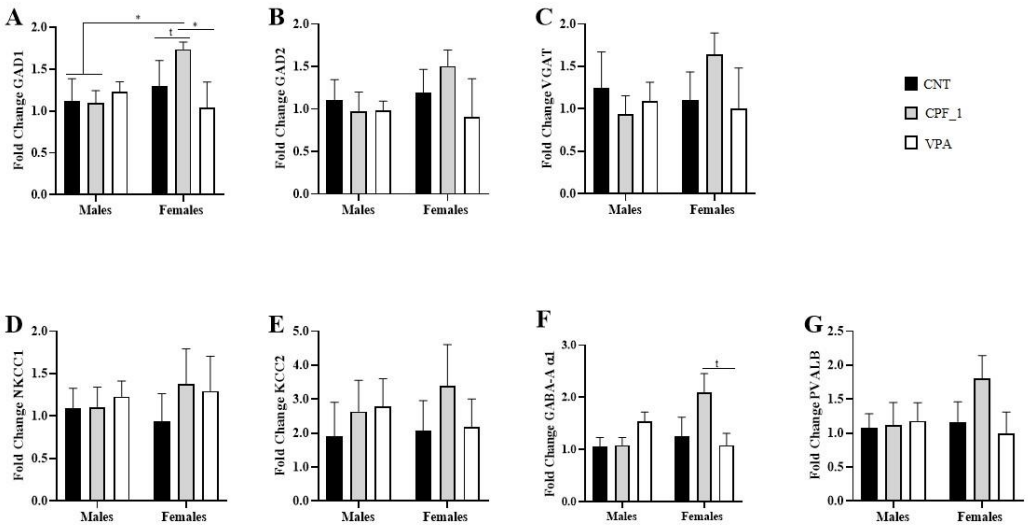


Fig. 5. Hippocampal relative gene expression related to PC 1 of the PCA. GAD1 (**A**). GAD2 (**B**). VGAT (**C**). NKCC1 (**D**). KCC2 (**E**). GABA-A α1 (**F**) and PVALB (**G**). An asterisk indicates differences between treatments at $p < 0.05$, while tendencies are indicated with a t.

3.3.2. Genes clustered in PC2

GABAergic- (GABA-A α2, GABA-A α5 and GABA-A β3 subunits) and glutamatergic- (GluN2A and GluN2B) related genes were clustered in PC 2 (Fig. 6). The composition of ionotropic GABA receptors establishes their distribution and regulation. In PC 2, we found an extrasynaptic subunit (i.e., GABA-A α5) that leads to tonic activation; a postsynaptic and presynaptic subunit (GABA-A α2) that, respectively, leads to phasic activation or acts as controllers of GABA release (autoreceptors), and a GABA-A β3 subunit, which is located in postsynapse and extrasynapse.

Moreover, the ionotropic glutamatergic (NMDA) receptor subunits, which were mainly postsynaptic receptors, were also clustered in PC 2.

We observed a general increase in treated males of all genes clustered in PC 2 (Fig. 6). Regarding $GABA_A$ subunits, a multivariate analysis of all genes clustered in this component showed a general trend of treatment in the GABA-A α2 [$F_{2,33} = 3.316$, $p = 0.051$] and GABA-A β3 subunits [$F_{2,33} = 3.170$, $p = 0.057$]. Moreover, we observed an interaction between sex and treatment in the GABA-A β3 subunit [$F_{2,33} = 3.877$, $p = 0.033$], whereas GABA-A α2 showed a tendency [$F_{2,33} = 3.237$, $p = 0.054$]. In both GABA subunits, *post-hoc* analysis showed significant differences in males, between CNT and both treated groups (GABA-A α2 subunit: CPF_1 ($p = 0.024$) and VPA ($p = 0.004$) and GABA-A β3 subunit: CPF_1 ($p = 0.011$) and VPA ($p = 0.007$)) (Fig. 6A and C).

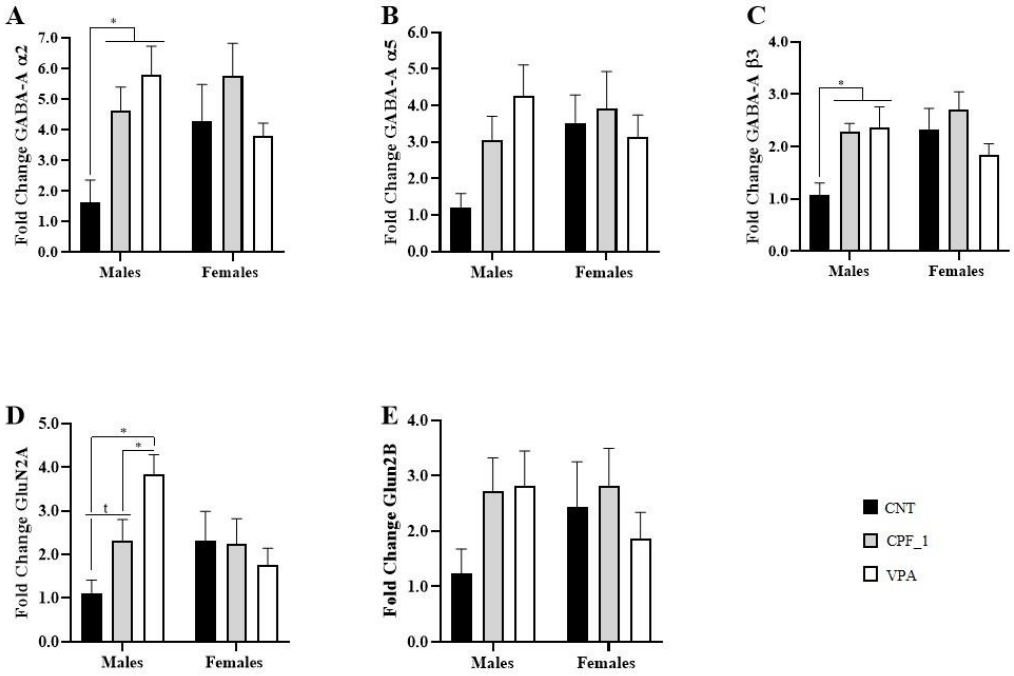


Fig. 6. Hippocampal relative gene expression related to PC 2 of the PCA. GABA-A α2 (**A**). GABA-A α5 (**B**). GABA-A β3 (**C**). GluN2A (**D**). GluN2B (**E**). An asterisk indicates differences between treatments at $p < 0.05$, while tendencies are indicated with a t.

However, in terms of glutamatergic expression, we only observed an interaction between sex and treatment [$F_{2,33} = 4.962$, $p = 0.014$] in the GluN2A subunit (Fig. 6D). *Post-hoc* analysis showed significant differences in males between VPA and CNT ($p = 0.001$), VPA and CPF_1 ($p = 0.023$), as well as a tendency between CNT and CPF_1 ($p = 0.079$) (Fig. 6D).

3.3.3. Genes clustered in PC3

The MMP9, a protease that mediates extracellular matrix degradation, was the only gene that clustered in PC 3. Although, results showed an upregulation of this gene, we did not observe significant differences due to their high variability (data not shown).

3.4. Hippocampal PVALB protein expression

Previous gene expression analysis showed an increase in GABAergic interneurons and synapse elements, but only in CPF-treated females. Intending to find a possible correlation between GABA upregulation and GABA neurons, we evaluated PVALB protein levels (Fig. 7). However, we did not find significant differences.

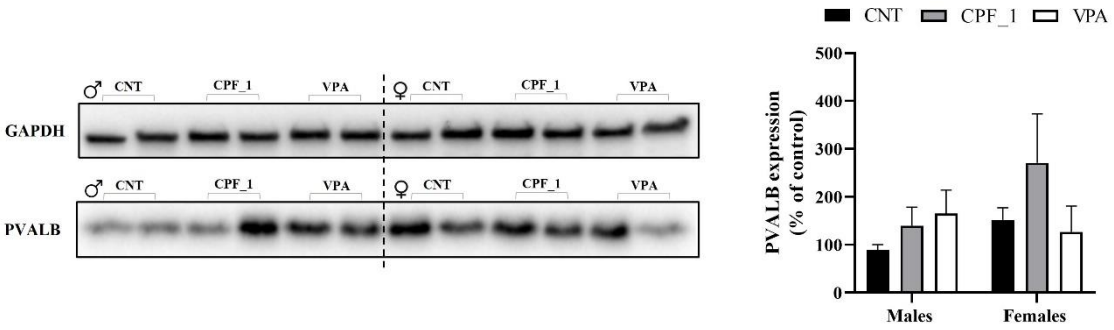


Fig. 7. Hippocampal relative PVALB protein expression.

4. Discussion

In the present investigation, we assessed the effects that low doses of CPF can have on social behavior when administered over different lifetime periods (prenatal or postnatal), as well as the effects of CPF on GABAergic and glutamatergic synapses by studying the gene expression, looking for an association between prenatal CPF exposure and neurodevelopmental disorders. For this reason, we also included mice prenatally exposed to VPA, as a mice autism model.

Adolescent mice did not show deficits in sociability in either experiment (prenatal or postnatal), but differences were observed when we evaluated the preference for the novelty phase. Males treated during prenatal period with CPF or VPA did not show a preference for the novel stimulus. Similarly, Lan et al. (2019) exposed mice between GD 12 and GD 15 to subtoxic doses of CPF ranging from 2 to 5 mg/kg/day. Then, they assessed their social behavior by conducting a Three-Chamber test in adult mice (PND 90), where only the males showed a reduction in the innate preference for a

conspecific (Lan et al., 2019). This suggests that prenatal exposure to CPF can disclose social behavior in the early stages of development which may persist into adulthood. Nevertheless, it should be borne in mind that our experiment was conducted with low doses of CPF according to the European Food Safety Authority (EFSA), which has established an acceptable daily intake of 0.001 mg/kg/day, whereas Lan et al. (2019) exposed mice to higher doses that cause adverse effects that perpetuate over time. On the other hand, a study conducted by Perez-Fernandez et al. (2020a) assessed postnatal exposure to CPF in rats by using the same protocol as our postnatal experiment and by evaluating social behavior at two different ages (adolescence and adulthood). In accordance with our results, Perez-Fernandez et al. (2020a) did not find social impairment on PND 35 (adolescence), but did observe differences during adulthood (around 5 months of age) where treated male rats reduced their novelty exploration. However, it must be noted that the difference between the two species in postnatal experiments could generate discrepancies in the results. These findings indicate that the CPF exposure period could condition adverse effects, suggesting that prenatal exposure could affect social behavior from an early stage, whereas postnatal exposure could disclose long-term social deficits. Anyway, further studies are needed to confirm this hypothesis. Nevertheless, it is clear that CPF exposure affects rodents' social behavior in a sex-dependent manner, with males being the most affected sex, in accordance with the incidence of autism in humans.

Behavioral outcomes observed in CPF- and VPA-treated males are related to nervous system development. Prenatal exposure takes place from GD 12 to GD 18. This timeframe refers to humans' second and third trimester of pregnancy (Azad et al., 2017) and encompasses the onset of neurogenesis, which starts at embryonic day 11 and ends before adolescence (around PND 20). Each brain region has a stage of neuron production. For example, in the cerebral cortex, neurogenesis occurs from GD 12 to GD 17, while in the hippocampus, neuronal production occurs in two stages; between GD 12 and GD 17 (neurogenesis of the CA2 region and pyramidal cells) and from GD 17 to PND 15 (neurogenesis of dentate gyrus) (Chen et al., 2017). Furthermore, this prenatal exposure encompasses other key brain developmental processes such as synaptogenesis or neuronal migration, which begin around GD 11 (Chen et al., 2017). Postnatal exposure starts on PND 10 and ends on PND 15, the equivalent to human brain development between the third trimester and the first month of age (Richard and Flamant, 2018). In mice, this timeframe covers the late stages of neuronal migration and synaptogenesis, in addition to developmental brain processes such as gliogenesis or myelination (Schepanski et al., 2018).

A great number of cases of developmental disorders, including autism, lack etiology or genetic basis. Recent biological evidence suggests that the autistic population presents alterations in the E/I balance, and more specifically, their glutamatergic system is upregulated, whereas their GABAergic system is downregulated (Uzunova et al., 2016). To find a plausible association between pesticides

and autism, we evaluated GABA- and glutamate-related genes only during the prenatal exposure experiment because this is where we found evidence for deficits in social behavior.

The GABA neurotransmitter has different important functions during development. It is involved in neuronal migration, neuronal differentiation, and synapse formation (Ben-Ari, 2002). To the best of our knowledge, this is the first time that a study has covered the hippocampal expression of a large variety of GABAergic genes in relation to CPF exposure and the evaluation of behavior related to autism. Although general upregulation of GABAergic synaptic genes was observed in CPF-treated females, only the GAD1 and GABA-A α1 subunit showed significant differences. GAD1 and GAD2 are the major rate-limiting enzymes that regulate GABA synthesis from a pool of L-glutamate (Tao et al., 2018). In contrast to our results, postmortem studies carried out on humans with autism showed a downregulation of both genes in the frontal cortex (Zhubi et al., 2017) and cerebellum (Fatemi et al., 2002), indicating a deficiency in GABA availability. GAD1 is located in interneurons and cytoplasm and is present during development in order to maintain metabolic activity, whereas GAD2 could be more involved in the vesicular synthesis of GABA and it seems to be present when synaptic inhibition is frequent (mature nervous system) (Fatemi et al., 2002). Thus, the overexpression of the inhibitory system observed in the current study suggests that prenatal CPF exposure increases the production of GABA neurotransmitter in females. In addition, prior to neuron maturation, GABA is excitatory and exerts a depolarizing action related to a high expression of neuronal co-transporter NKCC1. In adult neurons, GABA switches from excitatory to inhibitory action and, consequently, GABAergic neurons exert a hyperpolarizing action producing an upregulation of the co-transporter KCC2 and a downregulation of NKCC1 (Lemonnier et al., 2017). This switch from excitatory to inhibitory GABA transmission during the second week following birth coincides with the increase in cholinergic activity, which builds to adult levels during the third week following birth (Abreu-Villaça et al., 2011; Leonzino et al., 2016). Considering that ChE is the main target for CPF, the current study suggests that CPF exposure may interfere with GABA switching on the central nervous system. GABA's E/I effects are mediated by GABA-A receptors, whose expression varies over the course of development (Kang and Barnes, 2013). In our study, GABA-A α1-containing receptors were upregulated in CPF-treated females. These receptors are predominant in synapsis, giving rise to a phasic response which is fundamental for information transfer in the brain (Farrant and Nusser, 2005). The GABA-A α1 subunit was decreased in the parietal cortex (Fatemi et al., 2009) and cerebellum (Yip et al., 2007) of the autistic population, but little is known about its expression in the hippocampus. Therefore, the results obtained from synaptic GABAergic gene expression, suggest that CPF acts in a sex-dependent manner. CPF exposure increases GAD1 expression in females, suggesting an overproduction of the GABA neurotransmitter which is related to the increase observed in the GABA-A α1 subunit. Therefore, this indicates an increment in inhibitory neuronal activity, maybe because of an increase in GABAergic neurons.

Another gene expression pattern was observed in CPF- and VPA-treated males. Glutamatergic receptor subunits (GluN2A and GluN2B) and GABAergic receptor subunits (GABA-A α2, GABA-A α5 and GABA-A β3) were significantly increased compared to control males. Studies of GABA-A receptor subunits in rats brains showed that α2, α3, α5 and β3 predominate during embryonic development, while α1 and β2 increase after birth (Laurie et al., 1992). Similarly, during postnatal development, the GABA-A receptors (α1, α4, β1 and β2 subunits) increase in the mice's hippocampus (Kim et al., 2015). The GABA-A α2 subunit is present in axon terminals of the hippocampus (pre-synapsis), acting as an autoreceptor that regulates the release of GABA in the synapse (Ruiz and Kullmann, 2013), even though some postsynaptic α2 subunits were also found (Farrant and Nusser, 2005). In addition, the GABA-A α5 subunit is predominantly or exclusively extra-synaptic, giving rise to a tonic response which is evident in certain embryonic regions before synaptic formation (Farrant and Nusser, 2005), while the GABA-A β3 subunit could be postsynaptic and extra-synaptic (Herd et al., 2008). The GABA-A α2 subunit has been associated with disorders of altered neuronal excitability such as epilepsy (Butler et al., 2018), with which ASD shares neurological mechanisms. In relation to this, Perez-Fernandez et al. (2020b) found a significant upregulation of the α2 subunit in the frontal cortex of rats exposed postnatally to CPF. In the same lines, GABA-A α5 and GABA-A β3 subunits are clustered in chromosomes 7 and 15 (mouse and humans, respectively) (Kang and Barnes, 2013). Defects in that part of the genome are associated with Prader-Willi and Angelman syndrome, two disorders that share symptoms with autism, making both subunits important candidates for neurodevelopmental disorders. Therefore, as we explained above, the upregulation in the treated males of the three GABA subunits (α2, α5 and β3) would suggest that CPF and VPA affect the GABA system similarly during early developmental stages. Indeed, prenatal CPF exposure affects GABAergic neurotransmission in both sexes, but effects do not point in the same direction.

Glutamate ionotropic NMDA receptors, GluN2A and GluN2B subunits, have a similar molecular structure and function (Myers et al., 2019). These subunits appear at different stages during development. GluN2B is expressed early, whereas the GluN2A subunit is expressed postnatally (Myers et al., 2019). These subunits have been studied concerning social behavior. In this sense, Jacobs et al. (2015) found that mice's forebrain GluN2A overexpression impairs social memory, while overexpression of the GluN2B subunit enhances social memory, suggesting that GluN2B is crucial for social memory formation and consolidation. In this regard, we observed a significant upregulation in the GluN2A subunit (CPF and VPA treated male mice), which could be related to deficits in the preference for novelty that we observed in our experiment. Nevertheless, we did not find any differences in the GluN2B subunit. The increase in GluN2A may suggest an imbalance between these two subunits, leading to an E/I disequilibrium, which could be a consequence of a delay or incorrect switch during development.

Regarding the GABA system, we also evaluated the expression of PVALB to test a possible increase in GABAergic interneurons which had been involved in the consolidation of short- and long-term memory formation (Nahar et al., 2021). In fact, it has been suggested that PVALB coordinates neuronal communication after novel experiences (Ognjanovski et al., 2017). Furthermore, the activity of excitatory neurons is modulated by these inhibitory interneurons, which control postsynaptic activation (Filice et al., 2020). Studies done in postmortem brains of humans with autism observed a decrease in PVALB mRNA levels in cerebellar Purkinje cells (Soghomonian et al., 2017) and the cerebral cortex (Schwede et al., 2018). In the same way, mouse models expressing an autism phenotype, showed a decrease in PVALB cells in the hippocampus, striatum and cortex, which is related to PVALB downregulation (Peñagarikano et al., 2015). Moreover, Lauber et al. (2018) found a decrease in PVALB protein expression, only in the striatum. Despite this, our gene expression analysis did not show significant differences in both PVALB expression and protein levels. However, a non-significant increase in CPF-treated females was observed, suggesting that CPF enhances the inhibitory system in females, whereas, in male's treatment seems to not affect PVALB gene and protein expression. These findings indicate that CPF's toxic effects involve different pathways in males and females.

In conclusion, the results of the current study show that the period in which exposure to CPF takes place plays a key role in determining the adverse effects produced in the short, medium or long term. Our results also suggest that CPF exposure during pregnancy could lead to social behavioral deficits in a sex-dependent manner. The same-sex bias was also observed in gene expression, suggesting that females treated with CPF presented an upregulation in the GABAergic system, whereas treated males showed a more immature pattern in both the GABA and glutamate systems, which could be compatible with an increase in neuronal excitability.

Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability: Data will be made available on request.

Acknowledgments: The authors would like to thank Dr Celeste di Paolo, Esperanza Chernichero and Juan Valencia for their technical support in caring for the animals. Likewise, the authors would also like to thank Séfora Barberà Parada for her support in the development of western blots. This research was funded by the Martí Franquès Grant Program-Doctoral modality (Reference: 2019PMF-PIPF-28) and the Spanish Government (Ministry of the Economy and Competitiveness (MINECO, Spain)) (Reference: PSI 2017- 86847-C2-R).

References

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

Aguado, F., Carmona, M.A., Pozas, E., Aguiló, A., Martínez-Guijarro, F.J., Alcantara, S., Borrell, V., Yuste, R., Ibañez, C.F., Soriano, E., 2003. BDNF regulates spontaneous correlated activity at early developmental stages by increasing synaptogenesis and expression of the K+/Cl- co-transporter KCC2. Development 130, 1267–1280. https://doi.org/10.1242/dev.00351.

Arndt, T.L., Stodgell, C.J., Rodier, P.M., 2005. The teratology of autism. Int. J. Dev. Neurosci. 23, 189–199. https://doi.org/10.1016/j.ijdevneu.2004.11.001.

Azad, M.B., Moyce, B.L., Guillemette, L., Pascoe, C.D., Wicklow, B., McGavock, J.M., Halayko, A.J., Dolinsky, V.W., 2017. Diabetes in pregnancy and lung health in offspring: developmental origins of respiratory disease. Paediatr. Respir. Rev. 21, 19–26. https://doi.org/10.1016/j.prrv.2016.08.007.

Baird, G., 2003. Diagnosis of autism. BMJ 327, 488–493. https://doi.org/10.1136/ bmj.327.7413.488.

Ben-Ari, Y., 2002. Excitatory actions of gaba during development: the nature of the nurture. Nat. Rev. Neurosci. 3, 728–739. https://doi.org/10.1038/nrn920.

Biosca-Brull, J., Pérez-Fernández, C., Mora, S., Carrillo, B., Pinos, H., Conejo, N.M., Collado, P., Arias, J.L., Martín-Sánchez, F., Sánchez-Santed, F., Colomina, M.T., 2021. Relationship between autism spectrum disorder and pesticides: a systematic review of human and preclinical models. Int. J. Environ. Res. Publ. Health 18, 5190. https://doi.org/10.3390/ijerph18105190.

Bozzi, Y., Provenzano, G., Casarosa, S., 2018. Neurobiological bases of autism-epilepsy comorbidity: a focus on excitation/inhibition imbalance. Eur. J. Neurosci. 47, 534–548. https://doi.org/10.1111/ejn.13595.

Butler, K.M., Moody, O.A., Schuler, E., Coryell, J., Alexander, J.J., Jenkins, A., Escayg, A., 2018. De novo variants in GABRA2 and GABRA5 alter receptor function and contribute to early-onset epilepsy. Brain 141, 2392–2405. https://doi.org/ 10.1093/brain/awy171.

Casida, J.E., 2017. Organophosphorus xenobiotic toxicology. Annu. Rev. Pharmacol. Toxicol. 57, 309–327. https://doi.org/10.1146/annurev-pharmtox-010716-104926.

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

Chambers, J.E., Chambers, H.W., 1989. Oxidative desulfuration of chlorpyrifos, chlorpyrifos-methyl, and leptophos by rat brain and liver. J. Biochem. Toxicol. 4, 201–203. https://doi.org/10.1002/jbt.2570040310.

Chen, T., Zhang, B., Li, G., Chen, L., Chen, L., 2016. Simvastatin enhances NMDA receptor GluN2B expression and phosphorylation of GluN2B and GluN2A through increased histone acetylation and Src signaling in hippocampal CA1 neurons. Neuropharmacology 107, 411–421. https://doi.org/10.1016/j. neuropharm.2016.03.028.

Chen, V.S., Morrison, J.P., Southwell, M.F., Foley, J.F., Bolon, B., Elmore, S.A., 2017. Histology atlas of the developing prenatal and postnatal mouse central nervous system, with emphasis on prenatal days E7.5 to E18.5. Toxicol. Pathol. 45, 705–744. https://doi.org/10.1177/0192623317728134.

Crawley, J.N., 2007. Mouse behavioral assays relevant to the symptoms of autism. Brain Pathol. 17, 448–459. https://doi.org/10.1111/j.1750-3639.2007.00096.x.

Crawley, J.N., 2004. Designing mouse behavioral tasks relevant to autistic-like behaviors. Ment. Retard. Dev. Disabil. Res. Rev. 10, 248–258. https://doi.org/ 10.1002/mrdd.20039.

EFSA, 2019. Statement on the available outcomes of the human health assessment in the context of the pesticides peer review of the active substance chlorpyrifos. EFSA J. 17. https://doi.org/10.2903/j.efsa.2019.5809.

EPA, 2021. Chlorpyrifos; tolerance revocations. Fed. Regist./No. 165/Monday, August 30, 2021/Rules Regul. URL. https://www.epa. gov/ingredients-used-pesticide-products/chlorpyrifos#actions, 86.

Ergaz, Z., Weinstein-Fudim, L., Ornoy, A., 2016. Genetic and non-genetic animal models for autism spectrum disorders (ASD). Reprod. Toxicol. 64, 116–140. https://doi.org/ 10.1016/j.reprotox.2016.04.024.

Farrant, M., Nusser, Z., 2005. Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors. Nat. Rev. Neurosci. 6, 215–229. https://doi.org/ 10.1038/nrn1625.

Fatemi, S.H., Halt, A.R., Stary, J.M., Kanodia, R., Schulz, S.C., Realmuto, G.R., 2002. Glutamic acid decarboxylase 65 and 67 kDa proteins are reduced in autistic parietal and cerebellar cortices. Biol. Psychiatr. 52, 805–810. https://doi.org/10.1016/ S0006-3223(02)01430-0.

Fatemi, S.H., Reutiman, T.J., Folsom, T.D., Thuras, P.D., 2009. $GABA_A$ receptor downregulation in brains of subjects with autism. J. Autism Dev. Disord. 39, 223–230. https://doi.org/10.1007/s10803-008-0646-7.

Filice, F., Janickova, L., Henzi, T., Bilella, A., Schwaller, B., 2020. The parvalbumin hypothesis of autism spectrum disorder. Front. Cell. Neurosci. 14, 1–24. https://doi. org/10.3389/fncel.2020.577525.

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

Garrido Zinn, C., Clairis, N., Silva Cavalcante, L.E., Furini, C.R.G., de Carvalho Myskiw, J., Izquierdo, I., 2016. Major neurotransmitter systems in dorsal hippocampus and basolateral amygdala control social recognition memory. Proc. Natl. Acad. Sci. USA 113, E4914–E4919. https://doi.org/10.1073/ pnas.1609883113.

Godhe-Puranik, Y., Thorn, C.F., Lambra, J.K., Leeder, J.S., 2013. Valproic acid pathway: pharmacokinetics and pharmacodynamics. Pharmacogenetics Genom. 23, 236–241. https://doi.org/10.1097/FPC.0b013e32835ea0b2.Valproic.

Gómez-Giménez, B., Felipo, V., Cabrera-Pastor, A., Agustí, A., Hernández-Rabaza, V., Llansola, M., 2018. Developmental exposure to pesticides alters motor activity and coordination in rats: sex differences and underlying mechanisms. Neurotox. Res. 33, 247–258. https://doi.org/10.1007/s12640-017-9823-9.

Gultekin, F., Karakoyun, I., Sutcu, R., Savik, E., Cesur, G., Orhan, H., Delibas, N., 2007. Chlorpyrifos increases the levels of hippocampal NMDA receptor subunits NR2A and NR2B in juvenile and adult rats. Int. J. Neurosci. 117, 47–62. https://doi.org/ 10.1080/00207450500535719.

Havdahl, A., Niarchou, M., Starnawska, A., Uddin, M., van der Merwe, C., Warrier, V., 2021. Genetic contributions to autism spectrum disorder. Psychol. Med. 51, 2260–2273. https://doi.org/10.1017/S0033291721000192.

Herd, M.B., Haythornthwaite, A.R., Rosahl, T.W., Wafford, K.A., Homanics, G.E., Lambert, J.J., Belelli, D., 2008. The expression of GABA A β subunit isoforms in synaptic and extrasynaptic receptor populations of mouse dentate gyrus granule cells. J. Physiol. 586, 989–1004. https://doi.org/10.1113/jphysiol.2007.146746.

Hitti, F.L., Siegelbaum, S.A., 2014. The hippocampal CA2 region is essential for social memory. Nature 508, 88–92. https://doi.org/10.1038/nature13028.

Huo, C., Liu, X., Zhao, J., Zhao, T., Huang, H., Ye, H., 2018. Abnormalities in behaviour, histology and prefrontal cortical gene expression profiles relevant to schizophrenia in embryonic day 17 MAM-Exposed C57BL/6 mice. Neuropharmacology 140, 287–301. https://doi.org/10.1016/j.neuropharm.2018.07.030.

Jacobs, S., Wei, W., Wang, D., Tsien, J.Z., 2015. Importance of the GluN2B carboxy-terminal domain for enhancement of social memories. Learn. Mem. 22, 401–410. https://doi.org/10.1101/lm.038521.115.

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

Kang, J.-Q., Barnes, G., 2013. A common susceptibility factor of both autism and epilepsy: functional deficiency of $GABA_A$ receptors. J. Autism Dev. Disord. 43, 68–79. https://doi.org/10.1007/s10803-012-1543-7.

Karalliedde, L.D., Edwards, P., Marrs, T.C., 2003. Variables influencing the toxic response to organophosphates in humans. Food Chem. Toxicol. 41, 1–13. https:// doi.org/10.1016/S0278-6915(02)00232-6.

Kim, J., Son, Y., Kim, J., Lee, S., Kang, S., Park, K., Kim, S., Kim, J., Kim, J., Takayama, C., Im, H., Yang, M., Shin, T., Moon, C., 2015. Developmental and degenerative modulation of GABAergic transmission in the mouse hippocampus. Int. J. Dev. Neurosci. 47, 320–332. https://doi.org/10.1016/j.ijdevneu.2015.08.009.

Kim, K.-H., Kabir, E., Jahan, S.A., 2017. Exposure to pesticides and the associated human health effects. Sci. Total Environ. 575, 525–535. https://doi.org/10.1016/j. scitotenv.2016.09.009.

Kizaki, K., Ito, R., Okada, M., Yoshioka, K., Uchide, T., Temma, K., Mutoh, K., Uechi, M., Hara, Y., 2006. Enhanced gene expression of myocardial matrix metalloproteinases 2 and 9 after acute treatment with doxorubicin in mice. Pharmacol. Res. 53, 341–346. https://doi.org/10.1016/j.phrs.2006.01.001.

Lan, A., Stein, D., Portillo, M., Toiber, D., Kofman, O., 2019. Impaired innate and conditioned social behavior in adult C57Bl6/J mice prenatally exposed to chlorpyrifos. Behav. Brain Funct. 15, 1–9. https://doi.org/10.1186/s12993-019- 0153-3.

