SHORT, MEDIUM, AND LONG-TERM BEHAVIORAL AND MOLECULAR EFFECTS OF THE PREWEANING EXPOSURE TO CHLORPYRIFOS IN WISTAR RATS

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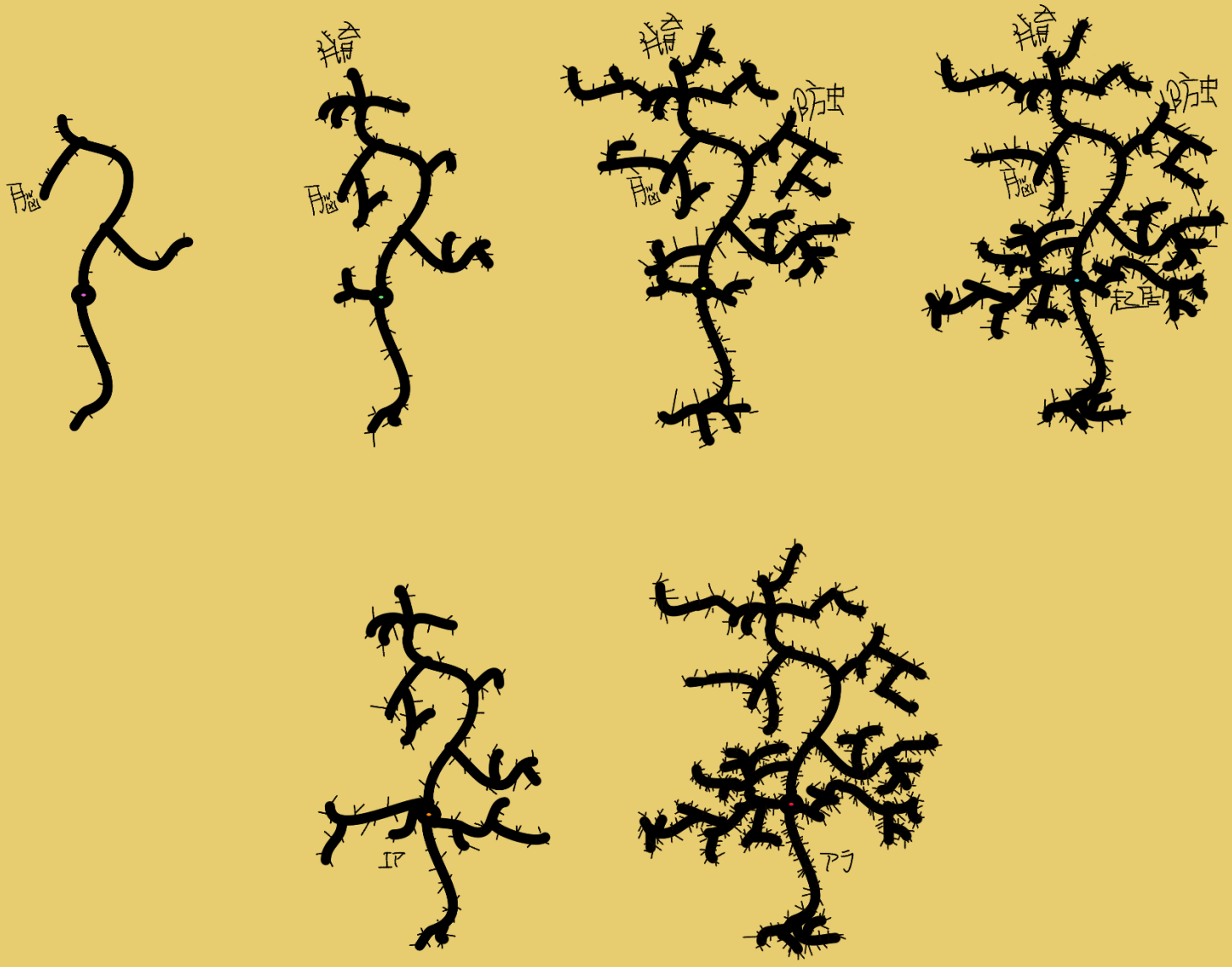

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“Every chess master was once a beginner”

Irving Chernev

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Abstract

Chlorpyrifos (CPF) is one of the most widely used Organophosphate pesticides (OP) in both developed and developing countries. Although its importance FOR human needs is unquestionable, its use has been systematically linked with several specific behavioral and physiological alterations, as well as with the incidence of numerous psychiatric, neurologic, neurodevelopmental, and neurodegenerative disorders. Even though the main mechanism of toxicology of CPF is the irreversible inhibition of Acetylcholinesterases (AChE) in the central nervous system (CNS), several authors have found alternative molecular targets (e.g., different components within the cholinergic system and from other neurotransmitter systems) without the implication of AChE by using doses well-bellow the range of systemic inhibition, defining a non-cholinesterase profile of neurotoxicology (NChEI). Interestingly, this profile has primarily been observed following developmental exposure protocols when the immature CNS appears to be more sensitive to OP intoxication. However, the different behavioral and physiological alterations induced by developmental exposure to NChEI doses of CPF can vary depending on the specific developmental stage, with different or opposing results for a specific variable according to whether the exposure occurs during gestation (early vs. late ages) or postnatally (neonatal vs. preweaning exposures). To date, preweaning ages are one of the least studied developmental periods. This is relevant, since the CNS of rodents at these ages is equivalent to the perinatal CNS in humans in developmental terms, an age known to be particularly sensitive to external stressors, including environmental chemicals. Based on this, we considered that the short, medium and long-term behavioral and molecular effects of exposure to NChEI doses of CPF must be analyzed in more depth, particularly at preweaning ages (Postnatal day 10 to 15) and using lower doses (1mg/kg/day) than those commonly reported in the current literature. In addition to this, we were also interested in studying the existence of other molecular targets that could explain the alternative mechanism of actions of CPF exposure. Following other authors, we also included both sexes, since the effects of CPF exposure have been shown to be sex-dependent for a multitude of behaviors. Our main results indicate that preweaning exposure to NChEI doses of CPF hypo and hypersensitized the cholinergic and the GABAergic systems, respectively when challenged with specific drugs. This was also accompanied by the up-regulation of both the muscarinic 2 receptor and the GABA-A-α2 subunit receptor genes in the dorsal striatum and the frontal cortex, respectively,

during early adulthood. A significant decrease in the mRNA expression of the brain-derived neurotrophic factor was also found at the dorsal striatum during adulthood. CPF exposure altered motricity by both increasing (adolescence) and decreasing (adulthood and late-adulthood) various locomotor outcomes, and also hypersensitized females to stressors (following an i.p. injection of saline) during adolescence. CPF exposure had no effect on sociability (adolescence and adulthood) or dominance (adulthood), whilst it did not alter basal attention (adulthood), inhibitory control (adulthood), spatial learning/memory (late-adulthood) skills or anxiety state. However, exposed females showed enhanced learning during adulthood in sustained attention-based tasks, which was not observed later in aging rats in spatial-memory-based mazes, whilst the exposed animals showed increased impulsive action behaviors, which were revealed when the contextual contingencies were manipulated (increasing the inter-trial interval in the 5-choice serial reaction time task). In addition to these behavioral, pharmacological, and brain gene expression effects, CPF also induced both medium (adolescence) and long-term (adulthood) gut dysbiosis involving several bacteria at both genus and species levels. CPF exposure also altered the levels of numerous plasma metabolites by inducing a long-term (adulthood) hyperlipidemic, hypoglycemic, and an apparent decreased cell energy production exclusively in adult female rats. All of the empirical data derived from the present doctoral thesis is novel and relevant with regard to the exposure protocol used, completely new regarding the exposure to CPF at any developmental stage, or even the exposure to CPF at any moment of the life-span. However, future research concerning this exposure protocol should include a more in-depth analysis of components of the cholinergic and GABAergic systems, at the both pharmacological and molecular levels, and we emphasize the necessity to analyze the protein expression level along with mRNA outcomes. The physiological basis of the different motoric and impulsive-like behaviors should also be further explored.

Resumen

El Clorpirifós (CPF) es uno de los pesticidas Organofosforados (OP) más ampliamente usados tanto en países desarrollados como en vías de desarrollo. Aunque su importancia en relación con las necesidades humanas es incuestionable, su uso se ha asociado sistemáticamente con multitud de alteraciones conductuales y fisiológicas, así como con la incidencia de muchas patologías psiquiátricas, neurológicas, del neurodesarrollo y neurodegenerativas. A pesar de que el principal mecanismo toxicológico del CPF es la inhibición irreversible de la Acetilcolinesterasa (AChE) en el sistema nervioso central (CNS), muchos autores han encontrado mecanismos moleculares alternativos (p.ej. diferentes componentes dentro del sistema colinérgico y de otros sistemas de neurotransmisión) sin la implicación de la AChE mediante el uso de dosis bajo el rango de inhibición sistémica, definiendo un perfil neuro toxicológico no colinesterásico (NChEI). Este perfil se ha visto eminentemente siguiendo protocolos de exposición durante el desarrollo, cuando el CNS parece ser más sensible a la intoxicación por OP. Sin embargo, las diferentes alteraciones tanto conductuales como fisiológicas inducidas por la exposición durante el desarrollo a dosis NChEI de CPF varían dependiendo del periodo específico del desarrollo, con resultados diferentes o incluso opuestos para variables específicas cuando la exposición acontece durante la gestación (exposición temprana o tardía) o posnatalmente (exposición neonatal o pre deteste). Hoy en día, la edad pre-destete es uno de los periodos del desarrollo menos estudiados. Esto es relevante ya que el CNS de los roderos a esas edades es equivalente al periodo perinatal in términos de desarrollo del CNS en humanos, una edad conocida por ser especialmente sensible a estresores externos, incluyendo agentes químicos presentes en el medioambiente. En base a esto, consideramos que los efectos conductuales y moleculares a corto, medio y largo plazo de la exposición a dosis NChEI de CPF debe ser analizado en mayor profundidad en relación con las edades pre-deteste (desde el día postnatal 10 al 15), usando dosis más bajas (1mg/kg/día) de las que se observan comúnmente en la literatura. Junto a esto, estamos también interesados en el estudio de la existencia de otras moléculas que puedan explicar mecanismos de acción moleculares alternativos de la exposición a CPF. Siguiendo a otros autores, hemos incluido el estudio de ambos sexos ya que los efectos de la exposición a CPF se han observado como dependientes del sexo in relación a multitud de comportamientos. Nuestros resultados principales muestran que la exposición durante la edad pre-destete a dosis NChEI de CPF hipo e hiper sensibilizó el sistema