Laporte, B., Gay-Quéheillard, J., Bach, V., Villégier, A.-S., 2018. Developmental neurotoxicity in the progeny after maternal gavage with chlorpyrifos. Food Chem. Toxicol. 113, 66–72. https://doi.org/10.1016/j.fct.2018.01.026.

Lauber, E., Filice, F., Schwaller, B., 2018. Dysregulation of parvalbumin expression in the Cntnap2 -/- mouse model of autism spectrum disorder. Front. Mol. Neurosci. 11, 1–15. https://doi.org/10.3389/fnmol.2018.00262.

Laurie, D., Wisden, W., Seeburg, P., 1992. The distribution of thirteen $GABA_A$ receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. J. Neurosci. 12, 4151–4172. https://doi.org/10.1523/JNEUROSCI.12-11- 04151.1992.

Lee, B., Zhang, Y., Kim, Y., Kim, S., Lee, Y., Han, K., 2017. Age-dependent decrease of GAD65/67 mRNAs but normal densities of GABAergic interneurons in the brain regions of Shank 3 -overexpressing manic mouse model. Neurosci. Lett. 649, 48–54. https://doi.org/10.1016/j.neulet.2017.04.016.

Lemonnier, E., Villeneuve, N., Sonie, S., Serret, S., Rosier, A., Roue, M., Brosset, P., Viellard, M., Bernoux, D., Rondeau, S., Thummler, S., Ravel, D., Ben-Ari, Y., 2017. Effects of bumetanide on neurobehavioral function in children and adolescents with autism spectrum disorders. Transl. Psychiatry 7, e1056. https://doi.org/10.1038/ tp.2017.10 e1056.

Leonzino, M., Busnelli, M., Antonucci, F., Verderio, C., Mazzanti, M., Chini, B., 2016. The timing of the excitatory-to-inhibitory GABA switch is regulated by the oxytocin receptor via KCC2. Cell Rep. 15, 96–103. https://doi.org/10.1016/j. celrep.2016.03.013.

Liu, Y., Chu, H., Chen, J., Zhou, L., Chen, Q., Yu, Y., Wu, Z., Wang, S., Lai, Y., Pan, C., Cui, Y., 2014. Age-related change in the expression of NKCC1 in the cochlear lateral wall of C57BL/6J mice. Acta Otolaryngol. 134, 1047–1051. https://doi.org/ 10.3109/00016489.2014.900704.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2 -ΔΔCT method. Methods 25, 402–408. https://doi. org/10.1006/meth.2001.1262.

Lopatina, O.L., Komleva, Y.K., Gorina, Y.V., Olovyannikova, R.Y., Trufanova, L.V., Hashimoto, T., Takahashi, T., Kikuchi, M., Minabe, Y., Higashida, H., Salmina, A.B., 2018. Oxytocin and excitation/inhibition balance in social recognition. Neuropeptides 72, 1–11. https://doi.org/10.1016/j.npep.2018.09.003.

Markram, K., Rinaldi, T., Mendola, D. La, Sandi, C., Markram, H., 2008. Abnormal fear conditioning and amygdala processing in an animal model of autism. Neuropsychopharmacology 33, 901–912. https://doi.org/10.1038/sj.npp.1301453.

Mitchell, S.J., Maguire, E.P., Cunningham, L., Gunn, B.G., Linke, M., Zechner, U., Dixon, C.I., King, S.L., Stephens, D.N., Swinny, J.D., Belelli, D., Lambert, J.J., 2018. Early-life adversity selectively impairs α2-$GABA_A$ receptor expression in the mouse nucleus accumbens and influences the behavioral effects of cocaine. Neuropharmacology 141, 98–112. https://doi.org/10.1016/j. neuropharm.2018.08.021.

Montagrin, A., Saiote, C., Schiller, D., 2018. The social hippocampus. Hippocampus 28, 672–679. https://doi.org/10.1002/hipo.22797.

Myers, S.J., Yuan, H., Kang, J.-Q., Tan, F.C.K., Traynelis, S.F., Low, C.-M., 2019. Distinct roles of GRIN2A and GRIN2B variants in neurological conditions. F1000Research 8, 1940. https://doi.org/10.12688/f1000research.18949.1.

Nahar, L., Delacroix, B.M., Nam, H.W., 2021. The role of parvalbumin interneurons in neurotransmitter balance and neurological disease. Front. Psychiatr. 12, 1–14. https://doi.org/10.3389/fpsyt.2021.679960.

Ognjanovski, N., Schaeffer, S., Wu, J., Mofakham, S., Maruyama, D., Zochowski, M., Aton, S.J., 2017. Parvalbumin-expressing interneurons coordinate hippocampal network dynamics required for memory consolidation. Nat. Commun. 8, 15039. https://doi.org/10.1038/ncomms15039.

Oka, Y., Ye, M., Zuker, C.S., 2015. Thirst driving and suppressing signals encoded by distinct neural populations in the brain. Nature 520, 349–352. https://doi.org/ 10.1038/nature14108.

Peñagarikano, O., Lázaro, M.T., Lu, X., Gordon, A., Dong, H., Lam, H.A., Peles, E., Maidment, N.T., Murphy, N.P., Yang, X.W., Golshani, P., Geschwind, D.H., 2015. Exogenous and evoked oxytocin restores social behavior in the Cntnap2 mouse model of autism. Sci. Transl. Med. 7, 1–26. https://doi.org/10.1126/ scitranslmed.3010257.

Perez-Fernandez, C., Morales-Navas, M., Aguilera-Sáez, L.M., Abreu, A.C., Guardia- Escote, L., Fernández, I., Garrido-Cárdenas, J.A., Colomina, M.T., Giménez, E., Sánchez-Santed, F., 2020a. Medium and long-term effects of low doses of Chlorpyrifos during the postnatal, preweaning developmental stage on sociability, dominance, gut microbiota and plasma metabolites. Environ. Res. 184, 109341. https://doi.org/10.1016/j.envres.2020.109341.

Perez-Fernandez, C., Morales-Navas, M., Guardia-Escote, L., Garrido-Cárdenas, J.A., Colomina, M.T., Giménez, E., Sánchez-Santed, F., 2020b. Long-term effects of low doses of Chlorpyrifos exposure at the preweaning developmental stage: a locomotor, pharmacological, brain gene expression and gut microbiome analysis. Food Chem. Toxicol. 135, 110865. https://doi.org/10.1016/j.fct.2019.110865.

Provenzano, G., Gilardoni, A., Maggia, M., Pernigo, M., Sgadò, P., Casarosa, S., Bozzi, Y., 2020. Altered expression of GABAergic markers in the forebrain of young and adult engrailed-2 knockout mice. Genes 11, 384. https://doi.org/10.3390/ genes11040384.

Ricceri, L., Markina, N., Valanzano, A., Fortuna, S., Cometa, M.F., Meneguz, A., Calamandrei, G., 2003. Developmental exposure to chlorpyrifos alters reactivity to environmental and social cues in adolescent mice. Toxicol. Appl. Pharmacol. 191, 189–201. https://doi.org/10.1016/S0041-008X(03)00229-1.

Richard, S., Flamant, F., 2018. Regulation of T3 availability in the developing brain: the mouse genetics contribution. Front. Endocrinol. 9, 1–11. https://doi.org/10.3389/ fendo.2018.00265.

Rowley, N.M., Madsen, K.K., Schousboe, A., Steve White, H., 2012. Glutamate and GABA synthesis, release, transport and metabolism as targets for seizure control. Neurochem. Int. 61, 546–558. https://doi.org/10.1016/j.neuint.2012.02.013.

Ruiz, A.J., Kullmann, D.M., 2013. Ionotropic receptors at hippocampal mossy fibers: roles in axonal excitability, synaptic transmission, and plasticity. Front. Neural Circ. 6, 1–12. https://doi.org/10.3389/fncir.2012.00112.

Sakai, A., Matsuda, T., Doi, H., Nagaishi, Y., Kato, K., Nakashima, K., 2018. Ectopic neurogenesis induced by prenatal antiepileptic drug exposure augments seizure susceptibility in adult mice. Proc. Natl. Acad. Sci. USA 115, 4270–4275. https://doi. org/10.1073/pnas.1716479115.

Sams, C., Cocker, J., Lennard, M.S., 2004. Biotransformation of chlorpyrifos and diazinon by human liver microsomes and recombinant human cytochrome P450s (CYP). Xenobiotica 34, 861–873. https://doi.org/10.1080/00498250400017273.

Schepanski, S., Buss, C., Hanganu-Opatz, I.L., Arck, P.C., 2018. Prenatal immune and endocrine modulators of offspring's brain development and cognitive functions later in life. Front. Immunol. 9, 1–15. https://doi.org/10.3389/fimmu.2018.02186.

Schwede, M., Nagpal, S., Gandal, M.J., Parikshak, N.N., Mirnics, K., Geschwind, D.H., Morrow, E.M., 2018. Strong correlation of downregulated genes related to synaptic transmission and mitochondria in post-mortem autism cerebral cortex. J. Neurodev. Disord. 10, 18. https://doi.org/10.1186/s11689-018-9237-x.

Silverman, J.L., Yang, M., Lord, C., Crawley, J.N., 2010. Behavioural phenotyping assays for mouse models of autism. Nat. Rev. Neurosci. 11, 490–502. https://doi.org/ 10.1038/nrn2851.

Soghomonian, J.-J., Zhang, K., Reprakash, S., Blatt, G.J., 2017. Decreased parvalbumin mRNA levels in cerebellar Purkinje cells in autism. Autism Res. 10, 1787–1796. https://doi.org/10.1002/aur.1835.

Suratman, S., Edwards, J.W., Babina, K., 2015. Organophosphate pesticides exposure among farmworkers: pathways and risk of adverse health effects. Rev. Environ. Health 30, 65–79. https://doi.org/10.1515/reveh-2014-0072.

Tan, S., Rudd, J.A., Yew, D.T., 2011. Gene expression changes in $GABA_A$ receptors and cognition following chronic ketamine administration in mice. PLoS One 6, e21328. https://doi.org/10.1371/journal.pone.0021328.

Tanimizu, T., Kenney, J.W., Okano, E., Kadoma, K., Frankland, P.W., Kida, S., 2017. Functional connectivity of multiple brain regions required for the consolidation of social recognition memory. J. Neurosci. 37, 4103–4116. https://doi.org/10.1523/ JNEUROSCI.3451-16.2017.

Tao, R., Davis, K.N., Li, C., Shin, J.H., Gao, Y., Jaffe, A.E., Gondré-Lewis, M.C., Weinberger, D.R., Kleinman, J.E., Hyde, T.M., 2018. GAD1 alternative transcripts and DNA methylation in human prefrontal cortex and hippocampus in brain development, schizophrenia. Mol. Psychiatr. 23, 1496–1505. https://doi.org/ 10.1038/mp.2017.105.

Tsang, K.H., Lai, S.K., Li, Q., Yung, W.H., Liu, H., Mak, P.H.S., Ng, C.C.P., McAlonan, G., Chan, Y.S., Chan, S.Y., 2015. The nucleosome assembly protein TSPYL2 regulates the expression of NMDA receptor subunits GluN2A and GluN2B. Sci. Rep. 4, 3654. https://doi.org/10.1038/srep03654.

Uzunova, G., Pallanti, S., Hollander, E., 2016. Excitatory/inhibitory imbalance in autism spectrum disorders: implications for interventions and therapeutics. World J. Biol. Psychiatr. 17, 174–186. https://doi.org/10.3109/15622975.2015.1085597.

Venerosi, A., Calamandrei, G., Ricceri, L., 2006. A social recognition test for female mice reveals behavioral effects of developmental chlorpyrifos exposure. Neurotoxicol. Teratol. 28, 466–471. https://doi.org/10.1016/j.ntt.2006.05.003.

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

Venerosi, A., Tait, S., Stecca, L., Chiarotti, F., De Felice, A., Cometa, M.F., Volpe, M.T., Calamandrei, G., Ricceri, L., 2015. Effects of maternal chlorpyrifos diet on social investigation and brain neuroendocrine markers in the offspring - a mouse study. Environ. Heal. A Glob. Access Sci. Source 14. https://doi.org/10.1186/s12940-015-0019-6.

Waye, M.M.Y., Cheng, H.Y., 2018. Genetics and epigenetics of autism: a Review. Psychiatr. Clin. Neurosci. 72, 228–244. https://doi.org/10.1111/pcn.12606.

Win-Shwe, T.T., Nway, N.C., Imai, M., Lwin, T.T., Mar, O., Watanabe, H., 2018. Social behavior, neuroimmune markers and glutamic acid decarboxylase levels in a rat model of valproic acid-induced autism. J. Toxicol. Sci. 43, 631–643. https://doi.org/ 10.2131/jts.43.631.

Yip, J., Soghomonian, J.-J., Blatt, G.J., 2007. Decreased GAD67 mRNA levels in cerebellar Purkinje cells in autism: pathophysiological implications. Acta Neuropathol. 113, 559–568. https://doi.org/10.1007/s00401-006-0176-3.

Zhubi, A., Chen, Y., Guidotti, A., Grayson, D.R., 2017. Epigenetic regulation of RELN and GAD1 in the frontal cortex (FC) of autism spectrum disorder (ASD) subjects. Int. J. Dev. Neurosci. 62, 63–72. https://doi.org/10.1016/j.ijdevneu.2017.02.003.

PUBLICATION IV

4.4. Publication IV

Exposure to chlorpyrifos during pregnancy differentially affects social behavior and GABA signaling elements in an *APOE*- and sex-dependent manner in a transgenic mouse model

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

Environmental Research 2023; 224; 115461
https://doi.org/10.1016/j.envres.2023.115461

Study IV overview

What do we already know?	In our laboratory, we have been studying the effects of CPF exposure throughout life. Prenatal exposure to the pesticide showed alterations in social behavior and an imbalance between excitatory and inhibitory neurotransmitters. We have also investigated the different vulnerabilities of the *APOE* genotype, and found that genetic background influences behavior, metabolisms and the cholinergic system.
What does the study add?	This study evaluates the effects of prenatal exposure to CPF on social behavior in apoE-TR mice. In addition, it studies the effects of prenatal exposure to the pesticide and the influence of the *APOE* genetic background on the gene expression of the main excitatory and inhibitory neurotransmitters in the hippocampus.
Highlights	Social novelty preference was impaired in apoE3 and apoE4 females prenatally exposed to CPF. Both apoE3 and apoE4 females treated with CPF showed an increase in GABA-A α1 subunit. Young apoE mice showed differences in the GABAergic system, specially, in the expression of GAD1, KCC2 and the GABA-A α2 and α5 subunits. Prenatal exposure to CPF increases the expression of GAD1 and the ionic cotransporter KCC2.

* No edited version of the online article

Exposure to chlorpyrifos during pregnancy differentially affects social behavior and GABA signaling elements in an *APOE*- and sex-dependent manner in a transgenic mouse model

Judit Biosca-Brull [abc], Laia Guardia-Escote [ab], Pia Basaure [a], Maria Cabré [ad], Jordi Blanco [ace], Cristian Pérez-Fernández [f], Fernando Sánchez-Santed [f], José L. Domingo [c], Maria Teresa Colomina [abc*]

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

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

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

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

[e] Universitat Rovira i Virgili, Department of Basic Medical Sciences, Reus, Spain.

[f] Department of Psychology, Health Research Center (CEINSA), Almeria University, 04120 Almeria, Spain.

Abstract: The massive use of chlorpyrifos (CPF) has been associated with an increased prevalence of neurodevelopmental disorders. Some previous studies have shown that prenatal, but not postnatal, CPF exposure cause social behavior deficits in mice depending on sex while others have found that in transgenic mice models carrying the human apolipoprotein E (*APOE*) ε3 and ε4 allele confer different vulnerabilities to either behavioral or metabolic disorders after CPF exposure. This study aims to evaluate, in both sexes, how prenatal CPF exposure and *APOE* genotype impact on social behavior and its relation to changes in GABAergic and glutamatergic systems. For this purpose, apoE3 and apoE4 transgenic mice were exposed through the diet to 0 or 1 mg/kg/day of CPF, between gestational day 12 and 18. A three-chamber test was used to assess social behavior on postnatal day (PND) 45. Then, mice were sacrificed, and hippocampal samples were analyzed to study the gene expression of GABAergic and glutamatergic elements. Results showed that prenatal exposure to CPF impaired social novelty preference and increased the expression of GABA-A α1 subunit in females of both genotypes. In addition, the expression of GAD1, the ionic cotransporter KCC2 and the GABA-A α2 and α5 subunits were increased in apoE3 mice, whereas CPF treatment only accentuated the expression of GAD1 and KCC2. Nevertheless, future research is needed to evaluate whether the influences detected in the GABAergic system are present and functionally relevant in adults and old mice.

Keywords: Chlorpyrifos, *APOE*, Autism, GABA, Glutamate, Social behavior

1. Introduction

The general population is constantly exposed to a wide variety of environmental toxics including pesticides (Huang et al., 2020). Organophosphate pesticides and, chlorpyrifos (CPF) in particular, are commonly used in many countries to control plants pathogens and promote agricultural production (Darko and Akoto, 2008; Wang et al., 2008). According to the US Environmental Protection Agency, each year approximately 5.1 million pounds of CPF are handled for agricultural proposes (EPA, 2020). This massive use has been associated with a wide variety of health problems, including cognitive and motor deficits (Burke et al., 2017; Eaton et al., 2008). Although environmental regulations have been published on CPF use in the last few years, the consequences on human health will persist for decades (EFSA, 2019; EPA, 2021).

The main target of CPF is the cholinergic system by irreversibly inhibiting the activity of the acetylcholinesterase (AChE) enzyme responsible for the hydrolysis of acetylcholine (ACh) to choline and acetate (Casida and Quistad, 2004; Flaskos, 2012). This inhibition produces an accumulation of ACh in the synaptic cleft, which overstimulates the postsynaptic cholinergic neurons, leading to cardiac or respiratory arrest, diarrhea, sweating or convulsions (Abou-Donia et al., 2016; Garcia et al., 2003). Apart from that, CPF have other non-cholinergic targets that trigger alterations in, for example, axonal transport (Terry, 2012), mitochondrial function (Middlemore-Risher et al., 2011), oxidative stress (Eftekhari et al., 2018) and inflammation (Mohammadzadeh et al., 2018). Notwithstanding this, low doses of CPF have been reported to give no signs of neurotoxicity (Abreu-Villaça and Levin, 2017; Casida, 2017).

Moreover, there is a growing body of clinical (Lan et al., 2019, 2017) and preclinical (Furlong et al., 2014; Millenson et al., 2017; Philippat et al., 2018) studies that have associated CPF exposure with neurodevelopmental disorders such as autism spectrum disorder (ASD), characterized by difficulties in communication, and social interaction, as well as the presence of stereotyped behaviors (Eaton et al., 2008; Takumi et al., 2020). ASD prevalence has increased in the last 30 years, which some authors believe is due to a gene-environment interaction (Matsui et al., 2018; Mottron and Bzdok, 2020; Zhang et al., 2018). A recent review conducted in our laboratory (Biosca-Brull et al., 2021) showed that experimental studies provide evidence that prenatal exposure to CPF, around gestational day (GD) 12, is associated with social and cognitive alterations in rodents. However, epidemiological studies were more variable, so it was difficult to draw conclusions (Biosca-Brull et al., 2021).

The gene of apolipoprotein E (*APOE*) is polymorphic in humans, and of the three human *APOE* alleles (ε2, ε3, ε4), ε4 increases the risk of cardiovascular and cognitive impairments, and neurodegeneration (Allen et al., 1997; Getz and Reardon, 2009). We also observed that the *APOE* genetic background contributes to neurobehavioral differences in mice from the early

developmental stages to adulthood (Basaure et al., 2019; Peris-Sampedro et al., 2016; Reverte et al., 2012) and modulates the effects of a variety of toxic agents such as CPF (Guardia-Escote et al., 2020), decabromodiphenyl ether (Reverte et al., 2013) or lead (Prada et al., 2016). The apoE protein has a key role in lipid transport and homeostasis in the nervous system (Yu and Foraker, 2015). The association between *APOE* and autism focuses on that apoE protein competes with Reelin (the protein of a well-established autism candidate gene) to join the very low-density lipoprotein receptor (VLDLR) and the apolipoprotein E receptor 2 (apoER2) (D'Arcangelo et al., 1999). The importance of cholesterol supply for normal brain development and function and the implication of Reelin in normal development suggest different vulnerabilities associated with *APOE* polymorphism for developing the symptomatology of autism.

On the other hand, a neurochemical hypothesis for autism points to a dysregulation of complementary systems such as gamma-aminobutyric acid (GABA) and glutamate (Ford et al., 2019; Vorstman et al., 2017). GABA neurotransmitter is synthesized from glutamate by the activity of glutamic acid decarboxylase (GAD). Both neurotransmitters are packed into synaptic vesicles and released in the synaptic cleft to join their respective receptors on the postsynaptic surface (Rowley et al., 2012). A disequilibrium between these neurotransmitters is known as excitatory/inhibitory (E/I) imbalance, suggesting an increase in glutamatergic activity alongside a decrease in GABAergic signaling, which leads to neuronal hyper-excitability (Canitano and Palumbi, 2021). The *APOE* gene interacts with the glutamatergic system (Chen et al., 2010; Zhang et al., 2020). In fact, Chen et al. (2010) associated the apoER2 with the activation of N-methyl-D-aspartate (NMDA) receptors at the neuronal surface, which are composed by two obligatory GluN1 subunits and two GluN2 or GluN3 subunits (Chazot et al., 1994; Vieira et al., 2020). Together with Reelin, apoER2 phosphorylates and enhances the activity of GluN2 subunits. Gómez-Giménez et al. (2018) demonstrated that exposure to low doses of CPF increases GABA neurotransmitter in cerebellum and hippocampus GluN2A and GluN2B NMDA receptor subunits, but only in males, indicating a sex-specific effect of CPF (Gómez-Giménez et al., 2018).

In addition, behavioral testing plays a crucial role in evaluating autistic-like behaviors. In this sense disruption in social behavior is one of the core symptoms of autism and it can be evaluated by a wide variety of tests. The three-chamber test, developed by Crawley (2004) is one of the most commonly used method to explore social behavior in mice including mice model of autism. Social behavior, especially the ability to remember an individual (social memory), is related to the medial temporal lobe of the brain, which includes the hippocampus (Okuyama, 2018). In particular, the CA1 hippocampal region has been associated with the ability to recognize and memorize a familiar conspecific because it encodes and stores the social recognition memory (Montagrin et al., 2018). Moreover, the CA1 region is interconnected with another hippocampal subfield, the CA2 region, indicating that both subparts play an important role in the formation and consolidation of social memory (Garrido Zinn et al., 2016; Montagrin et al., 2018).

Given that CPF exposure is associated with an increase in developmental disorders, in which genetic background may be a protective or risk factor, the present study was aimed to evaluate the effect of prenatal exposure to CPF on the human *APOE3* and *APOE4* polymorphism in transgenic mice. In particular, we were looking for its effect on social behaviors and gene expression of GABAergic and glutamatergic signaling elements, as well as a possible association between prenatal exposure to CPF, *APOE* genotype and autism symptomatology.

2. Material and Methods

2.1. Animals

Human apoE3 and apoE4 target replacement (TR) homozygote mice were obtained from Taconic Europe (Lille Skensved, Denmark). After a quarantine period, one male and two females were mated for 3 h. Once a vaginal plug was detected, the GD 0 was assigned. Animals were housed in plastic cages containing three to five animals until GD 12, when pregnant females were housed individually and randomly selected to receive one of the two treatments (Control [CNT] or CPF). The day of delivery was assigned as PND 0. Only litters with at least four live pups were used in this study. All mice were maintained in a 12 h light/dark automatic cycle (light ON at 8:00-20:00) with controlled temperature (22 ± 2 ºC) and humidity (50 ± 10 %). Food (SAFE® A04 diet, Panlab, Barcelona, Spain) and tap water were administered *ad libitum*. The present study followed the ARRIVE Guidelines (Percie du Sert et al., 2020) and complied with Spanish Royal Decree 53/2013 on the protection of experimental animals and the European Communities Council Directive (86/609/EEC). It was approved by the Animal Care and Use Committee of the Rovira i Virgili University and assigned an authorization code (number 10735) by the Government of Catalonia.

2.2. Treatment and experimental design

Pregnant female mice were exposed to 0 or 1 mg/kg/day of CPF (0,0-diethyl O-(3,5,6-trichloropyridin-2-yl) phosphorothioate) 99.5 % purity provided by Sigma-Aldrich (Madrid, Spain) through the diet from GD 12 to 18. The standard food was supplemented with 15 mg/kg (Pantlab, Barcelona, Spain) and calculated to deliver 1 mg/kg/day. Dams were daily monitored for body weight and food intake to verify the dose provided. The control group received the standard diet (Fig. 1). The dose was chosen because it is within the threshold for brain AChE inhibition (Silva, 2020), although it should be considered that many studies find a transient inhibition of approximately 24 h (Carr et al., 2013; Perez-Fernandez et al., 2020b, 2020a). The period of exposure was based on previous studies and include the sensitive period for adverse effects

produced by valproic acid (Biosca-Brull et al., 2022; Markram et al., 2008; Schneider and Przewłocki, 2005).

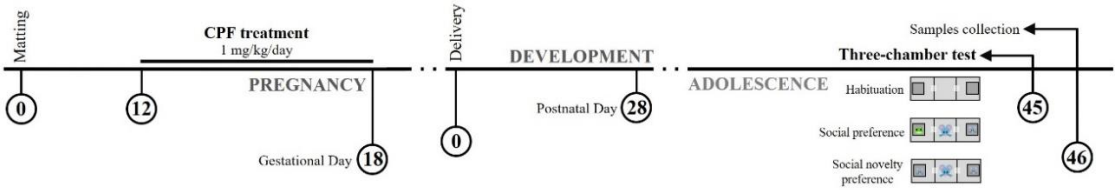


Fig. 1. Schema of the experimental design.

Litters were kept with their mothers until PND 28. A maximum of four pups (two males and two females) per litter were separated in order to assess social behavior during adolescence (PND 45). On PND 46, animals were sacrificed, and brain samples were flash-frozen and then stored at -80ºC until gene expression analysis. The unit of analysis was the litter. For this reason, values obtained from individuals of the same litter and sex were averaged to a litter value. The total number of litters and animals used are shown in Table 1.

Table 1

Animals used in this study. The number in parentheses refers to the total number of animals, whereas the other number refers to the litters used.

		Social behavior		Gene expression	
		CNT	CPF	CNT	CPF
apoE3					
	Males	8 (14)	9 (12)	5 (5)	5 (5)
	Females	8 (13)	8 (13)	6 (6)	6 (6)
apoE4					
	Males	10 (15)	9 (12)	6 (6)	6 (6)
	Females	9 (17)	8 (14)	6 (6)	6 (6)

CNT-Control; CPF-Chlorpyrifos

2.3. Behavioral assessment: Three-chamber test

The three-chamber test was used, as on other occasions in our laboratory, to assess social behavior (Basaure et al., 2019; Biosca-Brull et al., 2022). Males and females of 45 days of age were placed in a Plexiglas box (60x30x30 cm) with three interconnected chambers (20x30x30 cm) and two doors in the middle walls so that the mice could move freely between compartments. Both

lateral chambers contain an empty wire cup (7x7 cm). Before starting the test, the mice were brought to the testing room and left undisturbed for 10 min. Then, one mouse was placed in the central compartment and allowed to move freely for 10 minutes. After the habituation phase, sociability preference was evaluated by placing an inanimate object in one of the two wire cups (i.e., red plastic frog, 2.5x2.5 cm), while an unfamiliar mouse of the same sex and age was placed in the other (social chamber). Finally, we evaluated the social novelty preference by replacing the inanimate object in the non-social chamber with an unfamiliar mouse of the same sex and age (novel chamber). The familiar mouse was kept in the same wire cup in the now non-novel chamber. In the last two phases, animals had 6 minutes to explore freely. At the end of the test, the Plexiglas box was cleaned with ethanol 70 % in order to avoid olfactory clues. The time that the subject mouse spent in each compartment was recorded by a video camera (Sony CCD-IRIS) and computerized by a video-tracking program (Etho-Vision©, Noldus Information Technologies, Wageningen, The Netherlands). We used this recording to evaluate the preference for the social or novel stimulus, assessing the time that the animal spends in the social or novel chamber versus the time in the non-social or non-novel chamber. We also evaluated other social variables such as sociability ratio ([time that the animal spends in the social chamber - time in the non-social chamber]/Total time exploring) and novelty ratio ([time that the animal spends in the novel chamber - time in the non-novel chamber]/Total time exploring). A positive ratio indicates a preference for the social or novel stimulus. “Total time exploring” refers to the sum of time in both lateral compartments. Those animals that explored one of the three chambers for less than 60 seconds, in the social preference phase, were removed from the study. Only one apoE3-treated male mouse was removed.

2.4. Gene expression analysis

Hippocampal Hippocampal RNA was extracted with the SPEEDTOOLS Total RNA Extraction Kit from Biotools (Madrid, Spain). After each extraction, the concentration and purity of RNA was measured by a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Then, complementary RNA (cDNA) was synthesized from 0.70 µg of RNA samples using a Maxima First Strand cDNA Kit for RT-qPCR (ThermoFisher Scientific, Waltham, MA, USA). Quantitative real-time polymerase chain reaction (qPCR) analysis, which included triplicates and was performed with Maxima SYBR Green/ROX qPCR Master Mix (2X) Kit (ThermoFisher Scientific, Waltham, MA, USA) and the 7900HT Fast Real-Time PCR System (ThermoFisher Scientific, Waltham, MA, USA), was used to assess the gene expression of GABA- and glutamate-related genes such as glutamate decarboxylase 1 and 2 (*Gad1* and *Gad2*), vesicular GABA transporter (*Slc32a1*), glutamate ionotropic receptor NMDA type subunit 2A (*Grin2a*) and 2B (*Grin2b*), GABA-A receptor subunit alpha 1 (*Gabra1*), alpha 2 (*Gabra2*), alpha 5 (*Gabra5*) and beta 3 (*Gabrb3*), solute carrier

family 12-member 5 (*Slc12a5*) and 2 (*Slc12a2*), parvalbumin (*Pvalb*) and retinoic-acid related orphan receptor alpha (*Rora*), which is an hormone-dependent transcription factor that could help understand the differences between sexes. Each sample was then normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) (ΔCt) and standardized to the average of the apoE3 male control group (ΔΔCt) to assess the relative gene expression levels with the 2-ΔΔCt method (Livak and Schmittgen, 2001). Primer sequences are described in detail in Table 2.

Table 2

Primers used in the qPCR analysis.