colinérgico y el GABAérgico, respectivamente, una vez dichos sistemas fueron "retados" con drogas específicas. A esto lo acompañó una regulación al alza de los genes tanto del receptor muscarínico 2 como la subunidad α2 del receptor GABA-A en el estriado dorsal y el córtex frontal, respectivamente, durante la adultez temprana. También se observó una importante reducción en la expresión del mRNA del factor neurotrófico derivado del cerebro en el estriado dorsal durante la adultez. La exposición a CPF alteró la motricidad tanto incrementándola (adolescencia) como disminuyéndola (adultez y adultez tardía) evaluado mediante diferentes medidas locomotoras, así como hipersesibilizó las hembras ante estresores (siguiendo una inyección i.p. de salino) durante la adolescencia. La exposición a CPF no afectó ni a la sociabilidad (adolescencia y adultez) ni a la dominancia (adultez), así como tampoco alteró el estado basal de los procesos atencionales, el control inhibitorio, la memoria/aprendizaje espacial o la ansiedad. Sin embargo, las hembras expuestas mostraron un nivel de aprendizaje mejorado durante la adultez en tareas reguladas por la atención sostenida, algo no observado en edades más avanzadas en tareas de memoria espacial, así como los animales expuestos mostraron un aumento de su impulsividad motora, descubierto cuando ciertas contingencias contextuales fueron manipuladas (aumento del intervalo inter-estímulo en la tarea de 5-choice serial reaction time). Junto a estos datos conductuales, farmacológicos y de expresión genética, la exposición CPF también indujo una disbiosis intestinal tanto a medio (adolescencia) como a largo plazo (adultez) con relación a multitud de bacterias tanto a nivel de género como de especies. La exposición a CPF también alteró la composición de metabolitos plasmáticos induciendo un perfil hiperlipídico, hipoglicémico, y un aparente descenso en la producción de energía celular exclusivamente en las hembras en edad adulta. Todos estos datos empíricos derivados de la presente tesis doctoral son nuevos y relevantes con relación al protocolo de exposición usado, completamente nuevos en relación con la exposición a CPF independientemente de la edad de exposición o, incluso, en relación con la exposición a CPF en cualquier edad. Sin embargo, las investigaciones futuras deberían incluir un análisis en profundidad de los diferentes componentes del sistema colinérgico de GABAérgico, tanto a nivel farmacológico como molecular, enfatizando la necesidad de analizar a nivel de expresión proteica en conjunción con medidas de expresión de mRNA. La base fisiológica de los diferentes comportamientos motores e impulsivos también deben ser explorados en mayor profundidad.

Index

1. General Introduction

1.1. Why do we use pesticides?

Maintaining proper feeding rates is essential for the development of any mammalian. In the case of humans, our omnivorous diet has mainly been characterized by the high ingestion of fruits, vegetables, and cereals. The empirical data derived from the most important health agencies suggest that these basic nutrients must comprise most of our total food consumed daily (U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2015; World Health Organization, 2018) in order to maintain a healthy diet (**Image 1**). It is also important that our diet includes meat and other derivates of cattle, which are primarily fed with cereals (predominantly corn and soy). This information contains an essential message that we need to have access to fruits, vegetables, and cereals in order to survive and maintain a good health.

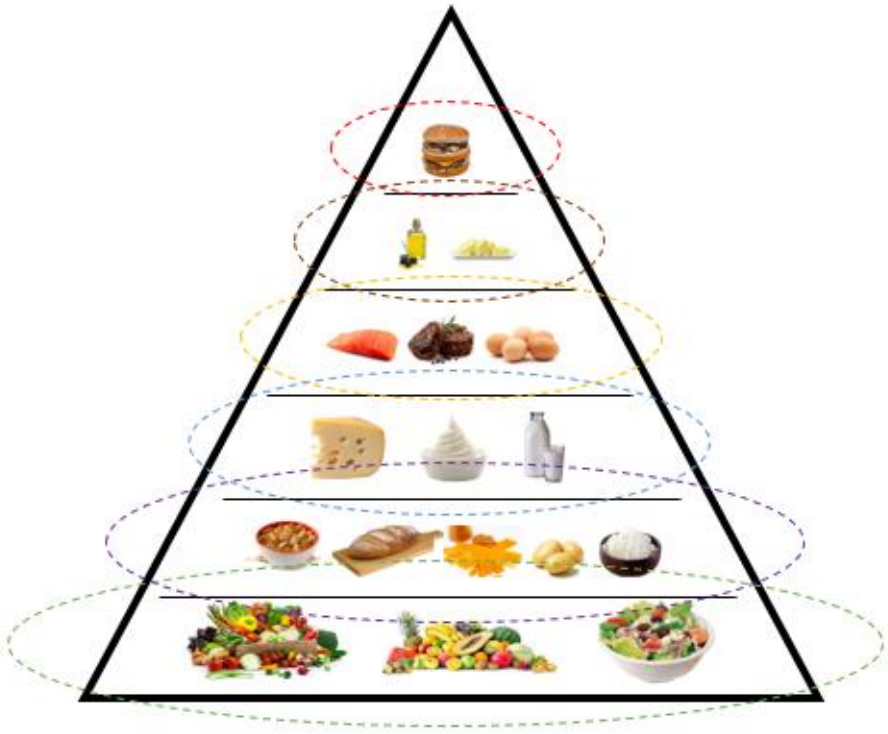

Image 1. The healthy feeding pyramid. From the bottom to the top, the daily healthy human diet should consist of fruits, vegetables and salads, cereals, bread, pasta, rice and potatoes, milk and milk-derived products, both animal and vegetable oils, and high-fat/sugar products.

However, these nutrients are not easy to produce, and, in this regard, a number of challenges are faced on a daily basis, including human contamination, natural disasters and, more frequently, infections from insects, fungus, and other plagues. In relation to this last category, humans have systematically attempted to develop both natural and

artificial products that eradicate, or at least mitigate, this effect, namely pest control. The compounds used for this purpose are known as pesticides.

The Food and Agricultural Organization defines a pesticide as: "[…] any substance or mixture of substance intended for preventing, destroying o controlling any pest […]" (Kaloyanova and El Batawi, 1991). Pesticides can be classified according to their chemical structure, physical properties, nature (artificial vs. natural compounds) or their final target (agricultural, residential, industrial, and pet care, amongst other purposes). In terms of the latter, pesticides are generally classified into insecticides (insects), fungicides (fungus), acaricides (mites), nematicide (nematodes), bactericides (bacteria), rodenticides (rodents), and avicides (birds), amongst others (Matthews GA, 2018).

However, the use of pesticides leads to two opposing effects; although they control a pest that is generally prejudicial to mankind, their toxic nature also means that they affect human health (Mostafalou and Abdollanhi, 2017). The United Nations Organization estimates that more than 3 million poisonings and more than 200,000 deaths per year are directly related to pesticides (United Nations Organization, 2017). However, this sort of statistic only reflects the most severe cases of pesticide poisoning, the visible summit of the iceberg, when death or acute exposure to very high doses (both accidental and suicide attempts) are the results (**Image 2**).

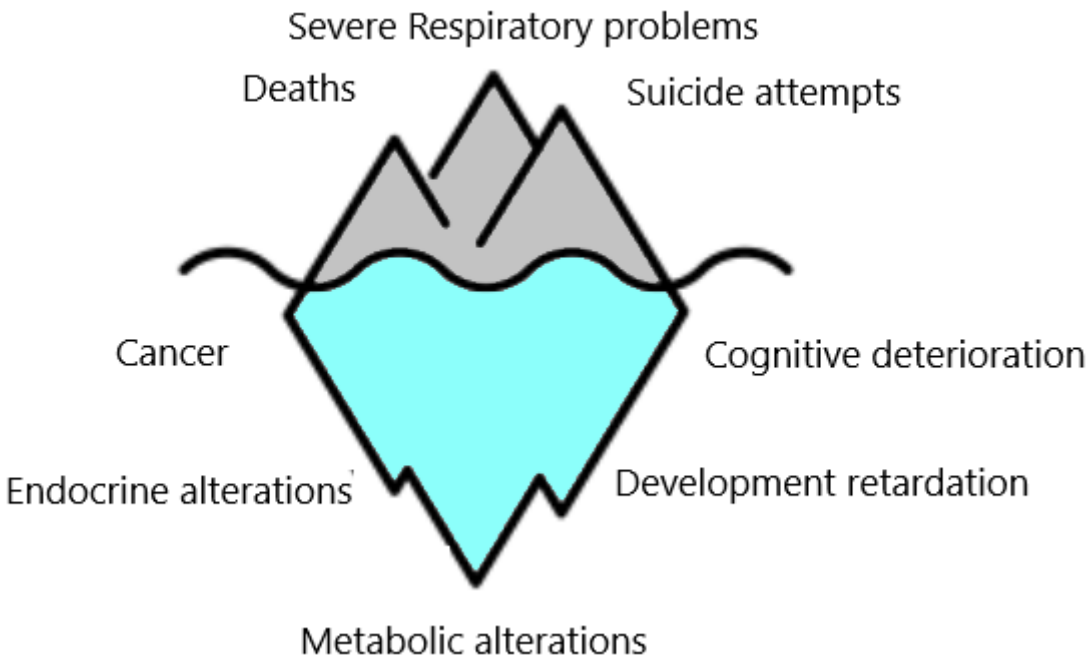

Image 2. Effects of the functions most commonly altered by pesticide exposure. The upper section of the iceberg (grey) represents the pathological outputs with the highest social/mediatic impact, but this is a minimum percentage of the total. The lower, hidden section (blue) represents those physiological and behavioral alterations derived from chronic exposure to low doses of pesticide, which is the most common outcome of harmful pesticides.

1.2. A brief journey through the history of pesticides

The nature of these pesticides and their usage has significantly evolved over time from the natural, uncontrolled products used by some ancient civilizations to the sophisticated artificial xenobiotics compounds found in the XXIth modern market.

From a historical point of view, the use of different natural, inorganic compounds for pest control by humans can almost be dated back to the time that they settled down and adopted a sedentary model of living. The earliest example of pesticide usage can be traced to Mesopotamia, where humans used elemental sulfur dusting 4500 years ago (Manyilizu WB, 2019). The Sumerians used sulfur compound mixes that were rubbed on their skin, which produced a protective shell against mosquitoes and other insects. The ancient Egyptians were known to use several recipes (included in the Ebers Papyrus) for functions related to insect control and protection. In Asia, the Chinese used different compounds based on mercury, arsenic, lime, dust, and other compounds for a number of functions. In Europe, and around this time (3000 years ago), the Greeks also used different compounds as pesticides such as burned Sulphur, in order to kill insects, later developing more

sophisticated methods for pest control both in the Greek and Roman empires. More recently, the maintenance of agricultural fields in the middle-ages in Europe was characterized by the use of several products such as mixes of wine and sulfur (for a more detailed description of all these uses, see Banaszkiewicz T, 2010).

However, the industrial revolution turned the bulk of these natural processes into more "sophisticated" toxins. With the acquisition of new knowledge and the scientific revolution that accompanied the industrial revolution, artificial chemicals gained prominence in terms of usage, since they showed a better capacity to control pests, higher efficiency and, ultimately, were more cost effective. Some of the most important chemical families used for these purposes included different inorganic compounds such as copper sulphate, sulphuric acid, and iron sulphate, which were widely used in the XIXth century (Banaszkiewicz T, 2010).

In the XXth century, three different chemical families have monopolized the pesticide industry worldwide: The Organochlorides (OC), the Carbamates (CM) and the Organophosphates (OP). OCs were the first group of pesticides to be used on a large scale (Pirsaheb et al., 2017), with dichlorodiphenyltrichloroethane (DDT) being the main representative. This was partially due to their relatively low production cost in comparison with coetaneous OPs, along with their wide range of use. However, OCs were banned or partially controlled in the late of '70s in Europe, Canada, and the USA because of their considerable potential to harm both humans and wildlife (Safe SH, 1994), along with their ability to widespread rapidly (El-Shahawi et al., 2010; Jantunen et al., 2015) and their high bioaccumulation (Jantunen et al., 2015; Jayaraj et al., 2016). DDT and the other 11 persistent organic pollutants (the dirty dozen) were finally prohibited worldwide following the agreements reached in the Stockholm Convention signed in 2001 (Stockholm Convention on Persistent Organic Pollutants, 2001).