***Mus musculus* gene**	**Article Symbology**	**Forward primer**	**Reverse primers**	**Source**
Gad1	GAD1	ATGATACTTGGTGTGGCGTAG	GACTCTTCTCTTCCAGGCTATTG	Lee et al. (2017)
Gad2	GAD2	CTCCGGCTTTTGGTCCTTCG	ATGCCGCCCGTGAACTTTTG	Lee et al. (2017)
Slc32a1	VGAT	TCATCGAGCTGGTGATGACG	CTTGGACACGGCCTTGAGAT	Oka et al. (2015)
Grin2a	GluN2A	CCATCAGCAGAGGTATCTAC	CAGTCTGAATGCGTGAAGCT	Chen et al. (2016)
Grin2b	GluN2B	TCCAGGAGTAATGGCACTGTTTC	CGAACATCATCACCCAGACAG	Tsang et al. (2015)
Gabra1	GABA-A α1	CACCATGAGGTTGACCGTGA	CTACAACCACTGAACGGGCT	Mitchell et al. (2018)
Gabra2	GABA-A α2	TTACAGTCCAAGCCGAATGTCCC	ACTTCTGAGGTTGTGTAAGCGTAGC	Tan et al. (2011)
Gabra5	GABA-A α5	CCCTCCTTGTCTTCTGTATTTCC	TGATGTTGTCATTGGTCTCGTCT	Tan et al. (2011)
Gabrb3	GABA-A β3	GAGGTCTTCACAAGCTCAAAATC	AGGCAGGGTAATATTTCACTCAG	Provenzano et al. (2020)
Slc12a5	KCC2	CTCAACAACCTGACGGACTG	GCACAACACCATTGGTT GCG	Aguado et al. (2003)
Slc12a2	NKCC1	AACCGCTTCGTGGTTACATC	TTGCAAGTGATGCATGGAAT	Liu et al. (2014)
Pvalb	PVALB	TGTCGATGACAGACGTGCTC	TTCTTCAACCCCAATCTTGC	Huo et al. (2018)
Rora	RORA	CCACCTACTCCTGTCCTCGTCAG	CTTCTGCACCTCGGCGTACAAG	Qin et al. (2021)

2.5. Statistical analysis

Statistical analysis was conducted by SPSS 27.0 software (IBM Corp. Chicago, IL, USA). A three-way analysis of variance (ANOVA) was conducted to assess the general effects of sex, treatment or genotype, and their interactions. Similarly, the general effects of variables that were evaluated over time (body weight) were assessed with repeated measures ANOVA (RMANOVA). The unit of analysis was for all cases the litter. A two-sample t-test was used so that the significant effects of prenatal treatment, genotype, and sex could be better analyzed when it was appropriate. The sociability and novelty ratio were assessed with a one-sample t-test. As a general gene screening, a

principal component analysis (PCA) of ΔCt was conducted. Correlations between genes were also assessed using Pearson or Spearman coefficients, depending on the homogeneity of the sample. The Levene test was used to study the homogeneity of variances. The Kruskal-Wallis or Mann-Whitney U test were performed when was appropriate. All data are presented as the mean ± S.E.M. Statistical significance was set at $p<0.05$.

3. Results

3.1. Body weight

Body weight was measured at three points in time: birth, the day of weaning and before social behavior was assessed. A three-way RMANOVA using the day as the within factor and sex, genotype and treatment as the between factor showed an increase in weight over the days [$F_{2,31}=3358.879$, $p<0.001$] and an interaction between PND and genotype [$F_{2,31}=6.786$, $p=0.004$] and PND and sex [$F_{2,31}=41.746$, $p<0.001$]. In order to define the general effect, each PND was studied with a two-sample t-test for each genotype and sex. The *APOE4* genotype showed a lower body weight on the day of weaning (PND 28 [$t_{38}=3.488$, $p=0.001$], while the females showed a lower body weight on the day of behavioral testing (PND 45) [$t_{38}=7.897$, $p<0.001$] (Fig. 2A and 2B).

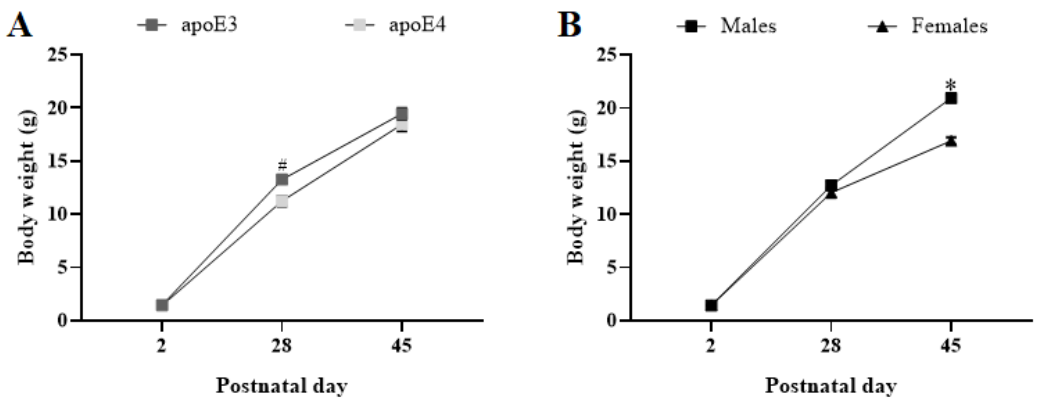


Fig. 2. Body weight depending on *APOE* genotype (**A**) or sex (**B**) at PND 2, 28 and 45. Symbols indicate differences between sexes (*) and genotypes (#) at $p<0.05$.

3.2. Social behavior in a three-chamber test

The results of social behavior are represented as a sociability or novelty ratio in which a value of 0, known as a chance value, indicates that mice spend the same time in each chamber. Positive

ratios indicate a preference for the social or novel stimulus, while negative ratios represent a preference for the non-social or non-novel stimulus (Fig. 3).

All groups showed a preference for the unfamiliar mouse in the sociability preference phase. A three-way ANOVA (sex, genotype, and treatment) showed a general effect of sex [$F_{1,68}$=4.335, p=0.042], which indicates that males had a greater preference for the unfamiliar mouse than females (Fig. 3A). The one-sample t-test analysis comparing the sociability ratio to the chance level showed significant social preference for all groups evaluated (males: apoE3-CNT [t_7=3.693, p=0.004], apoE3-CPF [t_8=8.432, p<0.001], apoE4-CNT [t_9=5.341, p<0.001], apoE4-CPF [t_8=11.935, p<0.001] and females: apoE3-CNT [t_7=3.477, p=0.005], apoE3-CPF [t_7=4.525, p=0.001], apoE4-CNT [t_8=2.999, p=0.009], apoE4-CPF [t_7=2.887, p=0.012]) (Fig. 3A).

Regarding the social novelty preference phase, a three-way (sex, genotype, and treatment) ANOVA showed a general effect of sex [$F_{1,68}$=9.525, p=0.003], and treatment [$F_{1,68}$=4.132, p=0.046], and an interaction between sex and treatment [$F_{1,68}$=4.191, p=0.045], indicating that male mice spent more time with the unfamiliar mouse than with the familiar one (Fig. 3B). Notably, prenatal CPF exposure affects social behavior in a sex-dependent manner, and neither apoE3 or apoE4 exposed females showed any preference for the novel subject. One-sample t-test analysis of the chance level indicated that all male groups showed a general preference for the new stimulus (apoE3-CNT [t_7=3.312, p=0.013], apoE3-CPF [t_8=2.805, p=0.023], apoE4-CNT [t_9=3.456, p=0.007], apoE4-CPF [t_8=6.335, p<0.001]), while only females in the apoE4-CNT group showed a significant preference [t_8=3.438, p=0.009]. However, apoE3-CNT females tended to show an interest in the unfamiliar mouse, which was not observed in the treated group [t_7=1.543, p=0.083] (Fig. 3B).

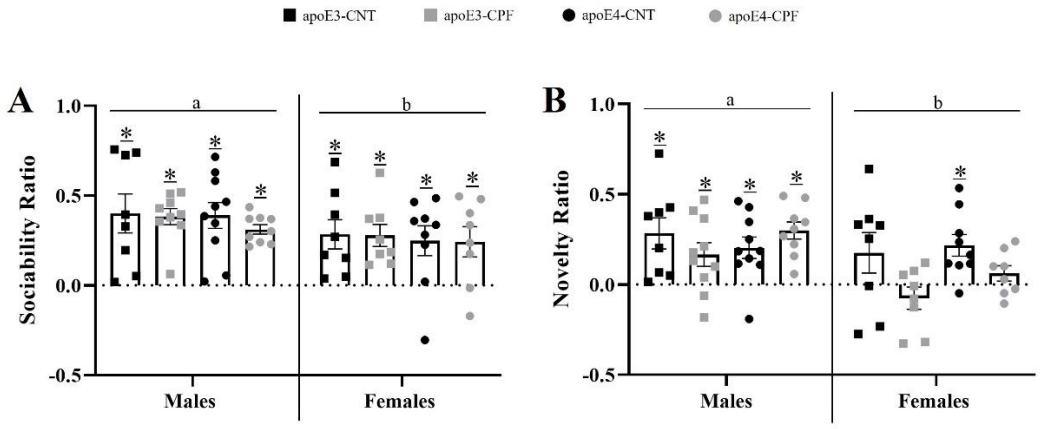


Fig. 3. Sociability and social novelty preferences assessed by the three-chamber test at PND 45. Sociability (**A**) and Novelty ratio (**B**) calculated as (time spent in (social or novel) chamber - time in (non-social or non-novel) chamber)/total time exploring. An asterisk indicates differences compared to the chance level (i.e., 0, equal time in each right or left compartment) (*), while different letters indicate differences between sexes at p<0.05.

3.3. Hippocampal gene expression

The expression of GABA and glutamate system genes was evaluated. Firstly, we performed a general screening of all the genes selected by means of a PCA analysis (Fig. 4). This analysis groups the genes into different principal components (PC). Each PC clusters the genes in terms of the expression patterns, so the genes in the same PC have a similar pattern and correlate positively between them (Supplementary Table 1). PC 1 explained 37.65 % of the variance and included genes from the two systems evaluated: GABA-A α2 (r=0.948), GABA-A α5 (r=0.825), GABA-A β3 (r=0.849), KCC2 (r=0.412), PVALB (r=-0.575), GluN2A (r=0.911) and GluN2B (r=0.894). PC 2 explained 22.56 % of the variance and was strongly correlated with the genes involved in the synthesis and release of GABA: GAD1 (r=0.850), GAD2 (r=0.890) and VGAT (r=0.899). Finally, PC 3 explained 10.81 % and included NKCC1 (r=0.791), GABA-A α1 (r=0.838) and RORA (r=0.660).

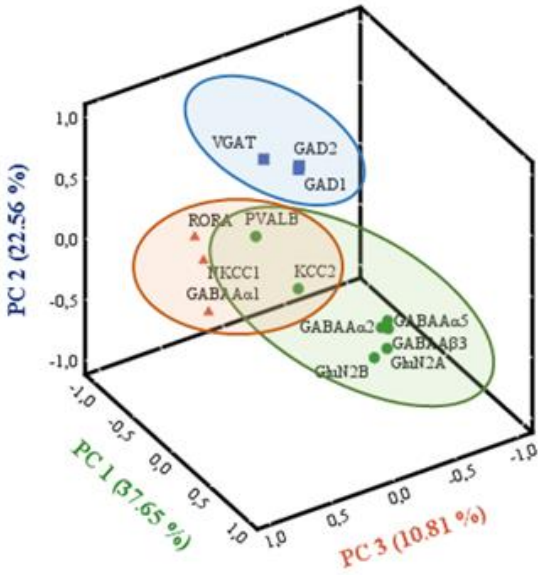


Fig. 4. Principal component analysis (PCA) of hippocampal gene expression related to GABAergic and glutamatergic systems, as well as RORA gene in adolescent homozygous apoE3- and apoE4-TR mice exposed prenatally to CPF.

3.3.1. PC 1 cluster: GABAergic and glutamatergic system-related genes and ionic cotransporter KCC2

Among the genes clustered in PC 1, we found two ionotropic glutamate N-methyl-D-aspartate (NMDA) receptor subunits, a GABAergic interneuron (PVALB), an ionic cotransporter (KCC2) and some ionotropic GABA receptor subunits (GABA-A α2, GABA-A α5 and GABA-A β3). As far as the GABA receptor is concerned, the GABA-A α5 subunit is located extrasynaptically causing a

tonic inhibition, the GABA-A α2 subunit is found in postsynaptic and presynaptic locations causing a phasic inhibition or acting as GABA controller, whereas the GABA-A β3 subunit is in postsynaptic or extrasynaptic locations.

Differences in the GABA system were observed depending on the *APOE* genotype and treatment, while the glutamate system showed differences depending on sex and genotype. A three-way (sex, genotype, and treatment) ANOVA showed that the ionic cotransporter KCC2 (Fig. 5A), GABA-A α2 and α5 subunits (Fig. 5D and 5F) were significantly influenced by the genotype (KCC2 [$F_{1,45}$=4.233, p=0.047], GABA-A α2 [$F_{1,45}$=8.465, p=0.006] and α5 [$F_{1,45}$=4.741, p=0.036]), indicating an increase of those elements in apoE3 mice in comparison with the *APOE* ε4 carriers (Fig. 5B, 5E and 5G). Moreover, an interaction between genotype and treatment [$F_{1,45}$=4.277, p=0.045] was also observed, indicating a greater expression of the ionic cotransporter in those apoE3 mice treated with CPF [t_{20}=-2.475, p=0.013]. It was also observed a non-significant trend of treatment [$F_{1,45}$=3.590, p=0.066] (Fig. 5C).

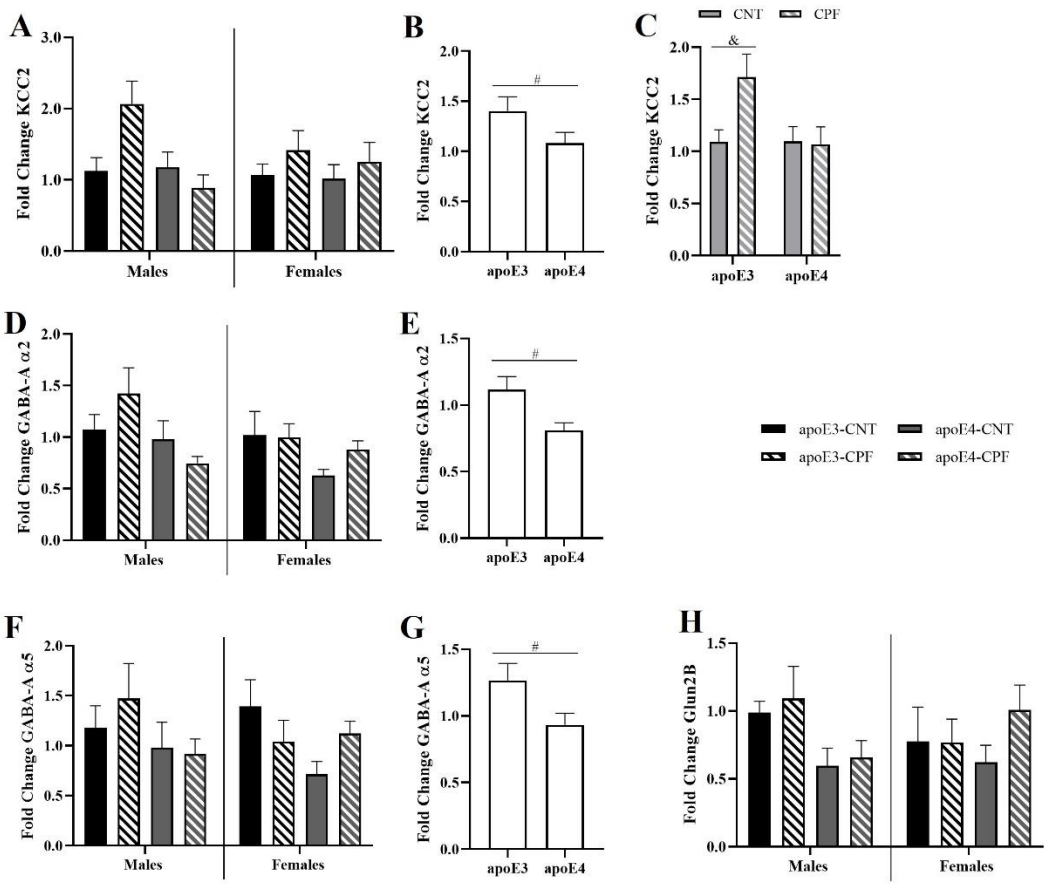


Fig. 5. Hippocampal gene expression clustered in PC 1. KCC2 (**A to C**), GABA-A α2 (**D and E**), GABA-A α5 (**F and G**) and GluN2B (**H**). Symbols indicate differences between genotype (#) and treatment (&) at p<0.05.

On the other hand, a three-way (sex, genotype and treatment) ANOVA analysis of ionotropic glutamate receptor subunits showed a non-significant interaction between genotype and sex [$F_{1,45}$=3.496, p=0.069] (Fig. 5H). Supplementary Table 2 shows the mean ± S.E.M of the genes that did not show significant differences.

3.3.2. PC 2 cluster: GABAergic system-related genes

As reported above, PC 2 covers those genes involved in the synthesis (GAD1 and GAD2) and the release (VGAT) of GABA to the synapse.

The expression of GAD1 depends on genotype and CPF exposure. A three-way (sex, genotype and treatment) ANOVA for each gene showed an overall effect of the genotype [$F1_{,45}$=4.963, p=0.032] and a non-statistical trend of the treatment [$F_{1,45}$=3.761, p=0.060] in GAD1 expression, indicating that apoE3 mice presented higher expression levels than apoE4 mice (Fig. 6A to 6C). Supplementary Table 2 shows the mean ± S.E.M of the genes that did not show significant differences.

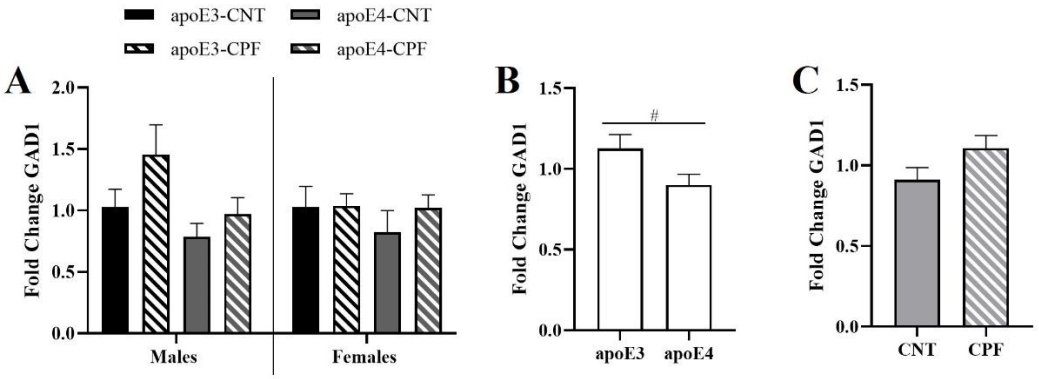


Fig. 6. Hippocampal gene expression clustered in PC 2. GAD 1 (**A to C**). The symbol # indicates differences between genotypes at p<0.05.

3.3.3. PC 3 cluster: GABAergic system-related genes, ionic cotransporter NKCC1 and RORA gene

The PC 3 cluster included the main ionotropic postsynaptic GABA subunit receptor (GABA-A α1), the ionic cotransporter NKCC1 and RORA.

The effects of prenatal CPF exposure on the GABAergic system depend on sex. A three-way (sex, genotype, and treatment) ANOVA showed an interaction between sex and treatment [$F_{1,45}$=4.442, p=0.042] in the GABA-A α1 subunit (Fig. 7A), indicating that prenatal CPF exposure

increases GABA-A α1 subunit expression in females [t_{22}=-2.195, p=0.019] (Fig. 7B). Supplementary Table 2 shows the mean ± S.E.M of the genes that did not show significant differences.

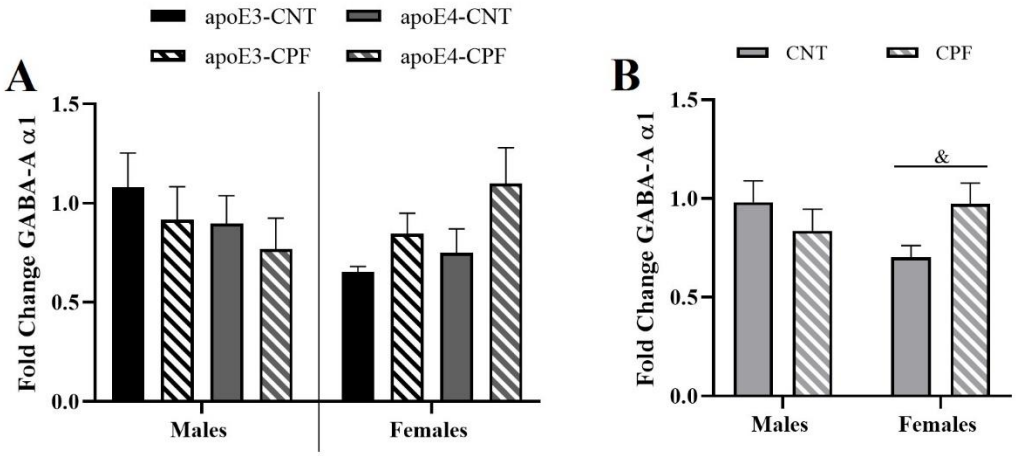


Fig. 7. Hippocampal gene expression clustered in PC 3. GABA-A α1 (**A and B**). The symbol & indicates differences between treatments at p<0.05.

3.3.4. GABA and glutamate developmental switch

As well as PCA, we also used the NKCC1/KCC2 ratio (Fig. 8) to explore the GABA switch from excitatory to inhibitory. It is well known that NKCC1 expression is increased during brain development, when GABA is excitatory, but when GABA switches to inhibitory (mature neurons), the KCC2 cotransporter is overexpressed. Statistical analysis showed a trend in the interaction between genotype and treatment [$F_{1,45}$=3.574, p=0.066] in the NKCC1/KCC2 ratio.

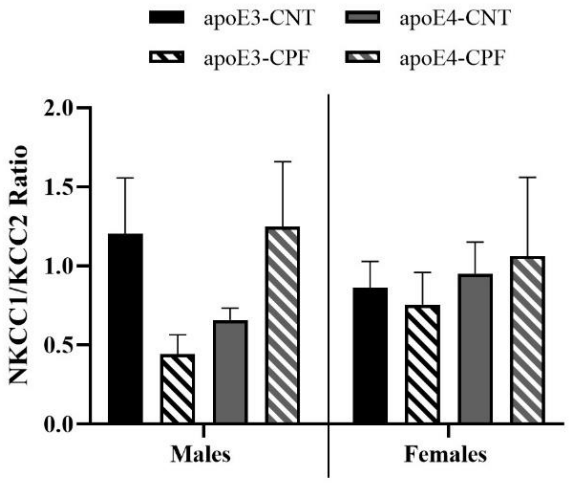


Fig. 8. Hippocampal gene expression of GABA cotransporters ratio.

Similarly, in the glutamatergic system, the GluN2A/GluN2B ratio is evaluated as a control of developmental maturation. The GluN2B subunit is highly expressed throughout embryonic life, while the expression of the GluN2A subunit increases during adulthood. This ratio has been observed to increase in mature neurons, although no differences were showed in this study [$F_{1,45}$=1.343, p=0.254] (Supplementary Fig. 1).

4. Discussion

The current study was aimed to provide new insights into the impact of *APOE* and gestational CPF exposure on developmental disorders. Hence, we investigated prenatal exposure to CPF in a humanized transgenic mouse model expressing the two human *APOE* alleles ε3 and ε4. We focused primarily on social behavior as an indicator of autistic-like behavior, along with hippocampal gene expression of the principal excitatory and inhibitory neurotransmitters. We observed a clear disruption in social novelty preference in females exposed to CPF regardless of the genotype, but no effects in males. Remarkably, the expression of some elements of the GABA and glutamate systems differed between genotypes, and there were some interactions between genotype and sex. CPF exposure mainly affects the expression of GABA elements and showed sporadic interactions with both genotype and sex.

Developmental exposure to the pesticide CPF has been associated with changes in social behavior in rodents. However, studies are not always consistent and both increased and decreased social activity has been reported. Studies conducted in C57BL/6J mice showed that prenatal exposure to doses below the threshold of observable signs of CPF toxicity evoked sex-dependent deficits in social behavior (Biosca-Brull et al., 2022; Lan et al., 2019). In this respect, Biosca-Brull et al. (2022) observed that the adolescent male offspring of dams treated with low doses of the pesticide (1 mg/kg/day) during gestation presented a decrease in the preference for the novel stimulus, but no effects in females. Another study conducted in C57BL/6J mice prenatally exposed to the pesticide at a dose of 5 mg/kg/day showed altered adult social behavior in male mice (a deficit in both innate and learned social preference), suggesting that alterations in social behavior were maintained over time (Lan et al., 2019). On the other hand, when CD-1 mice were prenatally exposed to CPF social investigation and ultrasonic vocalizations (USV) increased in females, while no effects were found in males (De Felice et al., 2014; Venerosi et al., 2006). Moreover, exposure during the perinatal period (from GD 15 to PND 14) in CD-1 mice has shown enhanced social recognition in males (Venerosi et al., 2015). Thus, it seems that the period of CPF exposure and the animal model plays an important role in behavioral social outcomes.

In this sense, our results showed that CPF exposure during gestation affects social behavior in adolescent homozygous females carrying the human *APOE* ε3 and ε4 allele, but no effects were

observed in males. In contrast, a previous study done in our laboratory with homozygous apoE3- and apoE4-TR male mice exposed to 1 mg/kg/day of CPF from PND 10 to 15 and then, at 5 months of age, re-exposed to the same doses for 15 days, showed that postnatal exposure to CPF enhanced the preference for the social stimulus in apoE4 males, whereas adult exposure enhanced this preference in apoE3 males (Basaure et al., 2019). These apparent discrepancies between postnatal and prenatal exposure may be because the exposure occurs during two different critical windows of the development of the central nervous system (CNS). It must also be taken into account that the period for social behavior assessment was different. In fact, we primarily considered prenatal exposure from GD 12 to 18 because this period corresponds to the second and third trimester of pregnancy in humans (Azad et al., 2017). In rodents, our prenatal exposure coincides with the beginning of CNS development. Processes such as neurogenesis begin on GD 9.5 and extend to PND 15 (Rice and Barone, 2000) and the peak of neuron formation in the hippocampus is well established between GD 14 and 17 (Semple et al., 2013).

As mentioned above, the GABA switch relies on the expression of both NKCC1 and KCC2 cotransporters. During brain maturation, NKCC1 is strongly expressed and causes chloride ions to enter through GABA-A receptors and increases the chloride concentration inside the neurons. Then, KCC2 becomes dominant and reduces intracellular chloride concentration by driving ion exit (Ben-Ari et al., 2012). In the present study, we did not observe any significant result, just a trend suggesting that the expression of both ionic cotransporters could be differently regulated by the *APOE* genetic background and prenatal CPF exposure. In this sense, whether differences related to basal GABA signaling between apoE3 and apoE4 isoforms, corresponds to differences on the maturation patterns during development (Basaure et al., 2018; Reverte et al., 2014) must be further explored. However, statistical values indicated that this parameter should be further studied.

To the best of our knowledge, this is the first investigation to focus on assessing the hippocampal gene expression of a wide variety of GABA-related genes in relation to *APOE* genotype, CPF exposure and social behavior. Hippocampus plays an important role in social behavior (Garrido Zinn et al., 2016; Montagrin et al., 2018). This structure together with other brain regions such as amygdala, hypothalamus, medial prefrontal cortex or anterior cingulate cortex contributes to the formation and consolidation of social memory (Barak and Feng, 2016; Tavares et al., 2015). The GABA neurotransmitter is highly present in various brain regions and it has a variety of important functions during development (Represa and Ben-Ari, 2005). GABA activity is mediated by GABA-A receptors. These ionotropic receptors are composed of different subunits, which condition their location and function (Fritschy and Panzanelli, 2014). Postsynaptic subunits such as GABA-A α1, α2, α3 and γ2 are related to fast and high-amplitude responses, whereas extrasynaptic subunits such as GABA-A α4, α5 and α6 are related to persistent low-amplitude responses (Fritschy and Panzanelli, 2014). The expression levels in each of these subunits change depending on the period of development and the brain region studied (Yu et al., 2006). In rats, Yu et al. (2006) showed an

increase of α2, α3 and α5 GABA-A subunits in the hippocampus during the first postnatal week, while in the adult brain these subunits decrease. In addition, the α1 subunit had lower expression levels in the neonatal brain, but reached high levels in adulthood (Lopez-Tellez et al., 2004; Yu et al., 2006). This indicates that an increase in the expression of both α2 and α5 could be associated with the formation of the inhibitory circuits in the hippocampus, promoting the switch of GABA during the first week of age. Our current findings showed that the *APOE3* genotype increased both α2 and α5 GABA-A subunits, indicating that GABA maturation in this genotype may occur earlier than in apoE4, in agreement with the results obtained for the chloride cotransporters ratio. In addition, an increase in GABA-A α1 subunit was observed in treated females, suggesting an upregulation of the GABA-A receptor or other direct or compensatory effect on the GABAergic system. However, we want to highlight that this subunit was the only modified of all the signaling elements evaluated in that group making difficult to draw solid conclusions between GABAergic system and social behavior. Although the statistical analysis conducted did not showed any significant effect of sex, our results suggest that both α2 and α5 subunits, as well as the ionic cotransporter KCC2 are differently regulated by sex. However, our sample size for this analysis is too small to detect significant differences.

The *APOE3* genotype also showed an increase in GAD1 expression due to CPF exposure. The GAD enzyme is responsible for the synthesis of GABA from glutamate. It is located in the GABAergic presynaptic neurons and, in particular, the GAD1 isoform is located in the cell soma (Naseri et al., 2017). GAD1 is expressed at low levels in fetuses, but expression increases during development, reaching its highest levels in adulthood (Hyde et al., 2011). The inhibition of this enzyme was strongly associated with a reduction in GABA release (Engel et al., 2001), indicating that GAD1 is important in the novo synthesis of GABA and plays an important role in the maintenance of inhibitory neurotransmission. Thus, our results suggest that CPF exposure contributes to the early maturation of the GABA system and increases the production of this neurotransmitter in apoE3 mice.