CM xenobiotic agents are chemical compounds derived from carbamic acids, and the first carbamate with insecticide properties introduced into the market was Carbaryl in 1956 (Tiwari et al., 2018), along with more than 50 different compounds such as Aldicarb, Carbofuran, Fenobucarb, and others, that share a common mechanism of action, the reversible inhibition of the Cholinesterases (ChEs). These compounds have been widely used for crop pest control as well as for industrial and household purposes, particularly in the case of Carbaryl (Fishel FM, 2011). Although Carbamates extensively used around

the world, their lower toxicological profile regarding ChEs inhibition led to the wider use of OP agents. OPs deserve special attention in this introduction.

1.3. Organophosphate xenobiotic compounds

The OPs were first discovered in the middle-late '30s by a group of German chemists (Jaga and Dharmani, 2003) and represent the most widely used family of pesticides, as well as being the largest group of commercially produced pesticides worldwide (Ghorab and Khalil, 2015) (**Table 1**). Since, OPs have a lower affinity for accumulation in the environment compared with OCs and are highly effective in terms of pest control, they rapidly became regarded as a good alternative. Although OPs are organic compounds derived mainly from phosphonic, phosphinic or phosphoric acid, most of them are derived from this latter molecule (Kulshreshtha and Shinde, 2014). The general chemical structure of OP compounds is displayed in **Image 3**.

OP	Commercial names	Molecular weight	Water solubility -25ºC-	Kow	Koc
Azamethiphos	Alfacron 10, Snip Fly Bait	324.68 g/mol	0.11mg/mL	1.05	25
Diazinon	Antigal, Diazol, Spectracide, Neocidol (…)	304.36 g/mol	40mg/L	$2.5x10^{4}$	$6.45x10^{3}$
Dichlorvos	Vapona, Dervisol, SD1750, Dichlorman (…)	220.98 g/mol	10mg/mL	$5x10^{-3}$	14.5
Malathion	Celthion, Malathion, Karbofos, Maltox (…)	330.36 g/mol	145mg/L	560	1800
Chlorpyrifos	Brodan, Detmol UA, Lorsban, Scout (…)	350.62 g/mol	2mg/L	$9.1x10^{4}$	6070

Table 1. Main OP compounds currently available in the market. Kow refers to the octanol-water coefficient of a substance, whilst Koc refers to the soil absorption coefficient of a substance. The information was extracted from Cecchine et al., (2000) and the Pesticide Properties Database of the National pesticide information center.

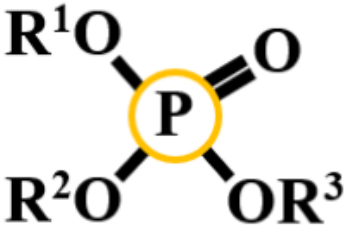

Image 3. General chemical structure of the OP agents

1.4. The special case of Chlorpyrifos

Chlorpyrifos (chlorpyrifos, O,0-diethyl O-3,5,6- trichloro-2-pyridyl phosphorothioate, chlorpyrifos-ethyl) (CPF) is one of the most important OP compounds based on its long-

standing widespread use from decades ago. Launched on the market in 1965 (Dow Chemical Company), CPF is one of the best-selling pesticides in the world, Europe and, more specifically, in Spain. Categorized as a moderately hazardous compound (World Health Organization), CPF is sold under a number of trademarks including Dursban, Lorsban, Suscon, Brosan, amongst others (Eaton et al., 2008). **Image 4** displays the general chemical structure of CPF.

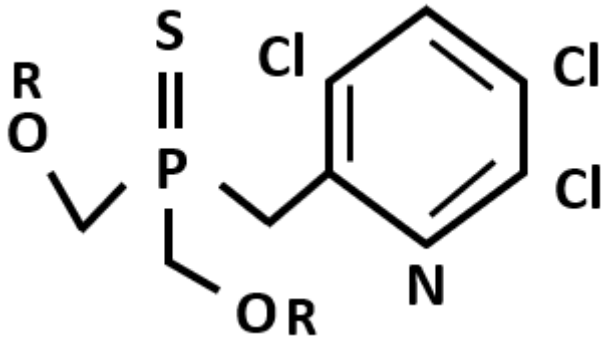

Image 4. Chemical structure of CPF

As can be observed, the CPF molecule is formed by an atom of phosphorus, double-bound to an atom of sulfur, two more ethyl unions, and an aromatic structure. This chemical structure defines the physical-chemical features of CPF, summarized in Table 2 from Eaton et al., 2008 and previously described in **Table 1** of the present thesis. Briefly, and in addition to the previously indicated properties, the melting point of CPF is around 42ºC, it decomposes at around 160ºC, its vapor pressure at 20-25ºC is $1.87x10^{-5}$ mm Hg and its Henry's law constant at 25ºC is $1.23x10^{-5}$ atm-m3/mol (Eaton et al., 2008).

1.4.1. Uses of Chlorpyrifos

Like most of the OP compounds, the use of CPF covers a wide range of fields and objectives. The majority of commercially produced CPFs products are used in agricultural purposes, particularly as an insecticide in crop production control, protecting corn, citrus, nuts, and tobacco, amongst others (Solomon et al., 2014). Moreover, its use in industrial, residential, and public spaces (e.g. termite and mosquito control, care of retail lawns, golf courses, and public fields, amongst others) reached its peak in western countries in 2001, when the United States government announced its partial banning at home. This was followed by a similar decision in the European Union just a few years later (Eaton et al., 2008). Although its use in developed countries is declining year upon year, the sales and

use (including residential use) of CPF-derived products continue to rise in developing nations.

1.4.2. Level of exposure to Chlorpyrifos

The wide range of applications of CPF provides an indirect indication of the ubiquitous and continuous risk of exposure that mankind and other mammalians face every single day, with special emphasis on those who live in or close to large areas of extensive or intensive agriculture production, as the south-east region of Spain. Although its persistence, as in the case of other OPs, is not particularly important in comparison with other xenobiotic compounds, CPF molecules have the capacity to remains in the environment (depending on the specific features of the soil and application), increasing the risk of exposure. Aside from the highly acute intoxications that could even lead to death, the normal exposure rates in humans are defined by a very-low daily exposure of just a few micrograms per kilogram per day (Eaton et al., 2008).

The effects of CPF vary according to the route of exposure (Smith et al., 2009). Currently, most of the exposure comes from the oral ingestion of fruits and vegetables which contain traces of pesticides, whilst inhalation (via the respiratory system) and dermal absorption are particularly prominent in farmers and people who live close to the agricultural production centers. CPF is readily absorbed by the enteric system, with rapid absorption and distribution to the central nervous system (CNS), whereas the absorption capacity following dermal exposure is somewhat lower than when it is ingested orally, and its later distribution is heavily marked by a high reservoir. Finally, CPF is generally well absorbed via inhalation and rapidly reaches the systemic system (Eaton et al., 2008).

1.4.3. Toxicokinetics of Chlorpyrifos

Once the CPF molecule is introduced into the organism, it undergoes several metabolic processes before it is distributed to various organs and tissues. **Image 5** displays the metabolic pathway of CPF biotransformation. In short, CPF can be metabolized in its -Oxon form CPF-Oxon (CPO) by the superfamily of enzymes Cytochromes P450 (CYP450) following a desulfurization process that substitutes the sulfur attached to the phosphorus atom for oxygen. CPO is then distributed to different tissues and has a high affinity for the serine section of the Acetylcholinesterase (AChE) which is recognized as the main mechanism of toxicity induced by CPF and the other OPs (described in the next section). Similarly, CYP450 can metabolize CPF molecules into 3,5,6-trichloro-2-

pyridinol (TCPy) and diethyl thiophosphate (DETP) following a dearylation metabolic process, whilst TCPy can be obtained from the action (hydrolysis) of paraoxonase 1 (PON1) on CPO, along with diethyl phosphate (DEP). TCPy and the remaining metabolites are then distributed and finally excreted by different routes such as urinary, biliary, fecal routes along with maternal milk elimination (Eaton et al., 2008), closing the cycle of CPF in the organism.

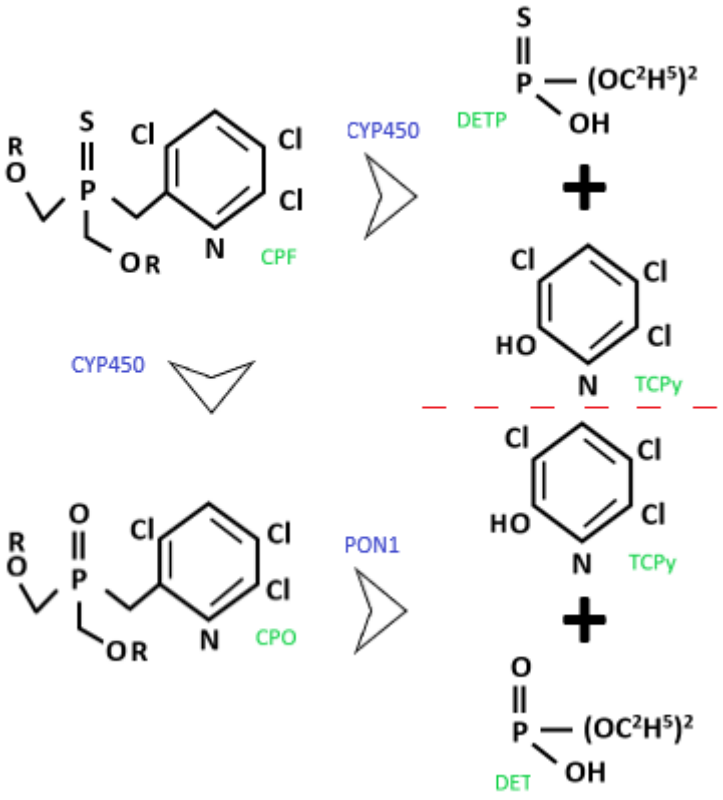

Image 5. Main components of the metabolic pathway involved in the biotransformation of CPF. The names of the molecules are displayed in green. The names of the enzymes involved in the metabolic processes are displayed in blue.

1.4.4. Toxicodinamics of Chlorpyrifos: when the objective is the Cholinesterases

The main toxic mechanism of action of CPF is the irreversible inhibition of the Cholinesterases both Butyrilcholinesterase (BuChE) and Acetylcholinesterases (AChE). However, the CPO metabolite is the molecule that actually shows this anticholinesterase profile; thus, CPF appears to work more as a pro-toxic than a toxic agent (US Department of Health and Human Services, 1997). The AChE is an essential enzyme, the main role of which is to hydrolyze the acetylcholine neurotransmitter in acetyl groups and choline. That is, the inhibition of AChE indirectly potentiates cholinergic activity in various

muscarinic and nicotinic receptors. When this cholinergic overstimulation is found following high doses of CPF or other OP compounds, it can induce the well-documented cholinergic syndrome (Jokanovic et al., 2011), leading to respiratory system disruptions, tremor, diarrhea, nausea, and confusion, amongst others. Cholinergic syndrome can lead to death by the acute depression of central and peripheral respiratory control centers.

1.4.5. Alternative molecular targets: when Cholinesterase is not the key

Although, unquestionably, the most important effects of CPF on pests are derived from the indirect cholinergic hyperstimulation of the muscarinic and nicotinic receptors that follows the inhibition of ChEs, exposure to CPF affects other molecules instead of/ in addition to these esterases. Some studies have proposed alternative molecular targets of the mammalian CNS following CPF exposure at low or very low doses that do not significatively inhibit the ChEs and that are far removed from the overt toxic effects (Burke et al., 2017). These non-cholinesterase related exposure protocols (NChEI) have generally been observed during development, when the immature CNS appears to be more sensitive to CPF toxicology. The most relevant studies regarding this exposure profile are systematically described in the following sections.