Inhibitory and excitatory neurotransmitters must be in balance if the brain is to develop properly. The main excitatory neurotransmitter in the adult CNS is glutamate which has a great variety of receptors (Thoreson, 1999). In the current study, we have focused on two ionotropic NMDA receptor subunits, which are predominant in the hippocampus (GluN2A and GluN2B subunits) (Shipton and Paulsen, 2014). The expression of both GluN2A and GluN2B subunits is regulated during development. GluN2B is highly expressed at birth but starts to decrease in the adult brain. On the other hand, the expression of GluN2A increases over the years, reaching its highest levels in adult life (Acutain et al., 2021). The switch between these subunits is commonly used as a parameter indicating synapse maturation, but in our study no differences were observed in the GluN2A/GluN2B ratio. In our results we observed that the expression of GluN2B increased in apoE3 males and decreased in apoE4 males, but no variations were observed in females. In accordance

with this, there are different studies that associated the apoE4 isoform with a down-regulation of the expression of the NMDA receptor subunits. Reelin activates the Src family non-receptor tyrosine kinases (SFKs) by binding to ApoE receptors. SKFs then phosphorylates and activates the GluN2 subunits of the NMDA receptors. However, the apoE3 and apoE4 isoforms differs in a single nucleotide which alter their innate intracellular trafficking properties. While the apoE3 isoform is readily endocytosed and recycled, the apoE4 remains in endosomes for a long period of time. This increases the possibility that ApoE receptor remains in the intracellular compartments being unable to interact with reelin and consequently activate glutamate receptors, reducing its expression in *APOE* ε4 carriers (Chen et al., 2010; Heeren et al., 2004; Liu et al., 2015; Zhang et al., 2020).

In conclusion, the results of the current study show that the long-lasting effects of prenatal exposure to low doses of CPF depend on *APOE* genotype and sex. Prenatal CPF exposure affects social behavior in a sex-dependent manner. Furthermore, gene expression analysis indicates differences between both genotypes in the expression of GABAergic system components, with higher levels in the apoE3 than in apoE4 mice, while females seem to be equally affected, especially in the expression of GABA-A α1 subunit. Nevertheless, we would like to point out that future research is needed to study the contribution of the *APOE* genotype to GABAergic and glutamatergic functions and related behaviors.

Credit author statement: Judit Biosca-Brull: Methodology, Formal analysis, Investigation, Writing-original draft, Writing-review & editing. Laia Guardia-Escote: Methodology, Investigation, Writing-review & editing. Pia Basaure: Methodology, Investigation, Writing-review & editing. Maria Cabré: Methodology, Investigation, Resources, Writing-review & editing. Jordi Blanco: Methodology, Investigation, Resources, Writing-review & editing. Cristian Pérez-Fernández: Writing-review & editing. Fernando Sánchez-Santed: Writing-review & editing. José L. Domingo: Writing-review & editing. Maria Teresa Colomina: Conceptualization, Resources, Writing-review & editing, Supervision, Funding acquisition.

Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationship that could have appeared to influence the work reported in this paper.

Data availability: Data will be made available on request.

Acknowledgments: The authors would like to thank Dr. Celeste di Paolo, Esperanza Chernichero and Juan Valencia for their technical support with the animal care. We also acknowledge Dr. Helena Torrell Galceran and Lorena García Alcalde of the Genomic facilities of the Center for Omics Sciences (COS) of the Rovira i Virgili University for their contribution to gene expression analysis. This research was funded by Martí Franquès Grant Program-Doctoral modality (Reference: 2019PMF-PIPF-28) and the Spanish Government (Ministry of the Economy and Competitiveness (MINECO, Spain)) (Reference: PSI2017- 86847-C2-R).

References

Abou-Donia, M.B., Siracuse, B., Gupta, N., Sobel Sokol, A., 2016. Sarin (GB, O-isopropyl methylphosphonofluoridate) neurotoxicity: critical review. Crit. Rev. Toxicol. 46, 845–875. https://doi.org/10.1080/10408444.2016.1220916

Abreu-Villaça, Y., Levin, E.D., 2017. Developmental neurotoxicity of succeeding generations of insecticides. Environ. Int. 99, 55–77. https://doi.org/10.1016/j.envint.2016.11.019

Acutain, M.F., Griebler Luft, J., Vazquez, C.A., Popik, B., Cercato, M.C., Epstein, A., Salvetti, A., Jerusalinsky, D.A., de Oliveira Alvares, L., Baez, M.V., 2021. Reduced Expression of Hippocampal GluN2A-NMDAR Increases Seizure Susceptibility and Causes Deficits in Contextual Memory. Front. Neurosci. 15, 1–15. https://doi.org/10.3389/fnins.2021.644100

Aguado, F., Carmona, M.A., Pozas, E., Aguiló, A., Martínez-Guijarro, F.J., Alcantara, S., Borrell, V., Yuste, R., Ibañez, C.F., Soriano, E., 2003. BDNF regulates spontaneous correlated activity at early developmental stages by increasing synaptogenesis and expression of the K+/Cl- co-transporter KCC2. Development 130, 1267–1280. https://doi.org/10.1242/dev.00351

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

Azad, M.B., Moyce, B.L., Guillemette, L., Pascoe, C.D., Wicklow, B., McGavock, J.M., Halayko, A.J., Dolinsky, V.W., 2017. Diabetes in pregnancy and lung health in offspring: developmental origins of respiratory disease. Paediatr. Respir. Rev. 21, 19–26. https://doi.org/10.1016/j.prrv.2016.08.007

Barak, B., Feng, G., 2016. Neurobiology of social behavior abnormalities in autism and Williams syndrome. Nat. Neurosci. 19, 647–655. https://doi.org/10.1038/nn.4276

Basaure, P., Guardia-Escote, L., Biosca-Brull, J., Blanco, J., Cabré, M., Peris-Sampedro, F., Sánchez-Santed, F., Domingo, J.L., Colomina, M.T., 2019. Exposure to chlorpyrifos at different ages triggers APOE genotype-specific responses in social behavior, body weight and hypothalamic gene expression. Environ. Res. 178, 108684. https://doi.org/10.1016/j.envres.2019.108684

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

Ben-Ari, Y., Khalilov, I., Kahle, K.T., Cherubini, E., 2012. The GABA Excitatory/Inhibitory Shift in Brain Maturation and Neurological Disorders. Neurosci. 18, 467–486. https://doi.org/10.1177/1073858412438697

Biosca-Brull, J., Guardia-Escote, L., Blanco, J., Basaure, P., Cabré, M., Sánchez-Santed, F., Domingo, J.L., Colomina, M.T., 2022. Prenatal, but not postnatal exposure to chlorpyrifos affects social behavior of mice and the excitatory-inhibitory balance in a sex-dependent manner. Food Chem. Toxicol. 169, 113423. https://doi.org/10.1016/j.fct.2022.113423

Biosca-Brull, J., Pérez-Fernández, C., Mora, S., Carrillo, B., Pinos, H., Conejo, N.M., Collado, P., Arias, J.L., Martín-Sánchez, F., Sánchez-Santed, F., Colomina, M.T., 2021. Relationship between Autism Spectrum Disorder and Pesticides: A Systematic Review of Human and Preclinical Models. Int. J. Environ. Res. Public Health 18, 5190. https://doi.org/10.3390/ijerph18105190

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

chlorpyrifos: from clinical findings to preclinical models and potential mechanisms. J. Neurochem. 142, 162–177. https://doi.org/10.1111/jnc.14077

Canitano, R., Palumbi, R., 2021. Excitation/Inhibition Modulators in Autism Spectrum Disorder: Current Clinical Research. Front. Neurosci. 15. https://doi.org/10.3389/fnins.2021.753274

Carr, R.L., Adams, A.L., Kepler, D.R., Ward, A.B., Ross, M.K., 2013. Induction of Endocannabinoid Levels in Juvenile Rat Brain Following Developmental Chlorpyrifos Exposure. Toxicol. Sci. 135, 193–201. https://doi.org/10.1093/toxsci/kft126

Casida, J.E., 2017. Organophosphorus Xenobiotic Toxicology. Annu. Rev. Pharmacol. Toxicol. 57, 309–327. https://doi.org/10.1146/annurev-pharmtox-010716-104926

Casida, J.E., Quistad, G.B., 2004. Organophosphate Toxicology: Safety Aspects of Nonacetylcholinesterase Secondary Targets. Chem. Res. Toxicol. 17, 983–998. https://doi.org/10.1021/tx0499259

Chazot, P.L., Coleman, S.K., Cik, M., Stephenson, F.A., 1994. Molecular characterization of N-methyl-D-aspartate receptors expressed in mammalian cells yields evidence for the coexistence of three subunit types within a discrete receptor molecule. J. Biol. Chem. 269, 24403–24409. https://doi.org/10.1016/S0021-9258(19)51098-5

Chen, T., Zhang, B., Li, G., Chen, L., Chen, L., 2016. Simvastatin enhances NMDA receptor GluN2B expression and phosphorylation of GluN2B and GluN2A through increased histone acetylation and Src signaling in hippocampal CA1 neurons. Neuropharmacology 107, 411–421. https://doi.org/10.1016/j.neuropharm.2016.03.028

Chen, Y., Durakoglugil, M.S., Xian, X., Herz, J., 2010. ApoE4 reduces glutamate receptor function and synaptic plasticity by selectively impairing ApoE receptor recycling. Proc. Natl. Acad. Sci. 107, 12011–12016. https://doi.org/10.1073/pnas.0914984107

Crawley, J.N., 2004. Designing mouse behavioral tasks relevant to autistic-like behaviors. Ment. Retard. Dev. Disabil. Res. Rev. 10, 248–258. https://doi.org/10.1002/mrdd.20039

D'Arcangelo, G., Homayouni, R., Keshvara, L., Rice, D.S., Sheldon, M., Curran, T., 1999. Reelin Is a Ligand for Lipoprotein Receptors. Neuron 24, 471–479. https://doi.org/10.1016/S0896-6273(00)80860-0

Darko, G., Akoto, O., 2008. Dietary intake of organophosphorus pesticide residues through vegetables from Kumasi, Ghana. Food Chem. Toxicol. 46, 3703–3706. https://doi.org/10.1016/j.fct.2008.09.049

De Felice, A., Venerosi, A., Ricceri, L., Sabbioni, M., Scattoni, M.L., Chiarotti, F., Calamandrei, G., 2014. Sex-dimorphic effects of gestational exposure to the organophosphate insecticide chlorpyrifos on social investigation in mice. Neurotoxicol. Teratol. 46, 32–39. https://doi.org/10.1016/j.ntt.2014.09.002

Eaton, D.L., Daroff, R.B., Autrup, H., Bridges, J., Buffler, P., Costa, L.G., Coyle, J., McKhann, G., Mobley, W.C., Nadel, L., Neubert, D., Schulte-Hermann, R., Spencer, P.S., 2008. Review of the Toxicology of Chlorpyrifos With an Emphasis on Human Exposure and Neurodevelopment. Crit. Rev. Toxicol. 38, 1–125. https://doi.org/10.1080/10408440802272158

EFSA, 2019. Statement on the available outcomes of the human health assessment in the context of the pesticides peer review of the active substance chlorpyrifos. EFSA J. 17. https://doi.org/10.2903/j.efsa.2019.5809

Eftekhari, A., Ahmadian, E., Azami, A., Johari-Ahar, M., Eghbal, M.A., 2018. Protective effects of coenzyme Q10 nanoparticles on dichlorvos-induced hepatotoxicity and mitochondrial/lysosomal injury. Environ. Toxicol. 33, 167–177. https://doi.org/10.1002/tox.22505

Engel, D., Pahner, I., Schulze, K., Frahm, C., Jarry, H., Ahnert-Hilger, G., Draguhn, A., 2001. Plasticity of rat central inhibitory synapses through GABA metabolism. J. Physiol. 535, 473–482. https://doi.org/10.1111/j.1469-7793.2001.00473.x

EPA, 2021. Chlorpyrifos; Tolerance Revocations. Fed. Regist. / Vol. 86, No. 165 / Monday, August 30, 2021 / Rules Regul. 86, 21–42.

EPA, 2020. Chlorpyrifos: Proposed Interim Registration Review Decision Case Number 0100.

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

Ford, T.C., Abu-Akel, A., Crewther, D.P., 2019. The association of excitation and inhibition signaling with the relative symptom expression of autism and psychosis-proneness: Implications for psychopharmacology. Prog. Neuro-Psychopharmacology Biol. Psychiatry 88, 235–242. https://doi.org/10.1016/j.pnpbp.2018.07.024

Fritschy, J.M., Panzanelli, P., 2014. GABA A receptors and plasticity of inhibitory neurotransmission in the central nervous system. Eur. J. Neurosci. 39, 1845–1865. https://doi.org/10.1111/ejn.12534

Furlong, M.A., Engel, S.M., Barr, D.B., Wolff, M.S., 2014. Prenatal exposure to organophosphate pesticides and reciprocal social behavior in childhood. Environ. Int. 70, 125–131. https://doi.org/10.1016/j.envint.2014.05.011

Garcia, S., Abu-Qare, A., Meeker-O'Connell, W., Borton, A., Abou-Donia, M., 2003. Methyl Parathion: A Review of Health Effects. J. Toxicol. Environ. Heal. Part B 6, 185–210. https://doi.org/10.1080/10937400306471

Garrido Zinn, C., Clairis, N., Silva Cavalcante, L.E., Furini, C.R.G., de Carvalho Myskiw, J., Izquierdo, I., 2016. Major neurotransmitter systems in dorsal hippocampus and basolateral amygdala control social recognition memory. Proc. Natl. Acad. Sci. 113, E4914–E4919. https://doi.org/10.1073/pnas.1609883113

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

Gómez-Giménez, B., Felipo, V., Cabrera-Pastor, A., Agustí, A., Hernández-Rabaza, V., Llansola, M., 2018. Developmental Exposure to Pesticides Alters Motor Activity and Coordination in Rats: Sex Differences and Underlying Mechanisms. Neurotox. Res. 33, 247–258. https://doi.org/10.1007/s12640-017-9823-9

Guardia-Escote, L., Basaure, P., Biosca-Brull, J., Cabré, M., Blanco, J., Pérez-Fernández, C., Sánchez-Santed, F., Domingo, J.L., Colomina, M.T., 2020. APOE genotype and postnatal chlorpyrifos exposure modulate gut microbiota and cerebral short-chain fatty acids in preweaning mice. Food Chem. Toxicol. 135, 110872. https://doi.org/10.1016/j.fct.2019.110872

Heeren, J., Grewal, T., Laatsch, A., Becker, N., Rinninger, F., Rye, K.-A., Beisiegel, U., 2004. Impaired Recycling of Apolipoprotein E4 Is Associated with Intracellular Cholesterol Accumulation. J. Biol. Chem. 279, 55483–55492. https://doi.org/10.1074/jbc.M409324200

Huang, X., Cui, H., Duan, W., 2020. Ecotoxicity of chlorpyrifos to aquatic organisms: A review. Ecotoxicol. Environ. Saf. 200, 110731. https://doi.org/10.1016/j.ecoenv.2020.110731

Huo, C., Liu, X., Zhao, J., Zhao, T., Huang, H., Ye, H., 2018. Abnormalities in behaviour, histology and prefrontal cortical gene expression profiles relevant to schizophrenia in embryonic day 17 MAM-Exposed C57BL/6 mice. Neuropharmacology 140, 287–301. https://doi.org/10.1016/j.neuropharm.2018.07.030

Hyde, T.M., Lipska, B.K., Ali, T., Mathew, S. V., Law, A.J., Metitiri, O.E., Straub, R.E., Ye, T., Colantuoni, C., Herman, M.M., Bigelow, L.B., Weinberger, D.R., Kleinman, J.E., 2011. Expression of GABA Signaling Molecules KCC2,

NKCC1, and GAD1 in Cortical Development and Schizophrenia. J. Neurosci. 31, 11088–11095. https://doi.org/10.1523/JNEUROSCI.1234-11.2011

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

Lan, A., Stein, D., Portillo, M., Toiber, D., Kofman, O., 2019. Impaired innate and conditioned social behavior in adult C57Bl6/J mice prenatally exposed to chlorpyrifos. Behav. Brain Funct. 15, 2. https://doi.org/10.1186/s12993-019-0153-3

Lee, B., Zhang, Y., Kim, Y., Kim, S., Lee, Y., Han, K., 2017. Age-dependent decrease of GAD65/67 mRNAs but normal densities of GABAergic interneurons in the brain regions of Shank3 -overexpressing manic mouse model. Neurosci. Lett. 649, 48–54. https://doi.org/10.1016/j.neulet.2017.04.016

Liu, D., Pan, X., Zhang, J., Shen, H., Collins, N.C., Cole, A.M., Koster, K.P., Ben Aissa, M., Dai, X., Zhou, M., Tai, L.M., Zhu, Y., LaDu, M.J., Chen, X., 2015. APOE4 enhances age-dependent decline in cognitive function by down-regulating an NMDA receptor pathway in EFAD-Tg mice. Mol. Neurodegener. 10, 7. https://doi.org/10.1186/s13024-015-0002-2

Liu, Y., Chu, H., Chen, J., Zhou, L., Chen, Q., Yu, Y., Wu, Z., Wang, S., Lai, Y., Pan, C., Cui, Y., 2014. Age-related change in the expression of NKCC1 in the cochlear lateral wall of C57BL/6J mice. Acta Otolaryngol. 134, 1047–1051. https://doi.org/10.3109/00016489.2014.900704

Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262

Lopez-Tellez, J.F., Vela, J., del Rio, J.C., Ramos, B., Baglietto-Vargas, D., Santa-Maria, C., Ruano, D., Gutierrez, A., Vitorica, J., 2004. Postnatal development of the α1 containing GABAA receptor subunit in rat hippocampus. Dev. Brain Res. 148, 129–141. https://doi.org/10.1016/j.devbrainres.2003.11.010

Markram, K., Rinaldi, T., Mendola, D. La, Sandi, C., Markram, H., 2008. Abnormal Fear Conditioning and Amygdala Processing in an Animal Model of Autism. Neuropsychopharmacology 33, 901–912. https://doi.org/10.1038/sj.npp.1301453

Matsui, F., Hecht, P., Yoshimoto, K., Watanabe, Y., Morimoto, M., Fritsche, K., Will, M., Beversdorf, D., 2018. DHA Mitigates Autistic Behaviors Accompanied by Dopaminergic Change in a Gene/Prenatal Stress Mouse Model. Neuroscience 371, 407–419. https://doi.org/10.1016/j.neuroscience.2017.12.029

Middlemore-Risher, M.L., Adam, B.L., Lambert, N.A., Terry, A. V., 2011. Effects of Chlorpyrifos and Chlorpyrifos-Oxon on the Dynamics and Movement of Mitochondria in Rat Cortical Neurons. J. Pharmacol. Exp. Ther. 339, 341–349. https://doi.org/10.1124/jpet.111.184762

Millenson, M.E., Braun, J.M., Calafat, A.M., Barr, D.B., Huang, Y.T., Chen, A., Lanphear, B.P., Yolton, K., 2017. Urinary organophosphate insecticide metabolite concentrations during pregnancy and children's interpersonal, communication, repetitive, and stereotypic behaviors at 8 years of age: The home study. Environ. Res. 157, 9–16. https://doi.org/10.1016/j.envres.2017.05.008

Mitchell, S.J., Maguire, E.P., Cunningham, L., Gunn, B.G., Linke, M., Zechner, U., Dixon, C.I., King, S.L., Stephens, D.N., Swinny, J.D., Belelli, D., Lambert, J.J., 2018. Early-life adversity selectively impairs α2-GABAA receptor expression in the mouse nucleus accumbens and influences the behavioral effects of cocaine. Neuropharmacology 141, 98–112. https://doi.org/10.1016/j.neuropharm.2018.08.021

Mohammadzadeh, L., Hosseinzadeh, H., Abnous, K., Razavi, B.M., 2018. Neuroprotective potential of crocin against malathion-induced motor deficit and neurochemical alterations in rats. Environ. Sci. Pollut. Res. 25, 4904–4914. https://doi.org/10.1007/s11356-017-0842-0

Montagrin, A., Saiote, C., Schiller, D., 2018. The social hippocampus. Hippocampus 28, 672–679. https://doi.org/10.1002/hipo.22797

Mottron, L., Bzdok, D., 2020. Autism spectrum heterogeneity: fact or artifact? Mol. Psychiatry 25, 3178–3185. https://doi.org/10.1038/s41380-020-0748-y

Naseri, M., Parham, A., Moghimi, A., 2017. The effect of sodium thiopental as a GABA mimetic drug in neonatal period on expression of GAD65 and GAD67 genes in hippocampus of newborn and adult male rats. Iran. J. Basic Med. Sci. 20, 996–1001. https://doi.org/10.22038/IJBMS.2017.9264

Oka, Y., Ye, M., Zuker, C.S., 2015. Thirst driving and suppressing signals encoded by distinct neural populations in the brain. Nature 520, 349–352. https://doi.org/10.1038/nature14108

Okuyama, T., 2018. Social memory engram in the hippocampus. Neurosci. Res. 129, 17–23. https://doi.org/10.1016/j.neures.2017.05.007

Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M.T., Baker, M., Browne, W.J., Clark, A., Cuthill, I.C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S.T., Howells, D.W., Karp, N.A., Lazic, S.E., Lidster, K., MacCallum, C.J., Macleod, M., Pearl, E.J., Petersen, O.H., Rawle, F., Reynolds, P., Rooney, K., Sena, E.S., Silberberg, S.D., Steckler, T., Würbel, H., 2020. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLOS Biol. 18, e3000410. https://doi.org/10.1371/journal.pbio.3000410

Perez-Fernandez, C., Morales-Navas, M., Guardia-Escote, L., Colomina, M.T., Giménez, E., Sánchez-Santed, F., 2020a. Postnatal exposure to low doses of Chlorpyrifos induces long-term effects on 5C-SRTT learning and performance, cholinergic and GABAergic systems and BDNF expression. Exp. Neurol. 330, 113356. https://doi.org/10.1016/j.expneurol.2020.113356

Perez-Fernandez, C., Morales-Navas, M., Guardia-Escote, L., Garrido-Cárdenas, J.A., Colomina, M.T., Giménez, E., Sánchez-Santed, F., 2020b. Long-term effects of low doses of Chlorpyrifos exposure at the preweaning developmental stage: A locomotor, pharmacological, brain gene expression and gut microbiome analysis. Food Chem. Toxicol. 135, 110865. https://doi.org/10.1016/j.fct.2019.110865

Peris-Sampedro, F., Reverte, I., Basaure, P., Cabré, M., Domingo, J.L., Colomina, M.T., 2016. Apolipoprotein E (APOE) genotype and the pesticide chlorpyrifos modulate attention, motivation and impulsivity in female mice in the 5-choice serial reaction time task. Food Chem. Toxicol. 92, 224–235. https://doi.org/10.1016/j.fct.2016.03.029

Philippat, C., Barkoski, J., Tancredi, D.J., Elms, B., Barr, D.B., Ozonoff, S., Bennett, D.H., Hertz-Picciotto, I., 2018. Prenatal exposure to organophosphate pesticides and risk of autism spectrum disorders and other non-typical development at 3 years in a high-risk cohort. Int. J. Hyg. Environ. Health 221, 548–555. https://doi.org/10.1016/j.ijheh.2018.02.004

Prada, D., Colicino, E., Power, M.C., Weisskopf, M.G., Zhong, J., Hou, L., Spiro, A., Vokonas, P., Brenan, K., Herrera, L.A., Schwartz, J., Baccarelli, A.A., 2016. APOE ε4 allele modifies the association of lead exposure with age-related cognitive decline in older individuals. Environ. Res. 151, 101–105. https://doi.org/10.1016/j.envres.2016.07.034

Provenzano, G., Gilardoni, A., Maggia, M., Pernigo, M., Sgadò, P., Casarosa, S., Bozzi, Y., 2020. Altered Expression of GABAergic Markers in the Forebrain of Young and Adult Engrailed-2 Knockout Mice. Genes (Basel). 11, 384. https://doi.org/10.3390/genes11040384

Qin, F., Liu, N., Nie, J., Shen, T., Xu, Y., Pan, S., Pei, H., Zhou, G., 2021. Circadian effects of ionizing radiation on reproductive function and clock genes expression in male mouse. Environ. Health Prev. Med. 26, 103. https://doi.org/10.1186/s12199-021-01021-4

Represa, A., Ben-Ari, Y., 2005. Trophic actions of GABA on neuronal development. Trends Neurosci. 28, 278–283. https://doi.org/10.1016/j.tins.2005.03.010

Reverte, I., Domingo, J.L., Colomina, M.T., 2014. Neurodevelopmental effects of decabromodiphenyl ether (BDE-209) in APOE transgenic mice. Neurotoxicol. Teratol. 46, 10–17. https://doi.org/10.1016/j.ntt.2014.08.003

Reverte, I., Klein, A.B., Domingo, J.L., Colomina, M.T., 2013. Long term effects of murine postnatal exposure to decabromodiphenyl ether (BDE-209) on learning and memory are dependent upon APOE polymorphism and age. Neurotoxicol. Teratol. 40, 17–27. https://doi.org/10.1016/j.ntt.2013.08.003

Reverte, I., Klein, A.B., Ratner, C., Domingo, J.L., Colomina, M.T., 2012. Behavioral phenotype and BDNF differences related to apoE isoforms and sex in young transgenic mice. Exp. Neurol. 237, 116–125. https://doi.org/10.1016/j.expneurol.2012.06.015

Rice, D., Barone, S., 2000. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ. Health Perspect. 108, 511–533. https://doi.org/10.1289/ehp.00108s3511

Rowley, N.M., Madsen, K.K., Schousboe, A., Steve White, H., 2012. Glutamate and GABA synthesis, release, transport and metabolism as targets for seizure control. Neurochem. Int. 61, 546–558. https://doi.org/10.1016/j.neuint.2012.02.013

Schneider, T., Przewłocki, R., 2005. Behavioral Alterations in Rats Prenatally Exposed to Valproic Acid: Animal Model of Autism. Neuropsychopharmacology 30, 80–89. https://doi.org/10.1038/sj.npp.1300518

Semple, B.D., Blomgren, K., Gimlin, K., Ferriero, D.M., Noble-Haeusslein, L.J., 2013. Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. Prog. Neurobiol. 106–107, 1–16. https://doi.org/10.1016/j.pneurobio.2013.04.001

Shipton, O. a, Paulsen, O., 2014. GluN2A and GluN2B subunit-containing NMDA receptors in hippocampal plasticity. Philos. Trans. R. Soc. B Biol. Sci. 369, 20130163. https://doi.org/10.1098/rstb.2013.0163

Silva, M.H., 2020. Effects of low-dose chlorpyrifos on neurobehavior and potential mechanisms: A review of studies in rodents, zebrafish, and Caenorhabditis elegans. Birth Defects Res. 112, 445–479. https://doi.org/10.1002/bdr2.1661

Takumi, T., Tamada, K., Hatanaka, F., Nakai, N., Bolton, P.F., 2020. Behavioral neuroscience of autism. Neurosci. Biobehav. Rev. 110, 60–76. https://doi.org/10.1016/j.neubiorev.2019.04.012

Tan, S., Rudd, J.A., Yew, D.T., 2011. Gene Expression Changes in GABAA Receptors and Cognition Following Chronic Ketamine Administration in Mice. PLoS One 6, e21328. https://doi.org/10.1371/journal.pone.0021328

Tavares, R.M., Mendelsohn, A., Grossman, Y., Williams, C.H., Shapiro, M., Trope, Y., Schiller, D., 2015. A Map for Social Navigation in the Human Brain. Neuron 87, 231–243. https://doi.org/10.1016/j.neuron.2015.06.011

Terry, A.V., 2012. Functional consequences of repeated organophosphate exposure: Potential non-cholinergic mechanisms. Pharmacol. Ther. 134, 355–365. https://doi.org/10.1016/j.pharmthera.2012.03.001

Thoreson, W., 1999. Glutamate receptors and circuits in the vertebrate retina. Prog. Retin. Eye Res. 18, 765–810. https://doi.org/10.1016/S1350-9462(98)00031-7

Tsang, K.H., Lai, S.K., Li, Q., Yung, W.H., Liu, H., Mak, P.H.S., Ng, C.C.P., McAlonan, G., Chan, Y.S., Chan, S.Y., 2015. The Nucleosome Assembly Protein TSPYL2 Regulates the Expression of NMDA Receptor Subunits GluN2A and GluN2B. Sci. Rep. 4, 3654. https://doi.org/10.1038/srep03654

Venerosi, A., Calamandrei, G., Ricceri, L., 2006. A social recognition test for female mice reveals behavioral effects of developmental chlorpyrifos exposure. Neurotoxicol. Teratol. 28, 466–471. https://doi.org/10.1016/j.ntt.2006.05.003

Venerosi, A., Tait, S., Stecca, L., Chiarotti, F., De Felice, A., Cometa, M.F., Volpe, M.T., Calamandrei, G., Ricceri, L., 2015. Effects of maternal chlorpyrifos diet on social investigation and brain neuroendocrine markers in the offspring – a mouse study. Environ. Heal. 14, 32. https://doi.org/10.1186/s12940-015-0019-6

Vieira, M., Yong, X.L.H., Roche, K.W., Anggono, V., 2020. Regulation of NMDA glutamate receptor functions by the GluN2 subunits. J. Neurochem. 154, 121–143. https://doi.org/10.1111/jnc.14970

Vorstman, J.A.S., Parr, J.R., Moreno-De-Luca, D., Anney, R.J.L., Nurnberger Jr, J.I., Hallmayer, J.F., 2017. Autism genetics: opportunities and challenges for clinical translation. Nat. Rev. Genet. 18, 362–376. https://doi.org/10.1038/nrg.2017.4

Wang, L., Liang, Y., Jiang, X., 2008. Analysis of Eight Organophosphorus Pesticide Residues in Fresh Vegetables Retailed in Agricultural Product Markets of Nanjing, China. Bull. Environ. Contam. Toxicol. 81, 377–382. https://doi.org/10.1007/s00128-008-9498-2

Yu, C.E., Foraker, J., 2015. Epigenetic considerations of the APOE gene. Biomol. Concepts 6, 77–84. https://doi.org/10.1515/bmc-2014-0039

Yu, Z.Y., Wang, W., Fritschy, J.M., Witte, O.W., Redecker, C., 2006. Changes in neocortical and hippocampal GABAA receptor subunit distribution during brain maturation and aging. Brain Res. 1099, 73–81. https://doi.org/10.1016/j.brainres.2006.04.118

Zhang, J., Zhang, F., Wu, J., Li, J., Yang, Z., Yue, J., 2020. Glutamate affects cholesterol homeostasis within the brain via the up-regulation of CYP46A1 and ApoE. Toxicology 432, 152381. https://doi.org/10.1016/j.tox.2020.152381

Zhang, R., Zhou, J., Ren, J., Sun, S., Di, Y., Wang, H., An, X., Zhang, K., Zhang, J., Qian, Z., Shi, M., Qiao, Y., Ren, W., Tian, Y., 2018. Transcriptional and splicing dysregulation in the prefrontal cortex in valproic acid rat model of autism. Reprod. Toxicol. 77, 53–61. https://doi.org/10.1016/j.reprotox.2018.01.008

Supplementary material

Supplementary Table 1

Correlations between GABergic and glutamatergic signaling elements and RORA gene.