The most significant alternative targets include direct effects on transcription factors, intra-cell signaling components, oxidative stress, lipid peroxidation, mitochondrial mismatch or the modulation of glial cell activity (e.g. Schuh et al., 2002; Saulsbury et al., 2009; Xu et al., 2017; Hussein et al., 2018). Added to this, CPF exposure appears to directly alter certain components of the main neurotransmitter systems at different levels (precursors, transporters, and pre and postsynaptic receptors, amongst others), but it is particularly determined by the developmental stage at which the organism is exposed. With regard to the latter, a number of developmental studies have reported empirical data on this relation for the cholinergic (Slotkin and Siedler 2007b; Basaure et al., 2018, 2019a; Guardia-Escote et al., 2018), dopaminergic (Dam et al., 1999; Slotkin et al., 2002; Aldridge et al., 2005c; Slotkin and Seidler, 2007a, c; Chen et al., 2011; Savy et al., 2015) and serotonergic (Raines et al., 2001; Aldridge et al., 2003, 2004, 2005a, b, c; Slotkin and Siedler, 2005, 2007a, c; Slotkin et al., 2014, 2015; Savy et al., 2015) systems, at a wide range of doses and developmental stages. Finally, the glutamatergic and the GABAergic systems appear to be also sensitive to developmental CPF exposure, although there is rather less empirical support for this possibility (Slotkin and Siedler, 2007b; Slotkin et al., 2010; Gómez-Gimenez et al., 2018).

In spite of the extensive body amount of empirical research on this issue, the specific effects of CPF exposure during preweaning stages (>PND7 to weaning) is sparse and primarily focuses on the endocannabinoid system (Carr et al., 2011, 2013, 2014, 2017).

1.4.6. Developmental Chlorpyrifos, behavior, and associated disorders

Exposure to CPF during development has been linked to numerous neurodevelopmental, neurodegenerative, and psychiatric disorders in humans, and several behavioral deficits in experimental animals. Examples include attentional deficit and hyperactivity disorder (ADHD) and autism spectrum disorders (ASD) (Mostafalou and Abdollahi, 2017), Alzheimer's disease (AD), idiopathic Parkinson disease (PD) amyotrophic lateral sclerosis (ALS) (Sánchez-Santed et al., 2016; Mostafalou and Abdollahi, 2017) and depression and anxiety (Chen et al., 2011).

Different preclinical models of developmental exposure to low doses of CPF has also linked this to the later development of alterations in various behavioral functions related to the symptomatology described in the above-mentioned disorders. That is, developmental CPF exposure triggers selective alterations in locomotor activity, motricity, learning, spatial memory, sustained attention, sociability, inhibitory control, anxiety, and depressive-like behaviors. In the following sections, we systematically describe the most important studies concerning these behaviors.

1.4.7. Effects of developmental Chlorpyrifos exposure on locomotor activity and motricity

Many of the most prominent neurodevelopmental and neurodegenerative disorders have motor alterations amongst their main clinical characteristics. These include ASD, ADHD, Parkinson's disease, and Alzheimer's disease, amongst others. Exposure to CPF and other OPs has been linked to these complex pathologies (e.g., Sagiv et al., 2010, 2018; Sánchez-Santed et al., 2016; Chang et al., 2018). In humans, this xenobiotic compound has also been associated with more specific alterations in motor functions such as visuo-motor skills, psychomotor delay, motor speed/coordination, and eye-hand coordination, amongst others (e.g., Steenland et al., 2000; Rauh et al., 2006; Harari et al., 2010; Malekirad et al., 2013; Rohlman et al., 2016; van Wendel de Joode et al., 2016).

Furthermore, there is a vast body of empirical literature on locomotor activity alterations following CPF exposure during adulthood (Pope et al., 1992; Carvajal et al., 2005; López-

Crespo et al., 2007; Yan et al., 2012; López-Granero et al., 2013, 2014, 2016; Peris-Sampedro et al., 2014; Savy et al., 2015; Adedara et al., 2018), adolescence (Chen et al., 2014; Avci et al., 2018; Singh et al., 2018) and particularly, during development. With regard to the latter category, **Table 2** summarizes the main studies conducted in rodents that link CPF exposure during development to locomotor behaviors.

Study	Strain/age/sex	Exposure dose/age/route	Exposure control	Behavioral test	Behavioral/pharmacological/physiological outcomes
Venerosi et al., 2015	Mice/>PND70/Both	6mg/kg/d GD15-PND14. Diet	AChE activity, weight, litter size, sex ratio	Open Field	= Locomotor activity. + Estrogen Receptor β at Hypothalamus (Males). – Oxytocin at Amygdala (males). + Vasopressin receptor 1a at amygdala.
De Felice et al., 2015	Mice BTBR/PND4-12/ Both	6mg/kg/d GD14-17 Gavage	Weight, reflexes	Motor reflexes	= righting reflex. + duration of pivoting. - head shaking and general locomotion.
Lan et al. 2017	Mice/PND6-12/M	2.5-5mg/kg/d. GD12-15. Gavage	Weight, reflexes, maternal care	Motor reflexes	Impaired motor reflexes (+ time to righting, - negative geotaxis, - cliff avoidance scores)
Lee et al. 2015	NMRI Mice/PNM2-4/M	0.1, 1-5mg/kg PND10 Gavage	AChE activity, weight, muscle fasciculation and convulsion	Novel home environment	- early locomotor activity (5mg/kg). - Calcium/calmodulin-dependent kinases II (Hippocampus) and Synaptophysin (Cortex) (5mg/kg)
Mullen et al. 2013	Mice/ PND30/Both	6mg/mL/d GD13-Delivery Pump	AChE activity	Open Field	- exploration of the center $^{+/+}$ (male)
Laporte et al., 2018	Rat/PND3-60/Both	1mg/kg/d GD1-PND21 Oral	Weight, eye opening	Motor reflexes; Open Field; Startle inhibition	Impaired motor reflexes (Males). – Locomotor activity (Males). Faster startle to prepulse+pulse.
Venerosi et al., 2008	Mice/PND40-45/Both	3mg/kg/d PND11-15 s.c.	Neurobehavioral battery	Three-chambers test (First phase)	- distance moved (spontaneous locomotor activity)
Ricceri et al., 2003	Mice/ PND25/Both	1-3mg/kg/d PND1-4, 11-14 s.c.	Neuromotor battery; AChE activity	Open Field	+ distanced travelled in preweaning exposed group (dose-dependent)