	GABA-A α2	GABA-A β3	GABA-A α5	KCC2	PVALB	GluN2A	GluN2B	GAD1	GAD2	VGAT	GABA-A α1	NKCC1	RORA
GABA-A α2		0.774***	0.777***	0.391**	-0.520***	0.806***	0.783***						-0.428**
GABA-A β3	0.774***		0.649***			0.641***	0.649***						-0.477**
GABA-A α5	0.717***	0.697***			-0.408**	0.863***	0.589***						
KCC2	0.409**					0.299*							
PVALB	-0.533***		-0.408**			-0.464**	-0.479**						
GluN2A	0.795***	0.726***	0.863***		-0.464**		0.820***			-0.372*			
GluN2B	0.842***	0.790***	0.589***	0.331*	-0.479**	0.820***				-0.294*			
GAD1									0.601***	0.710***		0.305*	
GAD2								0.601***		0.814***			
VGAT						-0.359*	-0.312*	0.644***	0.811***			0.449**	0.344*
GABA-A α1												0.467**	
NKCC1								0.305*		0.481**	0.467**		
RORA	-0.374*	-0.457**	-0.320*			-0.361*	-0.404**		0.317*	0.363*	0.380**	0.644***	

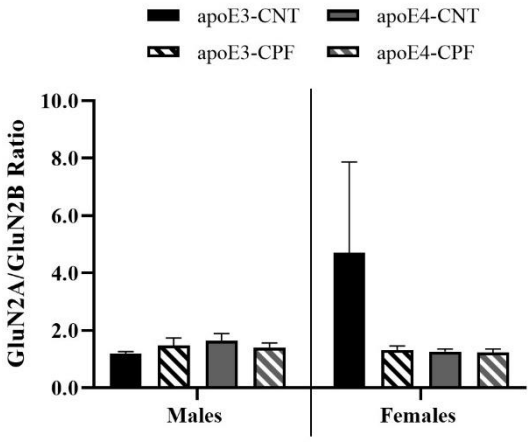
*Correlation is significant at $p<0.05$; ** Correlation is significant at $p<0.01$; *** Correlation is significant at $p<0.001$

Supplementary Table 2

Hippocampal GABAergic and glutamatergic signaling elements and RORA gene clustered in PC 1, PC 2 or PC 3 with non-significant differences.

Article Symbology	Treatment	apoE3		apoE4	
		♂	♀	♂	♀
PC1					
PVALB	CNT	1.043±0.196	1.101±0.363	0.778±0.118	1.170±0.227
	CPF	0.863±0.221	1.467±0.548	0.887±0.151	0.843±0.200
GluN2A	CNT	1.169±0.113	1.179±0.318	0.925±0.252	0.747±0.120
	CPF	1.169±0.113	1.179±0.318	0.925±0.252	0.747±0.120
GABA-A β3	CNT	1.122±0.219	1.048±0.298	0.915±0.219	0.906±0.122
	CPF	1.100±0.180	0.835±0.110	0.856±0.136	0.906±0.093
PC2					
GAD2	CNT	1.070±0.193	1.087±0.211	0.924±0.209	1.251±0.632
	CPF	1.441±0.286	1.130±0.271	1.128±0.281	0.988±0.201
VGAT	CNT	1.049±0.153	1.063±0.171	1.131±0.243	1.173±0.373
	CPF	1.655±0.493	1.413±0.276	0.969±0.153	1.045±0.136
PC3					
NKCC1	CNT	1.277±0.300	0.856±0.137	0.752±0.150	0.911±0.240
	CPF	0.851±0.277	0.985±0.354	0.926±0.269	0.927±0.243
RORA	CNT	1.191±0.242	0.825±0.209	0.912±0.286	0.803±0.183
	CPF	0.715±0.169	0.810±0.164	0.774±0.205	0.856±0.157

CNT-Control; CPF-Chlorpyrifos



Supplementary Fig. 1. Hippocampal gene expression of GluN2A/GluN2B ratio.

DISCUSSION

5. DISCUSSION

This thesis was designed to evaluate the effects of prenatal exposure to the pesticide CPF and the influence of the *APOE* polymorphism, as well as sex differences at different times of life. In addition, it also attempts to establish a relationship between these environmental and genetic factors and ASD in order to shed more light on the etiology of the disorder. To this end, a pharmacological mouse model of autism was included in this project, as a positive control for some autistic like behaviors. In addition, C57BL/6J and apoE-TR mice of both sexes were exposed to low doses of CPF during late gestation (from GD 12 to 18). Development, behavioral and biochemical changes were assessed in two different lifetime periods. On the one hand, we evaluated the effect of prenatal exposure to CPF and the influence of apoE3 and apoE4 isoforms on communication skills and physical/motor development, as well as late-onset anxiety behaviors. On the other hand, we assessed the mid-term effects of gestational exposure to CPF on social behavior in young adult mice and the involvement of the GABAergic and glutamatergic systems, as well as the different vulnerabilities conferred by the *APOE* genotype. However, the initial approach to this topic was to evaluate all published experimental evidence on ASD and pesticide exposure, with special attention to OP. This chapter dicusses all the results and offers a longitudinal perspective on the effects of prenatal exposure to CPF, and how these are modulated by the *APOE* genetic background or sex. Limitations of this thesis and future prospects are also discussed.

5.1. General discussion

Our comprehensive review of the evidence available and our assessment of the relationship between a wide variety of pesticides and ASD or associated symptomatology shows that there was little evidence on the interaction between pesticide exposure and genetic polymorphisms in either clinical or preclinical studies. Furthermore, although both types of study seem to be observe autistic symptomatology after exposure to OP, and in particular CPF, our review highlights the importance of investigating the contribution of this pesticide to autistic behaviors, as the studies are so different in many aspects such as the way, age or the source of exposure that it is difficult to draw conclusions. Therefore, we focused on evaluating the contribution of prenatal CPF exposure to two core symptoms of autism (communication skills and social behavior) and secondary clinical signs such as physical/motor development and anxiety behavior, as well as the biochemical effects produced. We also assessed the different responses of development and behavior to *APOE* genotype, and CPF exposure since previous studies in our laboratory have observed different vulnerabilities (Basaure et al., 2018; Peris-Sampedro et al., 2015b; Reverte et al., 2014).

Indeed, we observed basal differences between apoE3 and apoE4 isoforms in all experimental sections of this thesis. ApoE4 mice emitted vocalizations of greater duration and intensity and

showed delayed somatic growth, but their maturation was enhanced in terms of eye opening and climb ability. Along the same lines, adolescent *APOE* ε4 carriers showed more anxious behaviors than *APOE* ε3 carriers, whereas social behavior was influenced by sex and treatment. Females of both *APOE* genotypes showed a non-preference for the novel stimulus after being prenatally treated with CPF. At this stage of life, apoE3 mice showed higher expression levels of both GABA-A α2 and α5 subunits, as well as GAD1 and the ionic cotransporter KCC2. Furthermore, exposure to CPF during gestation increased the expression of KCC2 in apoE3-TR mice, while the expression of GABA-A α1 was increased in both apoE3- and apoE4-TR females prenatally treated with CPF. On the other hand, the study of prenatal CPF exposure in C57BL/6J mice showed that its effects were just the opposite of VPA exposure in terms of communicative behaviors. However, both groups of animals prenatally treated showed a delay in physical development. Young adult animals treated with VPA clearly showed anxious behavior, whereas social behavior was impaired by both CPF and VPA treatments in a sex-dependent manner. Gender differences were also observed in the gene expression of several GABA- and glutamate-related elements. Prenatal exposure to CPF or VPA increased the expression of GABA-A α2 and β3, as well as GluN2A subunit in males, whereas prenatal exposure to CPF increased the expression of GAD1 and GABA-A α1 in females.

According to the literature, the dose of CPF used in all the experiments of this project was near the inhibition threshold (1 mg/kg/day) (Silva, 2020). The assessment of cholinergic activity in pup brains at PND 2 showed no inhibition of the AChE enzyme. Likewise, studies that used the same dose found no alterations in activity 24 h after the last prenatal or postnatal exposure (Morales-Navas et al., 2020; Perez-Fernandez et al., 2020a, 2020b). However, a study with rats that evaluates maternal and fetal AChE activity after a single oral dose of CPF (50 mg/kg) during gestation observed that the peaks of maternal and fetal brain AChE activity are inhibited 48 h and 4 h after dosing, respectively (Ashry et al., 2002), whereas single dermal exposure to 30 mg/kg of CPF revealed maternal and fetal peak inhibition 24 h after the last exposure (Abu-Qare et al., 2001). Therefore, the dose and the administration route play an important role in cholinergic inhibition, as does the period of development in which exposure occurs, since young rodents recover AChE activity more rapidly than adults (Dam et al., 2000; Pope and Liu, 1997). This indicates that the time between maternal exposure and AChE determination in our study was long enough for cholinergic activity to recover. As well as this, several studies on AD patients (Eggers et al., 2006; Soininen et al., 1995b) and transgenic mice models (Basaure et al., 2018) have observed that brain cholinergic enzyme activity was influenced by the *APOE* genetic background. Even so, our results and those reported by Kálmán et al. (2004) disagreed.

Prior to the start of pup's evaluation, nest quality and maternal care were evaluated in order to rule out any possible effects of the treatment on dams, which would be indirectly conditioning the development of the litter. Maternal behavior is complex and onset depends on internal hormonal levels and the processing of a wide variety of external factors related to pups (Numan, 1988).

Appropiate maternal care is key for the proper development of pups. In this respect, the maternal behavior of our dams was not negatively affected by treatment or genotype, indicating that the developmental or behavioral differences observed in different stages of life are not related to differences in maternal care.

Rodent pups emit calls in the ultrasonic range when they are separated from their mother and siblings. These vocalizations are known as distress calls and induce maternal search and pup retrieval, which shortens the time taken to transport pups to the nest (Hashimoto et al., 2001; Smotherman et al., 1974). The calling rates are highest when the survival of pups is strictly dependent on dams, whereas the acquisition of locomotor and foraging abilities causes a decrease in vocalization (Caruso et al., 2020). The analysis of neonatal vocalizations can help to identify autistic-like disturbances during the early stages of development (Caruso et al., 2018). Alterations were observed in some features of pup vocalizations after a single dose of VPA (500 mg/kg) around the time of the fetal neuronal tube closure (GD 12.5). VPA-treated rats showed a reduction in the total number and average duration of calls, as well as an increase in the average frequency on PND 7 and 9 (Gzielo et al., 2020; Potasiewicz et al., 2020). Along these lines, CPF exposure during the late gestational period was associated with the same reduction in the total number and average duration of USVs on PND 7 in rats and on PND 10 in CD-1 mice, as well as with an increase in the time it took them to emit their first call (Morales-Navas et al., 2020; Venerosi et al., 2009). However, we should point out that conditions such as strain, age, sex or the responsiveness to environmental factors influence the communication of rodents (Caruso et al., 2022; Sasaki et al., 2020; Scattoni et al., 2009). Scattoni et al. (2008) compared three commonly used control strains to an inbred mouse strain (BTBR T+tf/J) which has several behavioral traits analogous to the three core symptoms of autism. Unlike the pharmacological model of autism, BTBR pups showed an increase in the number and average duration of calls on PND 4, 6, 8 and 12, as well as an increase in amplitude, although the frequency was lower on PND 6 and 8 (Scattoni et al., 2008). Since apoE4 animals also emitted longer calls with higher amplitudes, but lower frequencies on PND7, we suggest that the *APOE* genotype exhibits a different vocalization profile than that observed in C57BL/6J mice. Indeed, to the best of our knowledge, this is the first time that *APOE* genetic background effects have been studied in terms of USVs. Therefore, the absence of standardized USV sonograpms across strains of mice highlights the need for further research on the influence of the *APOE* genotype on communication behaviors and whether it follows a pattern similar to the pharmacological autism model or BTBR mice. However, prenatal exposure to CPF does not affect genotype response.

The general effects of treatment were observed during the assessment of physical and motor development. Both CPF and VPA treatment affected the weight of C57BL/6J mice and caused delayed eye opening, but no effects on motor skills were observed. In humans, Whyatt et al. (2004) observed that gestational exposure to subtoxic doses of CPF reduced infant body weight and length. In fact, subtoxic exposure to the pesticide is associated with alterations in the expression of genes

involved in fetal brain development such as genes related to neuronal communication, growth and plasticity (Stapleton and Chan, 2009). On the other hand, genotype differences were also manifested during developmental assessment. During the preweaning stage, mice carrying the *APOE* ε3 alelle had higher body weight than *APOE* ε4 carriers but showed delayed eye opening and reduced climbing ability. However, it appears that the differences observed between apoE isoforms could be due to differences in maturation patterns, which suggests earlier maturation in apoE4 mice (Basaure et al., 2018). In addition, Arbones-Mainar et al. (2016) showed differences in the metabolic profile of both isoforms. While apoE3 mice tends to accumulate white adipose tissue with a consequent increase in body weight, the presence of the *APOE* ε4 allele produces considerable oxidation of fatty acids and an increase in thermogenesis (Arbones-Mainar et al., 2016). Along these lines, Peris-Sampedro et al. (2018, 2015a, 2015b) reported that the *APOE3* genotype is more likely to develop obesity and related metabolic dysfuctions. Therefore, these metabolic differences could explain the alterations observed in body weight.

Early-life experiences play an important role in the development of neuronal systems involved in emotion and cognition (Easterbrooks et al., 2012; Sroufe, 2005). In rodents, individual differences in maternal care can predict long-term anxiety-like behaviors in offspring. Specifically, litters with higher values in maternal care, shows a lower risk of developing anxious behaviors in adulthood (Caldji et al., 1998; Zhang, 2005). Likewise, the number of USVs emitted during maternal separation and acoustic parameters such as amplitude and frequency may also predict anxious behaviors in adulthood (Gardner, 1985; Insel et al., 1986). Wöhr and Schwarting (2008) indicate that rats that emit rare vocalization patterns during infancy showed an increase in inactive behaviors as a response to shocks in adulthood. In the present study, we found that animals treated with VPA showed anxiety-like behaviors and altered patterns of USV. These animals travelled shorther distances and more slowly in the central area, which confirms that, in the face of adversity, they show immobilization behaviors. Interestingly, Insel et al. (1986) studied the effect of some anxiolytic and anxiogenic compounds on communication behaviors. The number and amplitude of vocalizations were reduced by anxiolytics, and increased by anxiogenics (Insel et al., 1986). Although no effects of CPF exposure were observed in our investigation, mice carrying the *APOE* ε4 allele showed vocalization patterns similar to those observed with anxiogenic compounds. In this respect, young apoE4 mice reduced their activity and the distance travelled in the center zone of the open field, which suggests fearful behaviors as a sign of anxiety. Hence, our results support the hypothesis that the alterations in communication patterns produced by pup isolation could be predicting long-term anxiety-like behaviors.

Although prenatal exposure to CPF did not produce autism symptomatology in terms of communication and led to few developmental impairments, deficits similar to those observed in VPA-treated mice were shown in social behaviors, specifically, in the evaluation of social novelty preference. C57BL/6J mice, prenatally treated with CPF and VPA, showed a loss of interest for the

novel stimulus, as did apoE3 and apoE4 CPF-treated females. Along these lines, Lan et al. (2017) orally exposed C57BL/6J mice to 2.5 or 5 mg/kg/day of CPF. The assessment of the social behavior of males during adulthood showed that exposure to the highest dose of CPF reduced the time spent with conspecifics, while both doses of CPF impaired the conditioned preference for social bedding (Lan et al., 2017). Later studies carried out by the same author (Lan et al., 2019) evaluated social behavior in both sexes. In this case, only the male offspring of mothers treated with 5 mg/kg/day of CPF presented a clear deficit in the innate and conditioned social domain (Lan et al., 2019). Thus, exposure to pesticide during late gestation could affect social behavior in a sex-dependent manner, since females have faster cerebral maturation than males and this leaves males more vulnerable to toxics (Taylor, 1969). In addition, these pieces of evidence suggest that the adverse effects observed during adolescence may persist over time, although we must consider that the dose and the time of CPF exposure differs between studies.

In parallel, we evaluated the effects of postnatal CPF exposure (from PND 10 to 15) at low doses. Unlike prenatal exposure, exposure to 1 mg/kg/day of CPF for six consecutive days in the early postnatal pre-weaning period does not affect the social or social novelty preference of adolescent mice. Likewise, studies that followed the same exposure protocol observed that social behaviors were not impaired during adolescence in rats, but in adulthood (PND 90), postnatal CPF exposure increased females interest in the novel stimulus but decreased males interest (Carr et al., 2020; Perez-Fernandez et al., 2020a). Therefore, as reported by other authors, these results suggest that prenatal exposure to the pesticide impairs social behavior from earlier stages, while postnatal exposure produces long-lasting effects. Along the same lines, a study with homozygous apoE3- and apoE4-TR male mice postnatally exposed to 1 mg/kg/day of CPF during the same postnatal period mentioned above and then, re-exposed to the same pesticide dose for 15 consecutive days at five months of age, showed that *APOE* ε4 carriers had a greater interest for the social stimulus after the first exposure, whereas *APOE* ε3 carriers had a greater interest after the re-exposure (Basaure et al., 2019a). In contrast, our prenatal CPF exposure reduced the interest in the novel stimulus in both apoE3- and apoE4-treated females but did not affect social preference. Nevertheless, these antagonistic effects between exposure periods may be because CPF exposure affects two critical windows in the development of the nervous system.

The timing of exposure to pesticides conditions the effects on neurodevelopment and brain function, as well as their associated behaviors throughout life. The populations of greatest concern for pesticide exposure are pregnant women and their children, because we found the most sensitive windows of exposure to be during pregnancy or between neonatal and adolescent development (Schepanski et al., 2018). Our exposure protocols span the second and third trimester of human pregnancy (prenatal exposure) and the third trimester and first months of age (postnatal exposure). In humans and rodents, the development of the CNS begins with the formation of the neural tube. This process occurs between GD 24 and 28 in humans and around GD 9.5 in mice. In parallel, on GD

9.5 neurogenesis starts, and does not end until PND 15 (Rice and Barone, 2000). However, the beginning and end of the brain maturation process varies between brain regions and cell type. For example, hippocampal pyramidal cells are generated during gestation, while granule cells are mostly generated during early postnatal development (Diamond, 1990). On the other hand, processes such as gliogenesis and myelination occur after birth. In fact, gliogenesis begins during the first three postnatal weeks and reaches its maximum during the second week (between PND 7 and 10). Coinciding with this peak, between PND 10 and 14, myelination begins, peaking around PND 20 (Semple et al., 2013).

Although the etiology of ASD is still unknown, one hypothesis proposes an imbalance between excitatory and inhibitory brain neuronal functions, which underlies the social, behavioral, cognitive and motor alterations observed in autism patients. In particular, E/I imbalance is due to an increase or decrease in glutamatergic or GABAergic signaling elements, respectively, and depends on the levels of ionotropic and metabotropic glutamate and GABA receptors, as well as their respective concentrations outside the neurons (Rubenstein and Merzenich, 2003; Uzunova et al., 2016).

In this thesis, we have studied some GABAergic and glutamatergic signaling elements that have previously been linked to ASD. Regarding the main inhibitory neurotransmitter in the adult brain, we observed that C57BL/6J females prenatally exposed to CPF overexpressed the GAD1 and GABA-A α1 subunit, whereas in the *APOE* genotype GAD1 expression increased in *APOE* ε3 carriers. Furthermore, in apoE mice, the GABA-A α1 subunit was affected by sex and treatment, with *APOE*-treated females showing higher expression levels. GAD1 and GAD2 are involved in the synthesis and regulation of GABA. Both are found in GABAergic presynaptic neurons but have different functions (Erlander et al., 1991; Tao et al., 2018). GAD1 is responsible for maintaining basal GABA levels, while GAD2 plays an important role in controlling the local release of GABA into the synapse (Esclapez and Houser, 1999). Although Dupuy and Houser (1996) observed that both isoforms are expressed as from GD 17 in the hippocampus of rats and are localized in similar areas, the GAD1 isoform predominates during early development, whereas the GAD2 isoform is more abundant during postnatal maturation (Jiang et al., 2022). Therefore, the increase observed in our results indicates that both C57BL/6J females treated with CPF and mice carrying the ε3 allele have a high uptake of GABA and this requires an increase in the production of the neurotransmitter to maintain its basal levels, which leads to an increase in GAD1 and a more active GABAergic system. Along these lines, the uptake of the main inhibitory neurotransmitter is given by its receptors. The GABA-A α1 subunit receptor is predominantly located in the postsynaptic membrane and mediates phasic responses. Its expression is low at birth but increases during brain development, peaking at PND 12 (Laurie et al., 1992). Hence, it is not surprising that C57BL/6J females treated with CPF also showed an increase in the GABA-A α1 subunit. However, *APOE* females exposed prenatally to the pesticide could show a deficit in the production of the GABA neurotransmitter, since the increase in this subunit is not reflected in any of the other signaling elements evaluated.

Apart from this subunit, GABA-A receptors in the hippocampus of rats are formed by the combination of α2, α5, β1, β3, γ1 and γ2 subunits. Unlike the GABA-A α1 subunit, the expression of α2 and α5 was detected in the hippocampus as from GD 17. However, the expression of both GABA subunits slightly decreases after PND 12 but remains high in adulthood. In contrast, the expression of the GABA-A β3 subunit is similar to that observed in α1. Levels of β3 are low at birth, but increase rapidly over time, and are high in adulthood. Therefore, GABA-A α2, α5 and β3 subunits, along with γ2, become the major subunits in the mature rat hippocampus (Laurie et al., 1992). Our results showed increased expression of GABA-A α2 and β3 subunits in both CPF- and VPA-treated C57BL/6J males. In addition, mice carrying the *APOE* ε3 allele showed an overexpression of GABA-A α2 and α5 subunits, with no treatment effects. The GABA-A α2 and β3 subunits, together with α1, α3, β2 and γ2 are predominantly localized at the postsynaptic membrane, while the GABA-A α5 subunit is localized at the extrasynapse, showing a high affinity for GABA and a slower but persistent response to its interaction (Brünig et al., 2002; Farrant and Nusser, 2005; Somogyi et al., 1996). Considering that the formation of mature synapses is accelerated after PND 12 and that during this process the expression of receptors located in GABAergic synapses increases (Aghajanian and Bloom, 1967), the overexpression observed in GABA-A α2 and β3 subunits indicates that both C57BL/6J treated mice and *APOE* ε3 carriers have a more mature GABAergic system. Nevertheless, the increase observed in the GABA-A α5 subunit of the *APOE3* genotype could indicate an inefficient uptake of GABA that led to an accumulation of the neurotransmitter in the extracellular space, which overstimulated this extrasynaptic receptor (Cellot and Cherubini, 2014).

Another important difference between the immature and adult brain is the change in the activity of the GABAergic system from excitatory to inhibitory. This change is related to the intracellular chloride concentration regulated by the ionic transporters NKCC1 and KCC2, which import and export this ion, respectively. In immature neurons, the expression of NKCC1 is high, while the expression of KCC2 is low, so there is an influx of chloride that increases the concentration inside the neuron. However, at the end of the first postnatal week, the expression of KCC2 increases, which cause the efflux of the chloride and a decrease in the intracellular concentration (Ben-Ari et al., 2012; Leonzino et al., 2016; Valeeva et al., 2013). Alterations in ion transporters were observed in mice carrying the *APOE* ε3 allele. Although the ratio between both transporters was not significantly altered, apoE3 mice showed an increase in the expression of the KCC2 transporter, which was enhanced by the prenatal exposure of CPF. This confirms the early maturation of the *APOE3* genotype.

Inhibitory and excitatory neurotransmitters must be in balance for proper brain development. In adult brain, the main excitatory neurotransmitter is glutamate. We have evaluated the expression of two NMDA receptor subunits that are highly expressed in the hippocampus (GluN2A and GluN2B) (Monyer et al., 1994). Significant results were observed in C57BL/6J mice, where both CPF- and

VPA-treated males showed an increase in the GluN2A subunit. Like GABA signaling elements, GluN2 subunits are age-dependent (Zhang and Luo, 2013). At birth, the GluN2B subunit is predominant in the brain. It peaks at PND 14 and then begins to decline. At the end of the first week of life the GluN2A subunit begins to increase its expression, and at PND 14 it exceeds the levels of GluN2B (Gambrill and Barria, 2011; Matta et al., 2011; Sheng et al., 1994). This developmental shift indicates glutamate maturation in brain. The results obtained in C57BL/6J males indicate that both treatments improved the maturation of the glutamate system, although we found no differences in the GluN2A/GluN2B ratio.

From our current results, we can state that the different apoE isoforms modulate differently all the developmental, behavioral and biochemical parameters studied, except for social behavior where both genotypes show differences according to sex and treatment. Interestingly, prenatal exposure to CPF oppositely influences communication skills and anxious behaviors in C57BL/6J mice, while both treatments affect development, social behavior and biochemical parameters assessed in the same ways, but with gender differences. Therefore, our results highlight the short- and mid-term impairments produced by gestational exposure to CPF, as well as the importance of studying genetic bajkground and gender in order to better understand their contribution to neurodevelopmental disorders.

5.2. Limitations

This investigation was conducted to assess the effects of CPF exposure during gestation, *APOE* genotype and sex, as individual factors of vulnerability, on neurodevelopmental behaviors. In an attempt to be comprehensive, we performed numerous physical, motor, behavioral and biochemical assessments during the first 45 days of mice life. Despite this, the study has several limitations.

First, although in humans prenatal exposure to VPA is clearly associated with a greater risk of a diagnosis of autism, the valproic acid animal model of autism differs from other autism models (i.e., BTBR mice). For this reason and due to the low viability of the offspring observed after VPA exposure, we must question whether this model is suitable for the study of autistic behavior.

Second, although in the different periods of this thesis we evaluated several core and secondary symptoms of ASD, we did not assess repetitive patterns of behavior in mice (third core symptom of autism). Nevertheless, we are aware that this analysis would provide further insight into the influence of pesticide, *APOE* genotype and sex on autism-like behaviors.

Third, we assessed the gene expression of some GABA and glutamate signaling elements. Although we observed that the *APOE* genetic background, sex and treatment led to some differences our

assessments could be improved by the evaluation of GABA and glutamate neurotransmitters levels, which would help understand the observed alterations in signaling elements analyzed.

Fourth, the behavioral and biochemical effects produced by prenatal exposure to CPF or VPA, *APOE* genotype or sex were assessed in a single time period. The evaluation of these parameters in adulthood would help to determine whether the observed alterations are transitory or are maintained over time.

5.3. Future perspectives

The results of this thesis suggest future needs for research that can extend its findings.

- The effects on communication skills of C57BL/6J individuals of prenatal exposure to the pesticide CPF do not resemble the effects of gestational exposure to VPA (Gzielo et al., 2020; Potasiewicz et al., 2020) or the effects of the apoE3 and apoE4 isoforms. This discrepancy between studies could be due to the mouse strain or the exposure method used. However, due to the lack of standardized USV sonograms across strains of mice, further research is needed to study the profile of mice vocalizations and determine if the *APOE* genotype has a communication profile similar to that observed in pharmacological or idiopathic autism animal models.

- The effect of the *APOE* genotype on the gene expression of some elements related to GABA and glutamate neurotransmitters should be further examined. We propose that these systems be evaluated at the time of weaning, adolescence and adulthood, to determine whether the differences between the apoE3 and apoE4 isoforms occurs as from the first stages of development or if they develop and are maintained over time.

- We studied the effects of gestational CPF exposure and its modulation by genotype and sex on the gene expression of some GABAergic and glutamatergic signaling elements in the hippocampus. Although the results showed differences between the *APOE* genotype, treatment and sex, we suggest including other features of the glutamate system such as glutamate transporters or AMPA or KA receptor subunits, which have prevously been associated with ASD in order to better understand the imbalance between excitatory and inhibitory neurotransmitters produced in the disorder (Nisar et al., 2022; Rojas, 2014).

CONCLUSIONS

6. CONCLUSIONS

Pesticides exposure and ASD:

i. Exposure to pesticides, and in particular organophosphates, promotes the onset of autistic behaviors in both clinical and preclinical studies. Even so, further research is needed in order to polish the differences observed between the current studies and to assess the influence of genetic polymorphisms on these exposures.

Prenatal exposure to chlorpyrifos or valproic acid in C57BL/6J mice:

→ Short-term effects:

ii. Exposure to chlorpyrifos or valproic acid during late gestation does not inhibit the activity of acetylcholinesterase in pup brain at postnatal day 2 and does not alter maternal behavior.

iii. Communication skills are influenced by prenatal exposure to valproic acid. The offspring of dams treated with valproic acid take longer to emit the first call and emit fewer vocalizations that are shorther and more intense than the vocalizations of the pups in the control and chlorpyrifos-treated group.

iv. Prenatal exposure to either chlorpyrifos or valproic acid reduces body weight and delays eye opening independently of sex, but no effect on motor development is observed.

→ Mid-term effects:

v. Exposure to valproic acid on gestational day 12 and 13 reduces the activity in the center of the open field and increases anxiety-like behavior in adolescent C57BL/6J mice.

vi. Prenatal exposure to chlorpyrifos and valproic acid reduces the social novelty preference in adolescent C57BL/6J males.

vii. Exposure to chlopyrifos during gestation affects the GABAergic system in a sex-dependent manner. C57BL/6J females prenatally treated with CPF show an increase in the gene expression of the GAD1 and GABA-A α1 subunit.

viii. Exposure to both prenatal chlorpyrifos and valproic acid increases the expression of some elements of the GABAergic and glutamatergic systems depending on sex. Male offspring of dams exposed to chlorpyrifos or valproic acid during pregnancy show an increase in the main GABAergic (GABA-A α2 and β3 subunit) and glutamatergic (GluN2A) signaling elements in the hippocampus.

Prenatal exposure to chlorpyrifos in apoE-TR mice:

→ Short-term effects:

ix. Exposure to chlorpyrifos from gestational day 12 to 18 does not inhibit the activity of acetylcholinesterase in pup brain at postnatal day 2 and does not alter maternal behavior.

x. Communication skills are influenced by the *APOE* genotype. The offspring of *APOE4* dams emit longer and more intense vocalizations than *APOE3* offspring.

xi. The *APOE* genotype influences the physical and motor development. Homozygous mice carrying the *APOE* ε3 allele show an increase in their body weight and strength but worse climbing ability.

→ Mid-term effects:

xii. The *APOE* genotype modulates anxiety-like behavior. Adolescent mice carrying the *APOE* ε4 allele show a reduction in the exploration of the center zone in comparison with *APOE* ε3 carriers.

xiii. Prenatal chlorpyrifos exposure influences the social novelty preference in adolescent *APOE* ε3 and ε4 carriers depending on sex. Female offpring of dams exposed to chlorpyrifos during pregnancy show a non-preference for the novel stimulus.

xiv. Adolescent apoE3- and apoE4-TR mice differs in the expression of the GABAergic system. Mice carrying the *APOE* ε3 allele shows faster maturation by overexpressing GAD1, the ionic cotransporter KCC2 and both GABA- A α2 and α5 receptor subunits.

xv. Exposure to chlorpyrifos during gestation affects some GABA elements in a sex-dependent manner. Homozygous females carrying either the *APOE* ε3 or ε4 allele show higher expression levels of the GABA-A α1 receptor subunit.

xvi. The expression of both GluN2A and GluN2B NMDA receptor subunits are not altered by prenatal exposure to chlorpyrifos, sex or the *APOE* genetic background.

6.2. General conclusion

The *APOE* genetic background influences communication skills, physical and motor development, anxiety-like behavior, and some signaling elements of the GABAergic system. On the other hand, gestational exposure to chlorpyrifos between gestational day 12 and 18 has mid-term sex-dependent behavioral consequences on both C57BL/6J and *APOE* mice. The effects of prenatal exposure to valproic acid on gestational day 12 and 13 on C57BL/6J mice are opposite to those of chlorpyrifos exposure in terms of communication and anxiety-like behavior, but similar in terms of physical development, social behavior and some GABAergic and glutamatergic signaling elements. All in all, these results highlight the importance of study sex and genetic differences in the population to assess the adverse effects produced by pesticide exposure and its association with ASD.

REFERENCES

7. REFERENCES

Abdel-Rahman, A., Blumenthal, G., Abou-Donia, S., Ali, F., A., A.M., Abou-Donia, M., 2002. Pharmacokinetic profile and placental transfer of a single intravenous injection of [14 C]chlorpyrifos in pregnant rats. Arch. Toxicol. 76, 452–459. https://doi.org/10.1007/s00204-002-0366-2

Abrahams, B.S., Geschwind, D.H., 2008. Advances in autism genetics: on the threshold of a new neurobiology. Nat. Rev. Genet. 9, 341–355. https://doi.org/10.1038/nrg2346

Abrams, P., Andersson, K.E., Buccafusco, J.J., Chapple, C., de Groat, W.C., Fryer, A.D., Kay, G., Laties, A., Nathanson, N.M., Pasricha, P.J., Wein, A.J., 2006. Muscarinic receptors: their distribution and function in body systems, and the implications for treating overactive bladder. Br. J. Pharmacol. 148, 565–578. https://doi.org/10.1038/sj.bjp.0706780

Abu-Qare, A.W., Abdel-Rahman, A., Brownie, C., Kishk, A.M., Abou-Donia, M.B., 2001. Inhibition of cholinesterase enzymes following a signles dermal dose of chlorpyrifos and methyl parathion, alone and in combination, in pregnant rats. J. Toxicol. Environ. Heal. Part A 63, 173–189. https://doi.org/10.1080/15287390151101529

Aghajanian, G.K., Bloom, F.E., 1967. The formation of synaptic junctions in developing rat brain: A quantitative electron microscopic study. Brain Res. 6, 716–727. https://doi.org/10.1016/0006-8993(67)90128-X

Albers, J.W., Cole, P., Greenberg, R.G., Mandel, J.S., Monson, R.R., Ross, J.H., Snodgrass, W.R., Spurgeon, A., van Gemet, M., 1999. Analysis of chlorpyrifos exposure and human health: expert panel report. J. Toxicol. Environ. Heal. Part B 2, 301–324. https://doi.org/10.1080/109374099281151

Alugubelly, N., Mohammed, A.N., Carr, R.L., 2021. Persistent proteomic changes in glutamatergic and GABAergic signaling in the amygdala of adolescent rats exposed to chlorpyrifos as juveniles. Neurotoxicology 85, 234–244. https://doi.org/10.1016/j.neuro.2021.05.012

Andrews-Zwilling, Y., Bien-Ly, N., Xu, Q., Li, G., Bernardo, A., Yoon, S.Y., Zwilling, D., Yan, T.X., Chen, L., Huang, Y., 2010. Apolipoprotein E4 Causes Age- and Tau-Dependent Impairment of GABAergic Interneurons, Leading to Learning and Memory Deficits in Mice. J. Neurosci. 30, 13707–13717. https://doi.org/10.1523/JNEUROSCI.4040-10.2010

Arbones-Mainar, J.M., Johnson, L.A., Torres-Perez, E., Garcia, A.E., Perez-Diaz, S., Raber, J., Maeda, N., 2016. Metabolic shifts toward fatty-acid usage and increased thermogenesis are associated with impaired adipogenesis in mice expressing human APOE4. Int. J. Obes. 40, 1574–1581. https://doi.org/10.1038/ijo.2016.93

Ashley-Koch, A.E., Jaworski, J., Ma, D.Q., Mei, H., Ritchie, M.D., Skaar, D.A., Robert Delong, G., Worley, G., Abramson, R.K., Wright, H.H., Cuccaro, M.L., Gilbert, J.R., Martin, E.R., Pericak-Vance, M.A., 2007. Investigation of potential gene–gene interactions between apoe and reln contributing to autism risk. Psychiatr. Genet. 17, 221–226. https://doi.org/10.1097/YPG.0b013e32809c2f75

Ashry, K.M., Abu-Qare, A.W., Saleem, F.R., Hussein, Y.A., Hamza, S.M., Kishk, A.M., Abou-Donia, M.B., 2002. Inhibition and recovery of maternal and fetal cholinesterase enzymes following a single oral dose of chlorpyrifos in rats. Arch. Toxicol. 76, 30–39. https://doi.org/10.1007/s00204-001-0314-6

Azad, M.B., Moyce, B.L., Guillemette, L., Pascoe, C.D., Wicklow, B., McGavock, J.M., Halayko, A.J., Dolinsky, V.W., 2017. Diabetes in pregnancy and lung health in offspring: developmental origins of respiratory disease. Paediatr. Respir. Rev. 21, 19–26. https://doi.org/10.1016/j.prrv.2016.08.007

Bahn, S., Volk, B., Wisden, W., 1994. Kainate receptor gene expression in the developing rat brain. J. Neurosci. 14, 5525–5547. https://doi.org/10.1523/JNEUROSCI.14-09-05525.1994

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

Balu, D., Karstens, A.J., Loukenas, E., Maldonado Weng, J., York, J.M., Valencia-Olvera, A.C., LaDu, M.J., 2019. The role of APOE in transgenic mouse models of AD. Neurosci. Lett. 707, 134285. https://doi.org/10.1016/j.neulet.2019.134285

Banerjee, S., Riordan, M., Bhat, M.A., 2014. Genetic aspects of autism spectrum disorders: insights from animal models. Front. Cell. Neurosci. 8, 1–18. https://doi.org/10.3389/fncel.2014.00058

Barman, D.N., Haque, M.A., Islam, S.M.A., Yun, H.D., Kim, M.K., 2014. Cloning and expression of ophB gene encoding organophosphorus hydrolase from endophytic Pseudomonas sp. BF1-3 degrades organophosphorus pesticide chlorpyrifos. Ecotoxicol. Environ. Saf. 108, 135–141. https://doi.org/10.1016/j.ecoenv.2014.06.023

Basaure, P., Guardia-Escote, L., Biosca-Brull, J., Blanco, J., Cabré, M., Peris-Sampedro, F., Sánchez-Santed, F., Domingo, J.L., Colomina, M.T., 2019a. Exposure to chlorpyrifos at different ages triggers APOE genotype-specific responses in social behavior, body weight and hypothalamic gene expression. Environ. Res. 178, 108684. https://doi.org/10.1016/j.envres.2019.108684

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

References

https://doi.org/10.1007/s00204-019-02387-9

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

Bekris, L.M., Yu, C.E., Bird, T.D., Tsuang, D.W., 2010. Genetics of Alzheimer Disease. J. Geriatr. Psychiatry Neurol. 23, 213–227. https://doi.org/10.1177/0891988710383571

Ben-Ari, Y., Gaiarsa, J.L., Tyzio, R., Khazipov, R., 2007. GABA: A Pioneer Transmitter That Excites Immature Neurons and Generates Primitive Oscillations. Physiol. Rev. 87, 1215–1284. https://doi.org/10.1152/physrev.00017.2006

Ben-Ari, Y., Khalilov, I., Kahle, K.T., Cherubini, E., 2012. The GABA Excitatory/Inhibitory Shift in Brain Maturation and Neurological Disorders. Neurosci. 18, 467–486. https://doi.org/10.1177/1073858412438697

Benson, G.S., Bauer, C., Hausner, L., Couturier, S., Lewczuk, P., Peters, O., Hüll, M., Jahn, H., Jessen, F., Pantel, J., Teipel, S.J., Wagner, M., Schuchhardt, J., Wiltfang, J., Kornhuber, J., Frölich, L., 2022. Don't forget about tau: the effects of ApoE4 genotype on Alzheimer's disease cerebrospinal fluid biomarkers in subjects with mild cognitive impairment—data from the Dementia Competence Network. J. Neural Transm. 129, 477–486. https://doi.org/10.1007/s00702-022-02461-0

Berg, E.L., Ching, T.M., Bruun, D.A., Rivera, J.K., Careaga, M., Ellegood, J., Lerch, J.P., Wöhr, M., Lein, P.J., Silverman, J.L., 2020. Translational outcomes relevant to neurodevelopmental disorders following early life exposure of rats to chlorpyrifos. J. Neurodev. Disord. 12, 40. https://doi.org/10.1186/s11689-020-09342-1

Bertomeu-Sánchez, J.R., 2019. Pesticides: Past and Present. HoST - J. Hist. Sci. Technol. 13, 1–27. https://doi.org/10.2478/host-2019-0001

Betancourt, A.M., Carr, R.L., 2003. The Effect of Chlorpyrifos and Chlorpyrifos-Oxon on Brain Cholinesterase, Muscarinic Receptor Binding, and Neurotrophin Levels in Rats Following Early Postnatal Exposure. Toxicol. Sci. 77, 63–71. https://doi.org/10.1093/toxsci/kfh003

Bohlhalter, S., Weinmann, O., Mohler, H., Fritschy, J., 1996. Laminar compartmentalization of GABAA-receptor subtypes in the spinal cord: an immunohistochemical study. J. Neurosci. 16, 283–297. https://doi.org/10.1523/JNEUROSCI.16-01-00283.1996

Bose, S., Kumar, P.S., Vo, D.V.N., 2021. A review on the microbial degradation of chlorpyrifos and its metabolite TCP. Chemosphere 283, 131447. https://doi.org/10.1016/j.chemosphere.2021.131447

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

Brahmand, M.B., Yunesian, M., Nabizadeh, R., Nasseri, S., Alimohammadi, M., Rastkari, N., 2019. Evaluation of chlorpyrifos residue in breast milk and its metabolite in urine of mothers and their infants feeding exclusively by breast milk in north of Iran. J. Environ. Heal. Sci. Eng. 17, 817–825. https://doi.org/10.1007/s40201-019-00398-3

Branchi, I., Santucci, D., Alleva, E., 2001. Ultrasonic vocalisation emitted by infant rodents: a tool for assessment of neurobehavioural development. Behav. Brain Res. 125, 49–56. https://doi.org/10.1016/S0166-4328(01)00277-7

Brünig, I., Scotti, E., Sidler, C., Fritschy, J.M., 2002. Intact sorting, targeting, and clustering of γ-aminobutyric acid A receptor subtypes in hippocampal neurons in vitro. J. Comp. Neurol. 443, 43–55. https://doi.org/10.1002/cne.10102

Caldji, C., Tannenbaum, B., Sharma, S., Francis, D., Plotsky, P.M., Meaney, M.J., 1998. Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. Proc. Natl. Acad. Sci. 95, 5335–5340. https://doi.org/10.1073/pnas.95.9.5335

Carr, R.L., Alugubelly, N., de Leon, K., Loyant, L., Mohammed, A.N., Patterson, M.E., Ross, M.K., Rowbotham, N.E., 2020. Inhibition of fatty acid amide hydrolase by chlorpyrifos in juvenile rats results in altered exploratory and social behavior as adolescents. Neurotoxicology 77, 127–136. https://doi.org/10.1016/j.neuro.2020.01.002

Caruso, A., Marconi, M.A., Scattoni, M.L., Ricceri, L., 2022. Ultrasonic vocalizations in laboratory mice: strain, age, and sex differences. Genes, Brain Behav. 21, 1–16. https://doi.org/10.1111/gbb.12815

Caruso, A., Ricceri, L., Scattoni, M.L., 2020. Ultrasonic vocalizations as a fundamental tool for early and adult behavioral phenotyping of Autism Spectrum Disorder rodent models. Neurosci. Biobehav. Rev. 116, 31–43. https://doi.org/10.1016/j.neubiorev.2020.06.011

Caruso, A., Sabbioni, M., Scattoni, M.L., Branchi, I., 2018. Quantitative and Qualitative Features of Neonatal Vocalizations in Mice, in: Handbook of Behavioral Neuroscience. Elsevier B.V., pp. 139–147. https://doi.org/10.1016/B978-0-12-809600-0.00013-5

Caselli, R.J., Dueck, A.C., Osborne, D., Sabbagh, M.N., Connor, D.J., Ahern, G.L., Baxter, L.C., Rapcsak, S.Z., Shi, J., Woodruff, B.K., Locke, D.E.C., Snyder, C.H., Alexander, G.E., Rademakers, R., Reiman, E.M., 2009. Longitudinal Modeling of Age-Related Memory Decline and the APOE ε4 Effect. N. Engl. J. Med. 361, 255–263. https://doi.org/10.1056/NEJMoa0809437

Casida, J.E., Quistad, G.B., 2005. Serine hydrolase targets of organophosphorus toxicants. Chem. Biol. Interact. 157–158, 277–

283. https://doi.org/10.1016/j.cbi.2005.10.036

Casida, J.E., Quistad, G.B., 2004. Organophosphate Toxicology: Safety Aspects of Nonacetylcholinesterase Secondary Targets. Chem. Res. Toxicol. 17, 983–998. https://doi.org/10.1021/tx0499259

Casida, J.E., Quistad, G.B., 1998. Golden Age of Insecticide Research: Past, Present, or Future? Annu. Rev. Entomol. 43, 1–16. https://doi.org/10.1146/annurev.ento.43.1.1

Caulfield, M.P., Birdsall, N.J.M., 1998. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol. Rev. 50, 279–90.

Cellot, G., Cherubini, E., 2014. GABAergic Signaling as Therapeutic Target for Autism Spectrum Disorders. Front. Pediatr. 2, 1–11. https://doi.org/10.3389/fped.2014.00070

Chaliha, D., Albrecht, M., Vaccarezza, M., Takechi, R., Lam, V., Al-Salami, H., Mamo, J., 2020. A Systematic Review of the Valproic-Acid-Induced Rodent Model of Autism. Dev. Neurosci. 42, 12–48. https://doi.org/10.1159/000509109

Champagne, D., Dupuy, J.B., Rochford, J., Poirier, J., 2002. Apolipoprotein E knockout mice display procedural deficits in the Morris water maze: analysis of learning strategies in three versions of the task. Neuroscience 114, 641–654. https://doi.org/10.1016/S0306-4522(02)00313-5

Changeux, J.P., Bertrand, D., Corringer, P.J., Dehaene, S., Edelstein, S., Léna, C., Le Novère, N., Marubio, L., Picciotto, M., Zoli, M., 1998. Brain nicotinic receptors: structure and regulation, role in learning and reinforcement. Brain Res. Rev. 26, 198–216. https://doi.org/10.1016/S0165-0173(97)00040-4

Chaste, P., Leboyer, M., 2012. Autism risk factors: genes, environment, and gene-environment interactions. Dialogues Clin. Neurosci. 14, 281–292. https://doi.org/10.31887/DCNS.2012.14.3/pchaste

Chen, V.S., Morrison, J.P., Southwell, M.F., Foley, J.F., Bolon, B., Elmore, S.A., 2017. Histology Atlas of the Developing Prenatal and Postnatal Mouse Central Nervous System, with Emphasis on Prenatal Days E7.5 to E18.5. Toxicol. Pathol. 45, 705–744. https://doi.org/10.1177/0192623317728134

Chen, Y., Durakoglugil, M.S., Xian, X., Herz, J., 2010. ApoE4 reduces glutamate receptor function and synaptic plasticity by selectively impairing ApoE receptor recycling. Proc. Natl. Acad. Sci. 107, 12011–12016. https://doi.org/10.1073/pnas.0914984107

Chiapella, G., Flores-Martín, J., Ridano, M.E., Reyna, L., Magnarelli de Potas, G., Panzetta-Dutari, G.M., Genti-Raimondi, S., 2013. The organophosphate chlorpyrifos disturbs redox balance and triggers antioxidant defense mechanisms in JEG-3 cells. Placenta 34, 792–798. https://doi.org/10.1016/j.placenta.2013.06.007

Corder, E.H., Ghebremedhin, E., Taylor, M.G., Thal, D.R., Ohm, T.G., Braak, H., 2004. The Biphasic Relationship between Regional Brain Senile Plaque and Neurofibrillary Tangle Distributions: Modification by Age, Sex, and APOE Polymorphism. Ann. N. Y. Acad. Sci. 1019, 24–28. https://doi.org/10.1196/annals.1297.005

Corder, E.H., Saunders, A.M., Risch, N.J., Strittmatter, W.J., Schmechel, D.E., Gaskell, P.C., Rimmler, J.B., Locke, P.A., Conneally, P.M., Schmader, K.E., Small, G.W., Roses, A.D., Haines, J.L., Pericak-Vance, M.A., 1994. Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer disease. Nat. Genet. 7, 180–184. https://doi.org/10.1038/ng0694-180

Corder, E.H., Saunders, A.M., Strittmatter, W.J., Schmechel, D.E., Gaskell, P.C., Small, G.W., Roses, A.D., Haines, J.L., Pericak-Vance, M.A., 1993. Gene Dose of Apolipoprotein E Type 4 Allele and the Risk of Alzheimer's Disease in Late Onset Families. Science. 261, 921–923. https://doi.org/10.1126/science.8346443

Costa, L.G., 2006. Current issues in organophosphate toxicology. Clin. Chim. Acta 366, 1–13. https://doi.org/10.1016/j.cca.2005.10.008

Costa, L.G., Cole, T.B., Vitalone, A., Furlong, C.E., 2005. Measurement of paraoxonase (PON1) status as a potential biomarker of susceptibility to organophosphate toxicity. Clin. Chim. Acta 352, 37–47. https://doi.org/10.1016/j.cccn.2004.09.019

Crawley, J.N., 2012. Translational animal models of autism and neurodevelopmental disorders. Dialogues Clin. Neurosci. 14, 293–305. https://doi.org/10.31887/DCNS.2012.14.3/jcrawley

Crawley, J.N., 2004. Designing mouse behavioral tasks relevant to autistic-like behaviors. Ment. Retard. Dev. Disabil. Res. Rev. 10, 248–258. https://doi.org/10.1002/mrdd.20039

Currenti, S.A., 2010. Understanding and Determining the Etiology of Autism. Cell. Mol. Neurobiol. 30, 161–171. https://doi.org/10.1007/s10571-009-9453-8

D'Arcangelo, G., Homayouni, R., Keshvara, L., Rice, D.S., Sheldon, M., Curran, T., 1999. Reelin Is a Ligand for Lipoprotein Receptors. Neuron 24, 471–479. https://doi.org/10.1016/S0896-6273(00)80860-0

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

References

Darstein, M., Petralia, R.S., Swanson, G.T., Wenthold, R.J., Heinemann, S.F., 2003. Distribution of Kainate Receptor Subunits at Hippocampal Mossy Fiber Synapses. J. Neurosci. 23, 8013–8019. https://doi.org/10.1523/JNEUROSCI.23-22-08013.2003

De Silva, H.J., Samarawickrema, N.A., Wickremasinghe, A.R., 2006. Toxicity due to organophosphorus compounds: what about chronic exposure? Trans. R. Soc. Trop. Med. Hyg. 100, 803–806. https://doi.org/10.1016/j.trstmh.2006.05.001

Deiana, S., Platt, B., Riedel, G., 2011. The cholinergic system and spatial learning. Behav. Brain Res. 221, 389–411. https://doi.org/10.1016/j.bbr.2010.11.036

Di, J., Li, J., O'Hara, B., Alberts, I., Xiong, L., Li, J., Li, X., 2020. The role of GABAergic neural circuits in the pathogenesis of autism spectrum disorder. Int. J. Dev. Neurosci. 80, 73–85. https://doi.org/10.1002/jdn.10005

Diamond, A., 1990. Rate of Maturation of the Hippocampus and the Developmental Progression of Children's Performance on the Delayed Non-Matching to Sample and Visual Paired Comparison Tasks. Ann. N. Y. Acad. Sci. 608, 394–433. https://doi.org/10.1111/j.1749-6632.1990.tb48904.x

Dietschy, J.M., Turley, S.D., 2004. Brain Lipids. Cholesterol metabolism in the central nervous system during early development and in the mature animal. J. Lipid Res. 45, 1375–1397. https://doi.org/10.1194/jlr.R400004-JLR200

Dolgin, E., 2017. The most popular genes in the human genome. Nature 551, 427–431. https://doi.org/10.1038/d41586-017-07291-9

Dong, L.M., Weisgraber, K.H., 1996. Human Apolipoprotein E4 Domain Interaction. J. Biol. Chem. 271, 19053–19057. https://doi.org/10.1074/jbc.271.32.19053

Dong, L.M., Wilson, C., Wardell, M.R., Simmons, T., Mahley, R.W., Weisgraber, K.H., Agard, D.A., 1994. Human apolipoprotein E. Role of arginine 61 in mediating the lipoprotein preferences of the E3 and E4 isoforms. J. Biol. Chem. 269, 22358–22365. https://doi.org/10.1016/S0021-9258(17)31797-0

Dupuy, S.T., Houser, C.R., 1996. Prominent Expression of Two Forms of Glutamate Decarboxylase in the Embryonic and Early Postnatal Rat Hippocampal Formation. J. Neurosci. 16, 6919–6932. https://doi.org/10.1523/JNEUROSCI.16-21-06919.1996

East, B.S., Fleming, G., Peng, K., Olofsson, J.K., Levy, E., Mathews, P.M., Wilson, D.A., 2018. Human Apolipoprotein E Genotype Differentially Affects Olfactory Behavior and Sensory Physiology in Mice. Neuroscience 380, 103–110. https://doi.org/10.1016/j.neuroscience.2018.04.009

Easterbrooks, M.A., Bureau, J.F., Lyons-Ruth, K., 2012. Developmental correlates and predictors of emotional availability in mother–child interaction: A longitudinal study from infancy to middle childhood. Dev. Psychopathol. 24, 65–78. https://doi.org/10.1017/S0954579411000666

Eaton, D.L., Daroff, R.B., Autrup, H., Bridges, J., Buffler, P., Costa, L.G., Coyle, J., McKhann, G., Mobley, W.C., Nadel, L., Neubert, D., Schulte-Hermann, R., Spencer, P.S., 2008. Review of the Toxicology of Chlorpyrifos With an Emphasis on Human Exposure and Neurodevelopment. Crit. Rev. Toxicol. 38, 1–125. https://doi.org/10.1080/10408440802272158

EFSA, 2019. Statement on the available outcomes of the human health assessment in the context of the pesticides peer review of the active substance chlorpyrifos. EFSA J. 17. https://doi.org/10.2903/j.efsa.2019.5809

EFSA, 2014. Conclusion on the peer review of the pesticide human health risk assessment of the active substance chlorpyrifos. EFSA J. 12, 1–34. https://doi.org/10.2903/j.efsa.2014.3640

Eggers, C., Herholz, K., Kalbe, E., Heiss, W.D., 2006. Cortical acetylcholine esterase activity and ApoE4-genotype in Alzheimer disease. Neurosci. Lett. 408, 46–50. https://doi.org/10.1016/j.neulet.2006.08.061

Eisenberg, D.T.A., Kuzawa, C.W., Hayes, M.G., 2010. Worldwide allele frequencies of the human apolipoprotein E gene: Climate, local adaptations, and evolutionary history. Am. J. Phys. Anthropol. 143, 100–111. https://doi.org/10.1002/ajpa.21298

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95. https://doi.org/10.1016/0006-2952(61)90145-9

Elsabbagh, M., Divan, G., Koh, Y.J., Kim, Y.S., Kauchali, S., Marcín, C., Montiel-Nava, C., Patel, V., Paula, C.S., Wang, C., Yasamy, M.T., Fombonne, E., 2012. Global Prevalence of Autism and Other Pervasive Developmental Disorders. Autism Res. 5, 160–179. https://doi.org/10.1002/aur.239

EPA, 2021. Chlorpyrifos; Tolerance Revocations. Fed. Regist. / Vol. 86, No. 165 / Monday, August 30, 2021 / Rules Regul. 86, 21–42.

Erlander, M.G., Tillakaratne, N.J.K., Feldblum, S., Patel, N., Tobin, A.J., 1991. Two genes encode distinct glutamate decarboxylases. Neuron 7, 91–100. https://doi.org/10.1016/0896-6273(91)90077-D

Esclapez, M., Houser, C.R., 1999. Up-regulation of GAD65 and GAD67 in remaining hippocampal GABA neurons in a model of temporal lobe epilepsy. J. Comp. Neurol. 412, 488–505. https://doi.org/10.1002/(SICI)1096-9861(19990927)412:3<488::AID-CNE8>3.0.CO;2-6

Fan, J., Tao, W., Li, X., Li, H., Zhang, J., Wei, D., Chen, Y., Zhang, Z., 2019. The Contribution of Genetic Factors to Cognitive

Impairment and Dementia: Apolipoprotein E Gene, Gene Interactions, and Polygenic Risk. Int. J. Mol. Sci. 20, 1177. https://doi.org/10.3390/ijms20051177

Farkhondeh, T., Amirabadizadeh, A., Samarghandian, S., Mehrpour, O., 2020. Impact of chlorpyrifos on blood glucose concentration in an animal model: a systematic review and meta-analysis. Environ. Sci. Pollut. Res. 27, 2474–2481. https://doi.org/10.1007/s11356-019-07229-w

Farrant, M., Nusser, Z., 2005. Variations on an inhibitory theme: phasic and tonic activation of GABAA receptors. Nat. Rev. Neurosci. 6, 215–229. https://doi.org/10.1038/nrn1625

Fenske, R.A., Farahat, F.M., Galvin, K., Fenske, E.K., Olson, J.R., 2012. Contributions of inhalation and dermal exposure to chlorpyrifos dose in Egyptian cotton field workers. Int. J. Occup. Environ. Health 18, 198–209. https://doi.org/10.1179/1077352512Z.00000000030

Fernell, E., Eriksson, Gillberg, C., 2013. Early diagnosis of autism and impact on prognosis: a narrative review. Clin. Epidemiol. 5, 33. https://doi.org/10.2147/CLEP.S41714

Ferri, S.L., Abel, T., Brodkin, E.S., 2018. Sex Differences in Autism Spectrum Disorder: a Review. Curr. Psychiatry Rep. 20, 9. https://doi.org/10.1007/s11920-018-0874-2

Fleisher, A., 2005. Sex, Apolipoprotein E ε4 Status, and Hippocampal Volume in Mild Cognitive Impairment. Arch. Neurol. 62, 953. https://doi.org/10.1001/archneur.62.6.953

Florey, E., 1991. GABA: history and perspectives. Can. J. Physiol. Pharmacol. 69, 1049–1056. https://doi.org/10.1139/y91-156

Fritschy, J., Paysan, J., Enna, A., Mohler, H., 1994. Switch in the expression of rat GABAA-receptor subtypes during postnatal development: an immunohistochemical study. J. Neurosci. 14, 5302–5324. https://doi.org/10.1523/JNEUROSCI.14-09-05302.1994

Gambrill, A.C., Barria, A., 2011. NMDA receptor subunit composition controls synaptogenesis and synapse stabilization. Proc. Natl. Acad. Sci. 108, 5855–5860. https://doi.org/10.1073/pnas.1012676108

Gardner, C.R., 1985. Distress vocalization in rat pups a simple screening method for anxiolytic drugs. J. Pharmacol. Methods 14, 181–187. https://doi.org/10.1016/0160-5402(85)90031-2

Georgieva, E., Yancheva, V., Stoyanova, S., Velcheva, I., Iliev, I., Vasileva, T., Bivolarski, V., Petkova, E., László, B., Nyeste, K., Antal, L., 2021. Which Is More Toxic? Evaluation of the Short-Term Toxic Effects of Chlorpyrifos and Cypermethrin on Selected Biomarkers in Common Carp (Cyprinus carpio, Linnaeus 1758). Toxics 9, 125. https://doi.org/10.3390/toxics9060125

Getman, D.K., Eubanks, J.H., Camp, S., Evans, G.A., Taylor, P., 1992. The human gene encoding acetylcholinesterase is located on the long arm of chromosome 7. Am. J. Hum. Genet. 51, 170–7.

Glynn, P., 1999. Neuropathy target esterase. Biochem. J. 344, 625. https://doi.org/10.1042/0264-6021:3440625

Gordon, I., Grauer, E., Genis, I., Sehayek, E., Michaelson, D.M., 1995. Memory deficits and cholinergic impairments in apolipoprotein E-deficient mice. Neurosci. Lett. 199, 1–4. https://doi.org/10.1016/0304-3940(95)12006-P

Gotti, C., Clementi, F., 2004. Neuronal nicotinic receptors: from structure to pathology. Prog. Neurobiol. 74, 363–396. https://doi.org/10.1016/j.pneurobio.2004.09.006

Gotti, C., Zoli, M., Clementi, F., 2006. Brain nicotinic acetylcholine receptors: native subtypes and their relevance. Trends Pharmacol. Sci. 27, 482–491. https://doi.org/10.1016/j.tips.2006.07.004

Grandjean, P., Landrigan, P.J., 2014. Neurobehavioural effects of developmental toxicity. Lancet Neurol. 13, 330–338. https://doi.org/10.1016/S1474-4422(13)70278-3

Graybeal, J.J., Bozzelli, P.L., Graybeal, L.L., Groeber, C.M., McKnight, P.E., Cox, D.N., Flinn, J.M., 2014. Human ApoE ε4 Alters Circadian Rhythm Activity, IL-1β, and GFAP in CRND8 Mice. J. Alzheimer's Dis. 43, 823–834. https://doi.org/10.3233/JAD-132009

Griffin, P., Mason, H., Heywood, K., Cocker, J., 1999. Oral and dermal absorption of chlorpyrifos: a human volunteer study. Occup. Environ. Med. 56, 10–13. https://doi.org/10.1136/oem.56.1.10

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

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

Grzadzinski, R., Huerta, M., Lord, C., 2013. DSM-5 and autism spectrum disorders (ASDs): an opportunity for identifying ASD subtypes. Mol. Autism 4, 12. https://doi.org/10.1186/2040-2392-4-12

Guo, J., Zhang, J., Wu, C., Lv, S., Lu, D., Qi, X., Jiang, S., Feng, C., Yu, H., Liang, W., Chang, X., Zhang, Y., Xu, H., Cao, Y., Wang, G., Zhou, Z., 2019. Associations of prenatal and childhood chlorpyrifos exposure with Neurodevelopment of 3-year-old children. Environ.

References

Pollut. 251, 538–546. https://doi.org/10.1016/j.envpol.2019.05.040

Gzielo, K., Potasiewicz, A., Hołuj, M., Litwa, E., Popik, P., Nikiforuk, A., 2020. Valproic acid exposure impairs ultrasonic communication in infant, adolescent and adult rats. Eur. Neuropsychopharmacol. 41, 52–62. https://doi.org/10.1016/j.euroneuro.2020.09.006

Hashimoto, H., Saito, T.R., Furudate, S., Takahashi, K.W., 2001. Prolactin Levels and Maternal Behavior Induced by Ultrasonic Vocalizations of the Rat Pup. Exp. Anim. 50, 307–312. https://doi.org/10.1538/expanim.50.307

Hatters, D.M., Peters-Libeu, C.A., Weisgraber, K.H., 2006. Apolipoprotein E structure: insights into function. Trends Biochem. Sci. 31, 445–454. https://doi.org/10.1016/j.tibs.2006.06.008

He, X., Tu, Y., Song, Y., Yang, G., You, M., 2022. The relationship between pesticide exposure during critical neurodevelopment and autism spectrum disorder: A narrative review. Environ. Res. 203, 111902. https://doi.org/10.1016/j.envres.2021.111902

Herriage, S., Chen, G., Pope, C., 2022. Concentration-dependent effects of chlorpyrifos oxon on peroxisome proliferator-activated receptor signaling in MCF-7 cells. Toxicol. Vitr. 78, 105268. https://doi.org/10.1016/j.tiv.2021.105268

Hollmann, M., Heinemann, S., 1994. Cloned Glutamate Receptors. Annu. Rev. Neurosci. 17, 31–108. https://doi.org/10.1146/annurev.ne.17.030194.000335

Hu, V.W., Sarachana, T., Sherrard, R.M., Kocher, K.M., 2015. Investigation of sex differences in the expression of RORA and its transcriptional targets in the brain as a potential contributor to the sex bias in autism. Mol. Autism 6, 7. https://doi.org/10.1186/2040-2392-6-7

Hu, Z., Yang, Y., Zhao, Y., Yu, H., Ying, X., Zhou, D., Zhong, J., Zheng, Z., Liu, J., Pan, R., Zhang, W., Cheng, F., Duan, S., 2018. APOE hypermethylation is associated with autism spectrum disorder in a Chinese population. Exp. Ther. Med. 15, 4749–4754. https://doi.org/10.3892/etm.2018.6069

Huang, Y., Mahley, R.W., 2014. Apolipoprotein E: Structure and function in lipid metabolism, neurobiology, and Alzheimer's diseases. Neurobiol. Dis. 72, 3–12. https://doi.org/10.1016/j.nbd.2014.08.025

ICD-11, 2022. URL https://icd.who.int/en (accessed 8.18.22).

Insel, T.R., Hill, J.L., Mayor, R.B., 1986. Rat pup ultrasonic isolation calls: Possible mediation by the benzodiazepine receptor complex. Pharmacol. Biochem. Behav. 24, 1263–1267. https://doi.org/10.1016/0091-3057(86)90182-6

Jentink, J., Loane, M.A., Dolk, H., Barisic, I., Garne, E., Morris, J.K., den Berg, L.T.W. de J., 2010. Valproic Acid Monotherapy in Pregnancy and Major Congenital Malformations. Obstet. Gynecol. Surv. 65, 619–620. https://doi.org/10.1097/OGX.0b013e3182021f65

Jiang, W., Kakizaki, T., Fujihara, K., Miyata, S., Zhang, Y., Suto, T., Kato, D., Saito, S., Shibasaki, K., Ishizaki, Y., Isoda, K., Yokoo, H., Obinata, H., Hirano, T., Miyasaka, Y., Mashimo, T., Yanagawa, Y., 2022. Impact of GAD65 and/or GAD67 deficiency on perinatal development in rats. FASEB J. 36, 1–10. https://doi.org/10.1096/fj.202101389R

Johnson, M., Glynn, P., 1995. Neuropathy target esterase (NTE) and organophosphorus-induced delayed polyneuropathy (OPIDP): recent advances. Toxicol. Lett. 82–83, 459–463. https://doi.org/10.1016/0378-4274(95)03495-1

Kálmán, J., Juhász, A., Rakonczay, Z., Ábrahám, G., Zana, M., Boda, K., Farkas, T., Penke, B., Janka, Z., 2004. Increased serum butyrylcholinesterase activity in type IIb hyperlipidaemic patients. Life Sci. 75, 1195–1204. https://doi.org/10.1016/j.lfs.2004.02.019

Kanner, L., 1968. Autistic disturbances of affective contact. Acta Paedopsychiatr. 35, 100–36.

Karalliedde, L., Edwards, P., Marrs, T., 2003. Variables influencing the toxic response to organophosphates in humans. Food Chem. Toxicol. 41, 1–13. https://doi.org/10.1016/S0278-6915(02)00232-6

Kim, J., Basak, J.M., Holtzman, D.M., 2009. The Role of Apolipoprotein E in Alzheimer's Disease. Neuron 63, 287–303. https://doi.org/10.1016/j.neuron.2009.06.026

Kleifeld, O., Diebler, M., Chapman, S., Oron, L., Michaelson, D.M., 1998. The effects of apolipoprotein e deficiency on braincholinergic neurons. Int. J. Dev. Neurosci. 16, 755–762. https://doi.org/10.1016/S0736-5748(98)00084-7

Kofman, O., Lan, A., Raykin, E., Zega, K., Brodski, C., 2020. Developmental and social deficits and enhanced sensitivity to prenatal chlorpyrifos in PON1-/- mouse pups and adults. PLoS One 15, e0239738. https://doi.org/10.1371/journal.pone.0239738

Kraeuter, A.K., Guest, P.C., Sarnyai, Z., 2019. The Open Field Test for Measuring Locomotor Activity and Anxiety-Like Behavior, in: Methods in Molecular Biology. pp. 99–103. https://doi.org/10.1007/978-1-4939-8994-2_9

Kruse, A.C., Kobilka, B.K., Gautam, D., Sexton, P.M., Christopoulos, A., Wess, J., 2014. Muscarinic acetylcholine receptors: novel opportunities for drug development. Nat. Rev. Drug Discov. 13, 549–560. https://doi.org/10.1038/nrd4295

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

References

Lan, A., Stein, D., Portillo, M., Toiber, D., Kofman, O., 2019. Impaired innate and conditioned social behavior in adult C57Bl6/J mice prenatally exposed to chlorpyrifos. Behav. Brain Funct. 15, 2. https://doi.org/10.1186/s12993-019-0153-3

Laurie, D., Wisden, W., Seeburg, P., 1992. The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. J. Neurosci. 12, 4151–4172. https://doi.org/10.1523/JNEUROSCI.12-11-04151.1992

Lee, V., Maguire, J., 2014. The impact of tonic GABAA receptor-mediated inhibition on neuronal excitability varies across brain region and cell type. Front. Neural Circuits 8, 1–27. https://doi.org/10.3389/fncir.2014.00003

Lemonnier, E., Villeneuve, N., Sonie, S., Serret, S., Rosier, A., Roue, M., Brosset, P., Viellard, M., Bernoux, D., Rondeau, S., Thummler, S., Ravel, D., Ben-Ari, Y., 2017. Effects of bumetanide on neurobehavioral function in children and adolescents with autism spectrum disorders. Transl. Psychiatry 7, e1056–e1056. https://doi.org/10.1038/tp.2017.10

Leonzino, M., Busnelli, M., Antonucci, F., Verderio, C., Mazzanti, M., Chini, B., 2016. The Timing of the Excitatory-to-Inhibitory GABA Switch Is Regulated by the Oxytocin Receptor via KCC2. Cell Rep. 15, 96–103. https://doi.org/10.1016/j.celrep.2016.03.013

Leung, L., Andrews-Zwilling, Y., Yoon, S.Y., Jain, S., Ring, K., Dai, J., Wang, M.M., Tong, L., Walker, D., Huang, Y., 2012. Apolipoprotein E4 Causes Age- and Sex-Dependent Impairments of Hilar GABAergic Interneurons and Learning and Memory Deficits in Mice. PLoS One 7, e53569. https://doi.org/10.1371/journal.pone.0053569

Lionetto, M.G., Caricato, R., Calisi, A., Giordano, M.E., Schettino, T., 2013. Acetylcholinesterase as a Biomarker in Environmental and Occupational Medicine: New Insights and Future Perspectives. Biomed Res. Int. 2013, 1–8. https://doi.org/10.1155/2013/321213

Liu, M., Kuhel, D.G., Shen, L., Hui, D.Y., Woods, S.C., 2012. Apolipoprotein E does not cross the blood-cerebrospinal fluid barrier, as revealed by an improved technique for sampling CSF from mice. Am. J. Physiol. Integr. Comp. Physiol. 303, R903–R908. https://doi.org/10.1152/ajpregu.00219.2012

Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262

Lord, C., Elsabbagh, M., Baird, G., Veenstra-Vanderweele, J., 2018. Autism spectrum disorder. Lancet 392, 508–520. https://doi.org/10.1016/S0140-6736(18)31129-2

Löscher, W., 2002. Basic Pharmacology of Valproate. CNS Drugs 16, 669–694. https://doi.org/10.2165/00023210-200216100-00003

Lowry, O., Rosebrough, N., Farr, A.L., Randall, R., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275. https://doi.org/10.1016/S0021-9258(19)52451-6

Luján, R., Shigemoto, R., López-Bendito, G., 2005. Glutamate and GABA receptor signalling in the developing brain. Neuroscience 130, 567–580. https://doi.org/10.1016/j.neuroscience.2004.09.042

Mackay, D., Giesy, J.P., Solomon, K.R., 2014. Fate in the Environment and Long-Range Atmospheric Transport of the Organophosphorus Insecticide, Chlorpyrifos and Its Oxon, in: Reviews of Environmental Contamination and Toxicology. pp. 35–76. https://doi.org/10.1007/978-3-319-03865-0_3

Mahley, R.W., 1988. Apolipoprotein E: Cholesterol Transport Protein with Expanding Role in Cell Biology. Science. 240, 622–630. https://doi.org/10.1126/science.3283935

Mahley, R.W., Huang, Y., 2012. Small-Molecule Structure Correctors Target Abnormal Protein Structure and Function: Structure Corrector Rescue of Apolipoprotein E4–Associated Neuropathology. J. Med. Chem. 55, 8997–9008. https://doi.org/10.1021/jm3008618

Mahley, R.W., Huang, Y., 2007. Atherogenic remnant lipoproteins: role for proteoglycans in trapping, transferring, and internalizing. J. Clin. Invest. 117, 1–5. https://doi.org/10.1172/JCI30889

Mahley, R.W., Huang, Y., Rall, S.C., 1999. Pathogenesis of type III hyperlipoproteinemia (dysbetalipoproteinemia): questions, quandaries, and paradoxes. J. Lipid Res. 40, 1933–1949. https://doi.org/10.1016/S0022-2275(20)32417-2

Mahley, R.W., Innerarity, T.L., Rall, S.C., Weisgraber, K.H., 1984. Plasma lipoproteins: apolipoprotein structure and function. J. Lipid Res. 25, 1277–94.

Mahley, R.W., Rall, S.C., 2000. Apolipoprotein E: Far More Than a Lipid Transport Protein. Annu. Rev. Genomics Hum. Genet. 1, 507–537. https://doi.org/10.1146/annurev.genom.1.1.507

Mahley, R.W., Stanley C. Rall, J., 2001. Type III Hyperlipoproteinemia (Dysbetalipoproteinemia): The Role of Apolipoprotein E in Normal and Abnormal Lipoprotein Metabolism, in: Metabolic & Molecular Bases of Inherited Disease. https://doi.org/10.1036/ommbid.148

Marais, A.D., 2019. Apolipoprotein E in lipoprotein metabolism, health and cardiovascular disease. Pathology 51, 165–176. https://doi.org/10.1016/j.pathol.2018.11.002

References

Marchant, N.L., King, S.L., Tabet, N., Rusted, J.M., 2010. Positive Effects of Cholinergic Stimulation Favor Young APOE ε4 Carriers. Neuropsychopharmacology 35, 1090–1096. https://doi.org/10.1038/npp.2009.214

Markram, K., Rinaldi, T., Mendola, D. La, Sandi, C., Markram, H., 2008. Abnormal Fear Conditioning and Amygdala Processing in an Animal Model of Autism. Neuropsychopharmacology 33, 901–912. https://doi.org/10.1038/sj.npp.1301453

Martin, L., Furuta, A., Blackstone, C., 1998. AMPA receptor protein in developing rat brain: glutamate receptor-1 expression and localization change at regional, cellular, and subcellular levels with maturation. Neuroscience 83, 917–928. https://doi.org/10.1016/S0306-4522(97)00411-9

Massoulié, J., 2002. The Origin of the Molecular Diversity and Functional Anchoring of Cholinesterases. Neurosignals 11, 130–143. https://doi.org/10.1159/000065054

Matta, J.A., Ashby, M.C., Sanz-Clemente, A., Roche, K.W., Isaac, J.T.R., 2011. mGluR5 and NMDA Receptors Drive the Experience- and Activity-Dependent NMDA Receptor NR2B to NR2A Subunit Switch. Neuron 70, 339–351. https://doi.org/10.1016/j.neuron.2011.02.045

Mauch, D.H., Nägler, K., Schumacher, S., Göritz, C., Müller, E.C., Otto, A., Pfrieger, F.W., 2001. CNS Synaptogenesis Promoted by Glia-Derived Cholesterol. Science. 294, 1354–1357. https://doi.org/10.1126/science.294.5545.1354

Meador, K.J., 2008. Effects of in Utero Antiepileptic Drug Exposure. Epilepsy Curr. 8, 143–147. https://doi.org/10.1111/j.1535-7511.2008.00273.x

Mehta, M. V., Gandal, M.J., Siegel, S.J., 2011. mGluR5-Antagonist Mediated Reversal of Elevated Stereotyped, Repetitive Behaviors in the VPA Model of Autism. PLoS One 6, e26077. https://doi.org/10.1371/journal.pone.0026077

Meldrum, B.S., 2000. Glutamate as a Neurotransmitter in the Brain: Review of Physiology and Pathology. J. Nutr. 130, 1007S–1015S. https://doi.org/10.1093/jn/130.4.1007S

Meng, F.T., Zhao, J., Fang, H., Liu, Y.J., 2015. The influence of chronic stress on anxiety-like behavior and cognitive function in different human GFAP-ApoE transgenic adult male mice. Stress 18, 419–426. https://doi.org/10.3109/10253890.2015.1040986

Meshorer, E., Toiber, D., Zurel, D., Sahly, I., Dori, A., Cagnano, E., Schreiber, L., Grisaru, D., Tronche, F., Soreq, H., 2004. Combinatorial Complexity of 5′ Alternative Acetylcholinesterase Transcripts and Protein Products. J. Biol. Chem. 279, 29740–29751. https://doi.org/10.1074/jbc.M402752200

Métin, C., Denizot, J.P., Ropert, N., 2000. Intermediate Zone Cells Express Calcium-Permeable AMPA Receptors and Establish Close Contact with Growing Axons. J. Neurosci. 20, 696–708. https://doi.org/10.1523/JNEUROSCI.20-02-00696.2000

Meuling, W.J.A., Ravensberg, L.C., Roza, L., Hemmen, J.J. van, 2005. Dermal absorption of chlorpyrifos in human volunteers. Int. Arch. Occup. Environ. Health 78, 44–50. https://doi.org/10.1007/s00420-004-0558-6

Meyer, M.R., Tschanz, J.T., Norton, M.C., Welsh-Bohmer, K.A., Steffens, D.C., Wyse, B.W., Breitner, J.C.S., 1998. APOE genotype predicts when — not whether — one is predisposed to develop Alzheimer disease. Nat. Genet. 19, 321–322. https://doi.org/10.1038/1206

Mileson, B., 1998. Common Mechanism of Toxicity: A Case Study of Organophosphorus Pesticides,. Toxicol. Sci. 41, 8–20. https://doi.org/10.1006/toxs.1997.2431

Millenson, M.E., Braun, J.M., Calafat, A.M., Barr, D.B., Huang, Y.T., Chen, A., Lanphear, B.P., Yolton, K., 2017. Urinary organophosphate insecticide metabolite concentrations during pregnancy and children's interpersonal, communication, repetitive, and stereotypic behaviors at 8 years of age: The home study. Environ. Res. 157, 9–16. https://doi.org/10.1016/j.envres.2017.05.008

Miller, D.R., McClain, E.S., Dodds, J.N., Balinski, A., May, J.C., McLean, J.A., Cliffel, D.E., 2021. Chlorpyrifos Disrupts Acetylcholine Metabolism Across Model Blood-Brain Barrier. Front. Bioeng. Biotechnol. 9, 1–13. https://doi.org/10.3389/fbioe.2021.622175

Mondadori, C.R.A., de Quervain, D.J.F., Buchmann, A., Mustovic, H., Wollmer, M.A., Schmidt, C.F., Boesiger, P., Hock, C., Nitsch, R.M., Papassotiropoulos, A., Henke, K., 2007. Better Memory and Neural Efficiency in Young Apolipoprotein E 4 Carriers. Cereb. Cortex 17, 1934–1947. https://doi.org/10.1093/cercor/bhl103

Monyer, H., Burnashev, N., Laurie, D.J., Sakmann, B., Seeburg, P.H., 1994. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12, 529–540. https://doi.org/10.1016/0896-6273(94)90210-0

Morales-Navas, M., Castaño-Castaño, S., Pérez-Fernández, C., Sánchez-Gil, A., Teresa Colomina, M., Leinekugel, X., Sánchez-Santed, F., 2020. Similarities between the Effects of Prenatal Chlorpyrifos and Valproic Acid on Ultrasonic Vocalization in Infant Wistar Rats. Int. J. Environ. Res. Public Health 17, 6376. https://doi.org/10.3390/ijerph17176376

Moser, V.C., 2000. Dose–response and time-course of neurobehavioral changes following oral chlorpyrifos in rats of different ages. Neurotoxicol. Teratol. 22, 713–723. https://doi.org/10.1016/S0892-0362(00)00087-8

Mullin, A.P., Gokhale, A., Moreno-De-Luca, A., Sanyal, S., Waddington, J.L., Faundez, V., 2013. Neurodevelopmental disorders: mechanisms and boundary definitions from genomes, interactomes and proteomes. Transl. Psychiatry 3, e329–e329.

https://doi.org/10.1038/tp.2013.108

Muñoz, S.S., Garner, B., Ooi, L., 2019. Understanding the Role of ApoE Fragments in Alzheimer's Disease. Neurochem. Res. 44, 1297–1305. https://doi.org/10.1007/s11064-018-2629-1

National Center for Biotechnology Information, 2022. Chlorpyrifos | C9H11Cl3NO3PS - PubChem. URL https://pubchem.ncbi.nlm.nih.gov/compound/2730 (accessed 7.28.22).

National Center for Biotechnology Information, 2022. Valproic acid | C8H16O2 - PubChem. URL https://pubchem.ncbi.nlm.nih.gov/compound/Valproic-acid (accessed 8.18.22).

Naughton, S.X., Terry, A. V., 2018. Neurotoxicity in acute and repeated organophosphate exposure. Toxicology 408, 101–112. https://doi.org/10.1016/j.tox.2018.08.011

Nelson, S.B., Valakh, V., 2015. Excitatory/Inhibitory Balance and Circuit Homeostasis in Autism Spectrum Disorders. Neuron 87, 684–698. https://doi.org/10.1016/j.neuron.2015.07.033

Nicolini, C., Fahnestock, M., 2018. The valproic acid-induced rodent model of autism. Exp. Neurol. 299, 217–227. https://doi.org/10.1016/j.expneurol.2017.04.017

Nisar, S., Bhat, A.A., Masoodi, T., Hashem, S., Akhtar, S., Ali, T.A., Amjad, S., Chawla, S., Bagga, P., Frenneaux, M.P., Reddy, R., Fakhro, K., Haris, M., 2022. Genetics of glutamate and its receptors in autism spectrum disorder. Mol. Psychiatry 27, 2380–2392. https://doi.org/10.1038/s41380-022-01506-w

Nolan, R.J., Rick, D.L., Freshour, N.L., Saunders, J.H., 1984. Chlorpyrifos: Pharmacokinetics in human volunteers. Toxicol. Appl. Pharmacol. 73, 8–15. https://doi.org/10.1016/0041-008X(84)90046-2

Numan, M., 1988. Neural basis of maternal behavior in the rat. Psychoneuroendocrinology 13, 47–62. https://doi.org/10.1016/0306-4530(88)90006-6

Olsen, R.W., Sieghart, W., 2008. International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Update. Pharmacol. Rev. 60, 243–60. https://doi.org/10.1124/pr.108.00505

Oriá, R.B., Patrick, P.D., Zhang, H., Lorntz, B., de Castro Costa, C.M., Brito, G.A.C., Barrett, L.J., Lima, A.A.M., Guerrant, R.L., 2005. APOE4 Protects the Cognitive Development in Children with Heavy Diarrhea Burdens in Northeast Brazil. Pediatr. Res. 57, 310–316. https://doi.org/10.1203/01.PDR.0000148719.82468.CA

Ostatníková, D., Lakatosová, S., Babková, J., Hodosy, J., Celec, P., 2021. Testosterone and the Brain: From Cognition to Autism. Physiol. Res. 69, S403–S419. https://doi.org/10.33549/10.33549/physiolres.934592

Otênio, J.K., Souza, K.D., Alberton, O., Alberton, L.R., Moreno, K.G.T., Gasparotto Junior, A., Palozi, R.A.C., Lourenço, E.L.B., Jacomassi, E., 2022. Thyroid-disrupting effects of chlorpyrifos in female Wistar rats. Drug Chem. Toxicol. 45, 387–392. https://doi.org/10.1080/01480545.2019.1701487

Owens, D.F., Kriegstein, A.R., 2002. Is there more to gaba than synaptic inhibition? Nat. Rev. Neurosci. 3, 715–727. https://doi.org/10.1038/nrn919

Pelch, K.E., Bolden, A.L., Kwiatkowski, C.F., 2019. Environmental Chemicals and Autism: A Scoping Review of the Human and Animal Research. Environ. Health Perspect. 127, 046001. https://doi.org/10.1289/EHP4386

Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M.T., Baker, M., Browne, W.J., Clark, A., Cuthill, I.C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S.T., Howells, D.W., Karp, N.A., Lazic, S.E., Lidster, K., MacCallum, C.J., Macleod, M., Pearl, E.J., Petersen, O.H., Rawle, F., Reynolds, P., Rooney, K., Sena, E.S., Silberberg, S.D., Steckler, T., Würbel, H., 2020. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLOS Biol. 18, e3000410. https://doi.org/10.1371/journal.pbio.3000410

Perez-Fernandez, C., Morales-Navas, M., Aguilera-Sáez, L.M., Abreu, A.C., Guardia-Escote, L., Fernández, I., Garrido-Cárdenas, J.A., Colomina, M.T., Giménez, E., Sánchez-Santed, F., 2020a. Medium and long-term effects of low doses of Chlorpyrifos during the postnatal, preweaning developmental stage on sociability, dominance, gut microbiota and plasma metabolites. Environ. Res. 184, 109341. https://doi.org/10.1016/j.envres.2020.109341

Perez-Fernandez, C., Morales-Navas, M., Guardia-Escote, L., Garrido-Cárdenas, J.A., Colomina, M.T., Giménez, E., Sánchez-Santed, F., 2020b. Long-term effects of low doses of Chlorpyrifos exposure at the preweaning developmental stage: A locomotor, pharmacological, brain gene expression and gut microbiome analysis. Food Chem. Toxicol. 135, 110865. https://doi.org/10.1016/j.fct.2019.110865

Peris-Sampedro, F., Basaure, P., Reverte, I., Cabré, M., Domingo, J.L., Colomina, M.T., 2015a. Chronic exposure to chlorpyrifos triggered body weight increase and memory impairment depending on human apoE polymorphisms in a targeted replacement mouse model. Physiol. Behav. 144, 37–45. https://doi.org/10.1016/j.physbeh.2015.03.006

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

References

Peris-Sampedro, F., Cabré, M., Basaure, P., Reverte, I., Domingo, J.L., Teresa Colomina, M., 2015b. Adulthood dietary exposure to a common pesticide leads to an obese-like phenotype and a diabetic profile in apoE3 mice. Environ. Res. 142, 169–176. https://doi.org/10.1016/j.envres.2015.06.036

Peris-Sampedro, F., Reverte, I., Basaure, P., Cabré, M., Domingo, J.L., Colomina, M.T., 2016. Apolipoprotein E (APOE) genotype and the pesticide chlorpyrifos modulate attention, motivation and impulsivity in female mice in the 5-choice serial reaction time task. Food Chem. Toxicol. 92, 224–235. https://doi.org/10.1016/j.fct.2016.03.029

Persico, A.M., D'Agruma, L., Zelante, L., Militerni, R., Bravaccio, C., Schneider, C., Melmed, R., Trillo, S., Montecchi, F., Elia, M., Palermo, M., Rabinowitz, D., Pascucci, T., Puglisi-Allegra, S., Reichelt, K.L., Muscarella, L., Guarnieri, V., Melgari, J.M., Conciatori, M., Keller, F., 2004. Enhanced APOE2 transmission rates in families with autistic probands. Psychiatr. Genet. 14, 73–82. https://doi.org/10.1097/01.ypg.0000128768.37838.17

Phiel, C.J., Zhang, F., Huang, E.Y., Guenther, M.G., Lazar, M.A., Klein, P.S., 2001. Histone Deacetylase Is a Direct Target of Valproic Acid, a Potent Anticonvulsant, Mood Stabilizer, and Teratogen. J. Biol. Chem. 276, 36734–36741. https://doi.org/10.1074/jbc.M101287200

Phillips, M.C., 2014. Apolipoprotein E isoforms and lipoprotein metabolism. IUBMB Life 66, 616–623. https://doi.org/10.1002/iub.1314

Piedrahita, J.A., Zhang, S.H., Hagaman, J.R., Oliver, P.M., Maeda, N., 1992. Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. Proc. Natl. Acad. Sci. 89, 4471–4475. https://doi.org/10.1073/pnas.89.10.4471

Pitas, R.E., Boyles, J.K., Lee, S.H., Hui, D., Weisgraber, K.H., 1987. Lipoproteins and their receptors in the central nervous system. Characterization of the lipoproteins in cerebrospinal fluid and identification of apolipoprotein B,E(LDL) receptors in the brain. J. Biol. Chem. 262, 14352–60.

Pitas, R.E., Ji, Z.S., Weisgraber, K.H., Mahley, R.W., 1998. Role of apolipoprotein E in modulating neurite outgrowth: potential effect of intracellular apolipoprotein E. Biochem. Soc. Trans. 26, 257–261. https://doi.org/10.1042/bst0260257

Pizzarelli, R., Cherubini, E., 2011. Alterations of GABAergic Signaling in Autism Spectrum Disorders. Neural Plast. 2011, 1–12. https://doi.org/10.1155/2011/297153

Plump, A.S., Smith, J.D., Hayek, T., Aalto-Setälä, K., Walsh, A., Verstuyft, J.G., Rubin, E.M., Breslow, J.L., 1992. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. Cell 71, 343–353. https://doi.org/10.1016/0092-8674(92)90362-G

Poirier, J., 1999. Apolipoprotein E4, cholinergic integrity and the pharmacogenetics of Alzheimer's disease. J. Psychiatry Neurosci. 24, 147–53.

Pontifex, M., Vauzour, D., Minihane, A.M., 2018. The effect of APOE genotype on Alzheimer's disease risk is influenced by sex and docosahexaenoic acid status. Neurobiol. Aging 69, 209–220. https://doi.org/10.1016/j.neurobiolaging.2018.05.017

Pope, C., Karanth, S., Liu, J., 2005. Pharmacology and toxicology of cholinesterase inhibitors: uses and misuses of a common mechanism of action. Environ. Toxicol. Pharmacol. 19, 433–446. https://doi.org/10.1016/j.etap.2004.12.048

Pope, C.N., Liu, J., 1997. Age-related differences in sensitivity to organophosphorus pesticides. Environ. Toxicol. Pharmacol. 4, 309–314. https://doi.org/10.1016/S1382-6689(97)10029-1

Potasiewicz, A., Gzielo, K., Popik, P., Nikiforuk, A., 2020. Effects of prenatal exposure to valproic acid or poly(I:C) on ultrasonic vocalizations in rat pups: The role of social cues. Physiol. Behav. 225, 113113. https://doi.org/10.1016/j.physbeh.2020.113113

Qiao, D., Seidler, F.J., Slotkin, T.A., 2001. Developmental neurotoxicity of chlorpyrifos modeled in vitro: comparative effects of metabolites and other cholinesterase inhibitors on DNA synthesis in PC12 and C6 cells. Environ. Health Perspect. 109, 909–913. https://doi.org/10.1289/ehp.01109909

Quistad, G.B., Nomura, D.K., Sparks, S.E., Segall, Y., Casida, J.E., 2002a. Cannabinoid CB1 receptor as a target for chlorpyrifos oxon and other organophosphorus pesticides. Toxicol. Lett. 135, 89–93. https://doi.org/10.1016/S0378-4274(02)00251-5

Quistad, G.B., Sparks, S.E., Casida, J.E., 2001. Fatty Acid Amide Hydrolase Inhibition by Neurotoxic Organophosphorus Pesticides. Toxicol. Appl. Pharmacol. 173, 48–55. https://doi.org/10.1006/taap.2001.9175

Quistad, G.B., Sparks, S.E., Segall, Y., Nomura, D.K., Casida, J.E., 2002b. Selective Inhibitors of Fatty Acid Amide Hydrolase Relative to Neuropathy Target Esterase and Acetylcholinesterase: Toxicological Implications. Toxicol. Appl. Pharmacol. 179, 57–63. https://doi.org/10.1006/taap.2001.9342

Rachinsky, T.L., Bryan Crenshaw, E., Taylor, P., 1992. Assignment of the gene for acetylcholinesterase to distal mouse chromosome 5. Genomics 14, 511–514. https://doi.org/10.1016/S0888-7543(05)80255-1

Raiford, K.L., Shao, Y., Allen, I.C., Martin, E.R., Menold, M.M., Wright, H.H., Abramson, R.K., Worley, G., DeLong, G.R., Vance, J.M., Cuccaro, M.L., Gilbert, J.R., Pericak-Vance, M.A., 2004. No association between theAPOE gene and autism. Am. J. Med. Genet. 125B, 57–60. https://doi.org/10.1002/ajmg.b.20104

Rathod, A.L., Garg, R.K., 2017. Chlorpyrifos poisoning and its implications in human fatal cases: A forensic perspective with reference to Indian scenario. J. Forensic Leg. Med. 47, 29–34. https://doi.org/10.1016/j.jflm.2017.02.003

Rauh, V., Arunajadai, S., Horton, M., Perera, F., Hoepner, L., Barr, D.B., Whyatt, R., 2011. Seven-Year Neurodevelopmental Scores and Prenatal Exposure to Chlorpyrifos, a Common Agricultural Pesticide. Environ. Health Perspect. 119, 1196–1201. https://doi.org/10.1289/ehp.1003160

Rauh, V.A., Garcia, W.E., Whyatt, R.M., Horton, M.K., Barr, D.B., Louis, E.D., 2015. Prenatal exposure to the organophosphate pesticide chlorpyrifos and childhood tremor. Neurotoxicology 51, 80–86. https://doi.org/10.1016/j.neuro.2015.09.004

Rauh, V.A., Garfinkel, R., Perera, F.P., Andrews, H.F., Hoepner, L., Barr, D.B., Whitehead, R., Tang, D., Whyatt, R.W., 2006. Impact of Prenatal Chlorpyrifos Exposure on Neurodevelopment in the First 3 Years of Life Among Inner-City Children. Pediatrics 118, e1845–e1859. https://doi.org/10.1542/peds.2006-0338

Reverte, I., Domingo, J.L., Colomina, M.T., 2014. Neurodevelopmental effects of decabromodiphenyl ether (BDE-209) in APOE transgenic mice. Neurotoxicol. Teratol. 46, 10–17. https://doi.org/10.1016/j.ntt.2014.08.003

Reverte, I., Klein, A.B., Domingo, J.L., Colomina, M.T., 2013. Long term effects of murine postnatal exposure to decabromodiphenyl ether (BDE-209) on learning and memory are dependent upon APOE polymorphism and age. Neurotoxicol. Teratol. 40, 17–27. https://doi.org/10.1016/j.ntt.2013.08.003

Reverte, I., Klein, A.B., Ratner, C., Domingo, J.L., Colomina, M.T., 2012. Behavioral phenotype and BDNF differences related to apoE isoforms and sex in young transgenic mice. Exp. Neurol. 237, 116–125. https://doi.org/10.1016/j.expneurol.2012.06.015

Reverte, I., Peris-Sampedro, F., Basaure, P., Campa, L., Suñol, C., Moreno, M., Domingo, J.L., Colomina, M.T., 2016. Attentional performance, impulsivity, and related neurotransmitter systems in apoE2, apoE3, and apoE4 female transgenic mice. Psychopharmacology (Berl). 233, 295–308. https://doi.org/10.1007/s00213-015-4113-9

Ricceri, L., Markina, N., Valanzano, A., Fortuna, S., Cometa, M.F., Meneguz, A., Calamandrei, G., 2003. Developmental exposure to chlorpyrifos alters reactivity to environmental and social cues in adolescent mice. Toxicol. Appl. Pharmacol. 191, 189–201. https://doi.org/10.1016/S0041-008X(03)00229-1

Ricceri, L., Venerosi, A., Capone, F., Cometa, M.F., Lorenzini, P., Fortuna, S., Calamandrei, G., 2006. Developmental Neurotoxicity of Organophosphorous Pesticides: Fetal and Neonatal Exposure to Chlorpyrifos Alters Sex-Specific Behaviors at Adulthood in Mice. Toxicol. Sci. 93, 105–113. https://doi.org/10.1093/toxsci/kfl032

Rice, D., Barone, S., 2000. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ. Health Perspect. 108, 511–533. https://doi.org/10.1289/ehp.00108s3511

Richard, S., Flamant, F., 2018. Regulation of T3 Availability in the Developing Brain: The Mouse Genetics Contribution. Front. Endocrinol. (Lausanne). 9, 1–11. https://doi.org/10.3389/fendo.2018.00265

Richardson, J.R., Fitsanakis, V., Westerink, R.H.S., Kanthasamy, A.G., 2019. Neurotoxicity of pesticides. Acta Neuropathol. 138, 343–362. https://doi.org/10.1007/s00401-019-02033-9

Rijpma, A., Jansen, D., Arnoldussen, I.A.C., Fang, X.T., Wiesmann, M., Mutsaers, M.P.C., Dederen, P.J., Janssen, C.I.F., Kiliaan, A.J., 2013. Sex Differences in Presynaptic Density and Neurogenesis in Middle-Aged ApoE4 and ApoE Knockout Mice. J. Neurodegener. Dis. 2013, 1–9. https://doi.org/10.1155/2013/531326

Roberts, E.M., English, P.B., Grether, J.K., Windham, G.C., Somberg, L., Wolff, C., 2007. Maternal Residence Near Agricultural Pesticide Applications and Autism Spectrum Disorders among Children in the California Central Valley. Environ. Health Perspect. 115, 1482–1489. https://doi.org/10.1289/ehp.10168

Rodier, P.M., Ingram, J.L., Tisdale, B., Nelson, S., Romano, J., 1996. Embryological origin for autism: Developmental anomalies of the cranial nerve motor nuclei. J. Comp. Neurol. 370, 247–261. https://doi.org/10.1002/(SICI)1096-9861(19960624)370:2<247::AID-CNE8>3.0.CO;2-2

Rojas, D.C., 2014. The role of glutamate and its receptors in autism and the use of glutamate receptor antagonists in treatment. J. Neural Transm. 121, 891–905. https://doi.org/10.1007/s00702-014-1216-0

Roullet, F.I., Lai, J.K.Y., Foster, J.A., 2013. In utero exposure to valproic acid and autism — A current review of clinical and animal studies. Neurotoxicol. Teratol. 36, 47–56. https://doi.org/10.1016/j.ntt.2013.01.004

Rowley, N.M., Madsen, K.K., Schousboe, A., Steve White, H., 2012. Glutamate and GABA synthesis, release, transport and metabolism as targets for seizure control. Neurochem. Int. 61, 546–558. https://doi.org/10.1016/j.neuint.2012.02.013

Rubenstein, J.L.R., Merzenich, M.M., 2003. Model of autism: increased ratio of excitation/inhibition in key neural systems. Genes, Brain Behav. 2, 255–267. https://doi.org/10.1034/j.1601-183X.2003.00037.x

Rylaarsdam, L., Guemez-Gamboa, A., 2019. Genetic Causes and Modifiers of Autism Spectrum Disorder. Front. Cell. Neurosci. 13, 1–15. https://doi.org/10.3389/fncel.2019.00385

Sagiv, S.K., Harris, M.H., Gunier, R.B., Kogut, K.R., Harley, K.G., Deardorff, J., Bradman, A., Holland, N., Eskenazi, B., 2018. Prenatal Organophosphate Pesticide Exposure and Traits Related to Autism Spectrum Disorders in a Population Living in Proximity to

References

Agriculture. Environ. Health Perspect. 126, 047012. https://doi.org/10.1289/EHP2580

Sakai, A., Matsuda, T., Doi, H., Nagaishi, Y., Kato, K., Nakashima, K., 2018. Ectopic neurogenesis induced by prenatal antiepileptic drug exposure augments seizure susceptibility in adult mice. Proc. Natl. Acad. Sci. 115, 4270–4275. https://doi.org/10.1073/pnas.1716479115

Sanghi, R., Pillai, M.K.K., Jayalekshmi, T.R., Nair, A., 2003. Organochlorine and organophosphorus pesticide residues in breast milk from Bhopal, Madhya Pradesh, India. Hum. Exp. Toxicol. 22, 73–76. https://doi.org/10.1191/0960327103ht321oa

Sarachana, T., Xu, M., Wu, R.C., Hu, V.W., 2011. Sex Hormones in Autism: Androgens and Estrogens Differentially and Reciprocally Regulate RORA, a Novel Candidate Gene for Autism. PLoS One 6, e17116. https://doi.org/10.1371/journal.pone.0017116

Sasaki, E., Tomita, Y., Kanno, K., 2020. Sex differences in vocalizations to familiar or unfamiliar females in mice. R. Soc. Open Sci. 7, 201529. https://doi.org/10.1098/rsos.201529

Saunders, M., Magnanti, B.L., Correia Carreira, S., Yang, A., Alamo-Hernández, U., Riojas-Rodriguez, H., Calamandrei, G., Koppe, J.G., Krayer von Krauss, M., Keune, H., Bartonova, A., 2012. Chlorpyrifos and neurodevelopmental effects: a literature review and expert elicitation on research and policy. Environ. Heal. 11, S5. https://doi.org/10.1186/1476-069X-11-S1-S5

Scattoni, M.L., Crawley, J., Ricceri, L., 2009. Ultrasonic vocalizations: A tool for behavioural phenotyping of mouse models of neurodevelopmental disorders. Neurosci. Biobehav. Rev. 33, 508–515. https://doi.org/10.1016/j.neubiorev.2008.08.003

Scattoni, M.L., Gandhy, S.U., Ricceri, L., Crawley, J.N., 2008. Unusual Repertoire of Vocalizations in the BTBR T+tf/J Mouse Model of Autism. PLoS One 3, e3067. https://doi.org/10.1371/journal.pone.0003067

Schepanski, S., Buss, C., Hanganu-Opatz, I.L., Arck, P.C., 2018. Prenatal Immune and Endocrine Modulators of Offspring's Brain Development and Cognitive Functions Later in Life. Front. Immunol. 9, 1–15. https://doi.org/10.3389/fimmu.2018.02186

Schneider, T., Przewłocki, R., 2005. Behavioral Alterations in Rats Prenatally Exposed to Valproic Acid: Animal Model of Autism. Neuropsychopharmacology 30, 80–89. https://doi.org/10.1038/sj.npp.1300518

Schroeder, J.C., Reim, D., Boeckers, T.M., Schmeisser, M.J., 2015. Genetic Animal Models for Autism Spectrum Disorder, in: Brain Imaging in Behavioral Neuroscience. pp. 311–324. https://doi.org/10.1007/7854_2015_407

Seitz, A., Gourevitch, D., Zhang, X.M., Clark, L., Chen, P., Kragol, M., Levenkova, N., Rux, J., Samulewicz, S., Heber-Katz, E., 2005. Sense and antisense transcripts of the apolipoprotein E gene in normal and ApoE knockout mice, their expression after spinal cord injury and corresponding human transcripts. Hum. Mol. Genet. 14, 2661–2670. https://doi.org/10.1093/hmg/ddi296

Semple, B.D., Blomgren, K., Gimlin, K., Ferriero, D.M., Noble-Haeusslein, L.J., 2013. Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. Prog. Neurobiol. 106–107, 1–16. https://doi.org/10.1016/j.pneurobio.2013.04.001

Shabani, Z., Mahmoudi, J., Farajdokht, F., Sadigh-Eteghad, S., 2020. An Overview of Nicotinic Cholinergic System Signaling in Neurogenesis. Arch. Med. Res. 51, 287–296. https://doi.org/10.1016/j.arcmed.2020.03.014

Shelton, J.F., Geraghty, E.M., Tancredi, D.J., Delwiche, L.D., Schmidt, R.J., Ritz, B., Hansen, R.L., Hertz-Picciotto, I., 2014. Neurodevelopmental Disorders and Prenatal Residential Proximity to Agricultural Pesticides: The CHARGE Study. Environ. Health Perspect. 122, 1103–1109. https://doi.org/10.1289/ehp.1307044

Shen, J., Yakel, J.L., 2009. Nicotinic acetylcholine receptor-mediated calcium signaling in the nervous system. Acta Pharmacol. Sin. 30, 673–680. https://doi.org/10.1038/aps.2009.64

Sheng, M., Cummings, J., Roldan, L.A., Jan, Y.N., Jan, L.Y., 1994. Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. Nature 368, 144–147. https://doi.org/10.1038/368144a0

Shore, V.G., Shore, B., 1973. Heterogeneity of human plasma very low density lipoproteins. Separation of species differing in protein components. Biochemistry 12, 502–507. https://doi.org/10.1021/bi00727a022

Silva, M.H., 2020. Effects of low-dose chlorpyrifos on neurobehavior and potential mechanisms: A review of studies in rodents, zebrafish, and *Caenorhabditis elegans*. Birth Defects Res. 112, 445–479. https://doi.org/10.1002/bdr2.1661

Silver, M.K., Shao, J., Zhu, B., Chen, M., Xia, Y., Kaciroti, N., Lozoff, B., Meeker, J.D., 2017. Prenatal naled and chlorpyrifos exposure is associated with deficits in infant motor function in a cohort of Chinese infants. Environ. Int. 106, 248–256. https://doi.org/10.1016/j.envint.2017.05.015

Silverman, J.L., Yang, M., Lord, C., Crawley, J.N., 2010. Behavioural phenotyping assays for mouse models of autism. Nat. Rev. Neurosci. 11, 490–502. https://doi.org/10.1038/nrn2851

Singleton, S.T., Lein, P.J., Dadson, O.A., McGarrigle, B.P., Farahat, F.M., Farahat, T., Bonner, M.R., Fenske, R.A., Galvin, K., Lasarev, M.R., Anger, W.K., Rohlman, D.S., Olson, J.R., 2015. Longitudinal assessment of occupational exposures to the organophosphorous insecticides chlorpyrifos and profenofos in Egyptian cotton field workers. Int. J. Hyg. Environ. Health 218, 203–211. https://doi.org/10.1016/j.ijheh.2014.10.005

Slotkin, T., Seidler, F., 2007. Prenatal chlorpyrifos exposure elicits presynaptic serotonergic and dopaminergic hyperactivity at adolescence: Critical periods for regional and sex-selective effects. Reprod. Toxicol. 23, 421–427. https://doi.org/10.1016/j.reprotox.2006.07.010

Slotkin, T.A., 2006. Developmental Neurotoxicity of Organophosphates: A Case Study of Chlorpyrifos, in: Toxicology of Organophosphate & Carbamate Compounds. Elsevier, pp. 293–314. https://doi.org/10.1016/B978-012088523-7/50022-3

Slotkin, T.A., Seidler, F.J., Fumagalli, F., 2007. Exposure to Organophosphates Reduces the Expression of Neurotrophic Factors in Neonatal Rat Brain Regions: Similarities and Differences in the Effects of Chlorpyrifos and Diazinon on the Fibroblast Growth Factor Superfamily. Environ. Health Perspect. 115, 909–916. https://doi.org/10.1289/ehp.9901

Smith, J.N., Hinderliter, P.M., Timchalk, C., Bartels, M.J., Poet, T.S., 2014. A human life-stage physiologically based pharmacokinetic and pharmacodynamic model for chlorpyrifos: Development and validation. Regul. Toxicol. Pharmacol. 69, 580–597. https://doi.org/10.1016/j.yrtph.2013.10.005

Smotherman, W.P., Bell, R.W., Starzec, J., Elias, J., Zachman, T.A., 1974. Maternal responses to infant vocalizations and olfactory cues in rats and mice. Behav. Biol. 12, 55–66. https://doi.org/10.1016/S0091-6773(74)91026-8

Sohal, V.S., Rubenstein, J.L.R., 2019. Excitation-inhibition balance as a framework for investigating mechanisms in neuropsychiatric disorders. Mol. Psychiatry 24, 1248–1257. https://doi.org/10.1038/s41380-019-0426-0

Soininen, H., Kosunen, O., Helisalmi, S., Mannermaa, A., Paljärvi, L., Talasniemi, S., Ryynänen, M., Riekkinen, P., 1995a. A severe loss of choline acetyltransferase in the frontal cortex of Alzheimer patients carrying apolipoprotein ε4 allele. Neurosci. Lett. 187, 79–82. https://doi.org/10.1016/0304-3940(95)11343-6

Soininen, H., Lehtovirta, M., Helisalmi, S., Linnaranta, K., Heinonen, O., Sr, P.R., 1995b. Increased acetylcholinesterase activity in the CSF of Alzheimer patients carrying apolipoprotein ε4 allele. Neuroreport 6, 2518–2520. https://doi.org/10.1097/00001756-199512150-00017

Soltaninejad, K., Abdollahi, M., 2009. Current opinion on the science of organophosphate pesticides and toxic stress: a systematic review. Med. Sci. Monit. 15, RA75-90.

Soltaninejad, K., Shadnia, S., 2014. History of the Use and Epidemiology of Organophosphorus Poisoning, in: Balali-Mood, M., Abdollahi, M. (Eds.), Basic and Clinical Toxicology of Organophosphorus Compounds. Springer London, London, pp. 25–43. https://doi.org/10.1007/978-1-4471-5625-3_2

Somogyi, P., Fritschy, J.M., Benke, D., Roberts, J.D., Sieghart, W., 1996. The γ2 Subunit of the GABA A Receptor is Concentrated in Synaptic Junctions Containing the α1 and β 23 Subunits in Hippocampus, Cerebellum and Globus Pallidus. Neuropharmacology 35, 1425–1444. https://doi.org/10.1016/S0028-3908(96)00086-X

Sroufe, L.A., 2005. Attachment and development: A prospective, longitudinal study from birth to adulthood. Attach. Hum. Dev. 7, 349–367. https://doi.org/10.1080/14616730500365928

Stanton, M., Peloso, E., Brown, K., Rodier, P., 2007. Discrimination learning and reversal of the conditioned eyeblink reflex in a rodent model of autism. Behav. Brain Res. 176, 133–140. https://doi.org/10.1016/j.bbr.2006.10.022

Stapleton, A.R., Chan, V.T., 2009. Subtoxic chlorpyrifos treatment resulted in differential expression of genes implicated in neurological functions and development. Arch. Toxicol. 83, 319–333. https://doi.org/10.1007/s00204-008-0346-2

Sullivan, P., Mace, B., Maeda, N., Schmechel, D., 2004. Marked regional differences of brain human apolipoprotein e expression in targeted replacement mice. Neuroscience 124, 725–733. https://doi.org/10.1016/j.neuroscience.2003.10.011

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

Sun, L., Margolis, F.L., Shipley, M.T., Lidow, M.S., 1998. Identification of a long variant of mRNA encoding the NR3 subunit of the NMDA receptor: its regional distribution and developmental expression in the rat brain. FEBS Lett. 441, 392–396. https://doi.org/10.1016/S0014-5793(98)01590-7

Suratman, S., Edwards, J.W., Babina, K., 2015. Organophosphate pesticides exposure among farmworkers: pathways and risk of adverse health effects. Rev. Environ. Health 30, 65–79. https://doi.org/10.1515/reveh-2014-0072

Sweet, R.A., MacDonald, M.L., Kirkwood, C.M., Ding, Y., Schempf, T., Jones-Laughner, J., Kofler, J., Ikonomovic, M.D., Lopez, O.L., Garver, M.E., Fitz, N.F., Koldamova, R., Yates, N.A., 2016. Apolipoprotein E*4 (APOE*4) Genotype Is Associated with Altered Levels of Glutamate Signaling Proteins and Synaptic Coexpression Networks in the Prefrontal Cortex in Mild to Moderate Alzheimer Disease. Mol. Cell. Proteomics 15, 2252–2262. https://doi.org/10.1074/mcp.M115.056580

Takai, H., Katayama, K., Uetsuka, K., Nakayama, H., Doi, K., 2003. Distribution of N-methyl-d-aspartate receptors (NMDARs) in the developing rat brain. Exp. Mol. Pathol. 75, 89–94. https://doi.org/10.1016/S0014-4800(03)00030-3

Tanvir, E., Afroz, R., Chowdhury, M., Gan, S., Karim, N., Islam, M., Khalil, M., 2016. A model of chlorpyrifos distribution and its biochemical effects on the liver and kidneys of rats. Hum. Exp. Toxicol. 35, 991–1004. https://doi.org/10.1177/0960327115614384

References

Tao, R., Davis, K.N., Li, C., Shin, J.H., Gao, Y., Jaffe, A.E., Gondré-Lewis, M.C., Weinberger, D.R., Kleinman, J.E., Hyde, T.M., 2018. GAD1 alternative transcripts and DNA methylation in human prefrontal cortex and hippocampus in brain development, schizophrenia. Mol. Psychiatry 23, 1496–1505. https://doi.org/10.1038/mp.2017.105

Taylor, D., 1969. Differental rates of cerebral maturation between sexes and between hemispheres. Lancet 294, 140–142. https://doi.org/10.1016/S0140-6736(69)92445-3

Terry, A.V., 2012. Functional consequences of repeated organophosphate exposure: Potential non-cholinergic mechanisms. Pharmacol. Ther. 134, 355–365. https://doi.org/10.1016/j.pharmthera.2012.03.001

Tidmarsh, L., Volkmar, F.R., 2003. Diagnosis and Epidemiology of Autism Spectrum Disorders. Can. J. Psychiatry 48, 517–525. https://doi.org/10.1177/070674370304800803

Timchalk, C., Busby, A., Campbell, J.A., Needham, L.L., Barr, D.B., 2007. Comparative pharmacokinetics of the organophosphorus insecticide chlorpyrifos and its major metabolites diethylphosphate, diethylthiophosphate and 3,5,6-trichloro-2-pyridinol in the rat. Toxicology 237, 145–157. https://doi.org/10.1016/j.tox.2007.05.007

Tsujino, N., Nakatani, Y., Seki, Y., Nakasato, A., Nakamura, M., Sugawara, M., Arita, H., 2007. Abnormality of circadian rhythm accompanied by an increase in frontal cortex serotonin in animal model of autism. Neurosci. Res. 57, 289–295. https://doi.org/10.1016/j.neures.2006.10.018

Utermann, G., 1975. Isolation and Partial Characterization of an Arginine-Rich Apolipoprotein from Human Plasma Very-Low-Density Lipoproteins: Apolipoprotein E. Hoppe-Seyler´s Zeitschrift für Physiol. Chemie 356, 1113–1122. https://doi.org/10.1515/bchm2.1975.356.2.1113

Uzunova, G., Pallanti, S., Hollander, E., 2016. Excitatory/inhibitory imbalance in autism spectrum disorders: Implications for interventions and therapeutics. World J. Biol. Psychiatry 17, 174–186. https://doi.org/10.3109/15622975.2015.1085597

Valeeva, G., Valiullina, F., Khazipov, R., 2013. Excitatory actions of GABA in the intact neonatal rodent hippocampus in vitro. Front. Cell. Neurosci. 7, 1–12. https://doi.org/10.3389/fncel.2013.00020

van den Berg, H., Hii, J., Soares, A., Mnzava, A., Ameneshewa, B., Dash, A.P., Ejov, M., Tan, S.H., Matthews, G., Yadav, R.S., Zaim, M., 2011. Status of pesticide management in the practice of vector control: a global survey in countries at risk of malaria or other major vector-borne diseases. Malar. J. 10, 125. https://doi.org/10.1186/1475-2875-10-125

van den Dries, M.A., Guxens, M., Pronk, A., Spaan, S., El Marroun, H., Jusko, T.A., Longnecker, M.P., Ferguson, K.K., Tiemeier, H., 2019. Organophosphate pesticide metabolite concentrations in urine during pregnancy and offspring attention-deficit hyperactivity disorder and autistic traits. Environ. Int. 131, 105002. https://doi.org/10.1016/j.envint.2019.105002

Vance, J.E., Hayashi, H., 2010. Formation and function of apolipoprotein E-containing lipoproteins in the nervous system. Biochim. Biophys. Acta - Mol. Cell Biol. Lipids 1801, 806–818. https://doi.org/10.1016/j.bbalip.2010.02.007

Venerosi, A., Calamandrei, G., Ricceri, L., 2006. A social recognition test for female mice reveals behavioral effects of developmental chlorpyrifos exposure. Neurotoxicol. Teratol. 28, 466–471. https://doi.org/10.1016/j.ntt.2006.05.003

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

Venerosi, A., Ricceri, L., Scattoni, M.L., Calamandrei, G., 2009. Prenatal chlorpyrifos exposure alters motor behavior and ultrasonic vocalization in cd-1 mouse pups. Environ. Heal. 8, 12. https://doi.org/10.1186/1476-069X-8-12

von Ehrenstein, O.S., Ling, C., Cui, X., Cockburn, M., Park, A.S., Yu, F., Wu, J., Ritz, B., 2019. Prenatal and infant exposure to ambient pesticides and autism spectrum disorder in children: population based case-control study. BMJ 364, l962. https://doi.org/10.1136/bmj.l962

Vorhees, C. V., 1987. Teratogenicity and developmental toxicity of valproic acid in rats. Teratology 35, 195–202. https://doi.org/10.1002/tera.1420350205

Vorstman, J.A.S., Parr, J.R., Moreno-De-Luca, D., Anney, R.J.L., Nurnberger Jr, J.I., Hallmayer, J.F., 2017. Autism genetics: opportunities and challenges for clinical translation. Nat. Rev. Genet. 18, 362–376. https://doi.org/10.1038/nrg.2017.4

Watanabe, M., Inoue, Y., Sakimura, K., Mishina, M., 1993. Distinct Spatio-temporal Distributions of the NMDA Receptor Channel Subunit mRNAs in the Brain. Ann. N. Y. Acad. Sci. 707, 463–466. https://doi.org/10.1111/j.1749-6632.1993.tb38099.x

Weisgraber, K.H., 1994. Apolipoprotein E: Structure-Function Relationships. pp. 249–302. https://doi.org/10.1016/S0065-3233(08)60642-7

Weisgraber, K.H., Rall, S.C., Mahley, R.W., 1981. Human E apoprotein heterogeneity. Cysteine-arginine interchanges in the amino acid sequence of the apo-E isoforms. J. Biol. Chem. 256, 9077–83.

Werling, D.M., Geschwind, D.H., 2013. Sex differences in autism spectrum disorders. Curr. Opin. Neurol. 26, 146–153. https://doi.org/10.1097/WCO.0b013e32835ee548

References

WHO, 2022. Autism. URL https://www.who.int/news-room/fact-sheets/detail/autism-spectrum-disorders (accessed 8.19.22).

Whyatt, R.M., Rauh, V., Barr, D.B., Camann, D.E., Andrews, H.F., Garfinkel, R., Hoepner, L.A., Diaz, D., Dietrich, J., Reyes, A., Tang, D., Kinney, P.L., Perera, F.P., 2004. Prenatal Insecticide Exposures and Birth Weight and Length among an Urban Minority Cohort. Environ. Health Perspect. 112, 1125–1132. https://doi.org/10.1289/ehp.6641

Williams, A.L., DeSesso, J.M., 2014. Gestational/Perinatal chlorpyrifos exposure is not associated with autistic-like behaviors in rodents. Crit. Rev. Toxicol. 44, 523–534. https://doi.org/10.3109/10408444.2014.907772

Wöhr, M., Schwarting, R.K.W., 2008. Maternal care, isolation-induced infant ultrasonic calling, and their relations to adult anxiety-related behavior in the rat. Behav. Neurosci. 122, 310–330. https://doi.org/10.1037/0735-7044.122.2.310

Wright, R.O., Hu, H., Silverman, E.K., Tsaih, S.W., Schwartz, J., Bellinger, D., Palazuelos, E., Weiss, S.T., Hernandez-Avila, M., 2003. Apolipoprotein E Genotype Predicts 24-Month Bayley Scales Infant Development Score. Pediatr. Res. 54, 819–825. https://doi.org/10.1203/01.PDR.0000090927.53818.DE

Wu, C., Sun, D., 2015. GABA receptors in brain development, function, and injury. Metab. Brain Dis. 30, 367–379. https://doi.org/10.1007/s11011-014-9560-1

Ye, S., Huang, Y., Müllendorff, K., Dong, L., Giedt, G., Meng, E.C., Cohen, F.E., Kuntz, I.D., Weisgraber, K.H., Mahley, R.W., 2005. Apolipoprotein (apo) E4 enhances amyloid β peptide production in cultured neuronal cells: ApoE structure as a potential therapeutic target. Proc. Natl. Acad. Sci. 102, 18700–18705. https://doi.org/10.1073/pnas.0508693102

Yu, P., Chen, K., Huang, X., Wang, X., Ren, Q., 2018. Production of γ-aminobutyric acid in Escherichia coli by engineering MSG pathway. Prep. Biochem. Biotechnol. 48, 906–913. https://doi.org/10.1080/10826068.2018.1514519

Zannis, V.I., Breslow, J.L., Utermann, G., Mahley, R.W., Weisgraber, K.H., Havel, R.J., Goldstein, J.L., Brown, M.S., Schonfeld, G., Hazzard, W.R., Blum, C., 1982. Proposed nomenclature of apoE isoproteins, apoE genotypes, and phenotypes. J. Lipid Res. 23, 911–4. https://doi.org/10.1080/10826068.2018.1514519

Zeidan, J., Fombonne, E., Scorah, J., Ibrahim, A., Durkin, M.S., Saxena, S., Yusuf, A., Shih, A., Elsabbagh, M., 2022. Global prevalence of autism: A systematic review update. Autism Res. 15, 778–790. https://doi.org/10.1002/aur.2696

Zhang, J., Lin, L., Dai, X., Xiao, N., Ye, Q., Chen, X., 2021. ApoE4 increases susceptibility to stress-induced age-dependent depression-like behavior and cognitive impairment. J. Psychiatr. Res. 143, 292–301. https://doi.org/10.1016/j.jpsychires.2021.09.029

Zhang, S.H., Reddick, R.L., Piedrahita, J.A., Maeda, N., 1992. Spontaneous Hypercholesterolemia and Arterial Lesions in Mice Lacking Apolipoprotein E. Science. 258, 468–471. https://doi.org/10.1126/science.1411543

Zhang, T.Y., 2005. Influence of Naturally Occurring Variations in Maternal Care on Prepulse Inhibition of Acoustic Startle and the Medial Prefrontal Cortical Dopamine Response to Stress in Adult Rats. J. Neurosci. 25, 1493–1502. https://doi.org/10.1523/JNEUROSCI.3293-04.2005

Zhang, X.M., Luo, J.H., 2013. GluN2A versus GluN2B: twins, but quite different. Neurosci. Bull. 29, 761–772. https://doi.org/10.1007/s12264-013-1336-9

UNIVERSITAT
ROVIRA i VIRGILI