Table 2 Continuation

Ricceri et al., 2006	Mice/PND70/M	3-6mg/kg/d GD15-18 Gavage 1-3mg/kg/d PND11-14 s.c.	AChE activity, weight, cholinergic intoxication	Open Field	+ locomotor activity following the highest dose (gestational). The highest postnatal dose blocked this effect.
Levin et al., 2002	SD Rats/PNW4-17/Both	1-5mg/kg/d GD17-20 s.c.	Weight, litter size	Fig. 8 Locomotor activity; T-maze; Radial-arm maze.	+ Locomotor activity (velocity in T-maze). + Locomotor activity exposed females (lower habituation to Fig. 8 test). + Locomotor activity (CPF exposed were faster than CNT in the Radial-arm maze). 1mg/kg/d CPF exposure increased the slowness (response latency) induced by Scopolamine in the radial-arm maze.
Icenogle et al., 2004	SD Rats/PNW4-13/Both	1-5mg/kg/d GD9-12 s.c.	Weight, litter size	Fig.8 Locomotor activity; T-maze; Radial-arm maze; Plus maze	+ Locomotor activity (velocity in T-maze). – Locomotor activity following 5mg/kg/d (increased motor habituation in Fig.8 test). + Locomotor activity following 5mg/kg/d (center crosses in Plus maze)
Silva et al., 2017	W rats/PND21-70/Both	0.01, 0.1, 1-10mg/kg/d GD14-20 Gavage	Weight, litter size	Open Field	+ Locomotor activity during early adolescence following all doses except the lowest one.
Venerosi et al., 2009	Mice/PND12/Both	6mg/kg/d GD15-18 Gavage	AChE activity, weight, neurobehavioral battery.	Spontaneous motor behavior	- Locomotor activity (increased immobility and decreased pivoting).
Dam et al., 2000	SD rats/PND21-30/Both	1mg/kg/d PND1-4 5mg/kg/d PND11-14 s.c.	ChE activity	Motor reflexes; Open Field	- Reflex righting and negative geotaxis (exposed females from PND1-4). – Locomotor activity (exposed males PND1-4, both PND21 and 30). + rearing behaviors (preweaning exposed males at PND30)
Levin et al., 2001	SD rats/PNW4-17/Both	1 mg/kg/d PND1-4 5mg/kg/d PND11-14 s.c.	Weight, litter size	Fig. 8 Locomotor activity; T-maze; Radial-arm maze.	- Velocity (increased response latency in T-Maze in males in neonatal exposure). + Locomotor activity (decreased habituation following the preweaning exposure protocol)
Carr et al., 2001	SD rats/PND10-30/Both	3 or 3+6 or 3+6+12mg/kg/d PND1-21 Gavage	AChE and BChE activity, death rate, weight	Open Field	- Locomotor activity in both sexes (medium and high dosage protocol at PND25 and 30)

Table 2 Continuation

Venerosi et al., 2006	Mice/PNM4/F	3-6mg/kg/d GD15-18 1-3mg/kg/d PND11-14	AChE activity, weigh, litter size	Social Recognition test	= Locomotor activity (frequencies)
Cole et al., 2012	PON KO mice/PND23-25/Both	0.15, 0.18-0.25 mg/kg/d CPO PND4-21 s.c.	AChE activity, weight, eye opening	Motor reflexes; rotarod; Open Field	= motor coordination and locomotor activity.
Gomez-Gimenez et al., 2017	W rats/PNM2-3/Both	0.1, 0.3-1mg/kg/d GD7-PND21 Sweet jelly	Weight, litter size,	Rotarod; Open Field	- Motor coordination in females (medium dosage). + Locomotor activity both sexes (lowest dosage). + extracellular GABA content in males' cerebellum (medium dosage). + expression of different glutamatergic NMDA receptor subunits in males' hippocampus (eminently at the lowest dose). – Expression of the 2B subunit in females' hippocampus.
Guardia-Escote et al., 2019	APOE3 and 4 mice/N.I./Both	1mg/kg/d PND10-15 Gavage	N.I.	Open field	+ Locomotor activity in exposed APOE4 mice.
Levin et al., 2014	SD Rats/PNW4-5/Both	1mg/kg/d PND1-14 s.c.	CPF levels, weight, litter size	Fig. 8 Locomotor activity; T-maze	+ Locomotor activity (exposed males), increased by gestational exposure to Dexamethasone.

Table 2. Main developmental studies concerning CPF exposure & locomotor behaviors in rodents. CPO: CPF-Oxon, PND= Postnatal day, M/F= Males/Females, B= Both sexes, PNM= Postnatal month, d= day, GD= Gestational day, s.c.= Sub cutaneous, AChE= Acetylcholinesterase, NI= Not indicated

A total of 21 developmental studies were reviewed. From these, twelve (57%) used mice as the experimental animals, and the remaining used rats. seven (33%) followed a gestational exposure protocol, nine (43%) exclusively postnatal and the remaining 5 (24%) both gestational and postnatal exposure regimens. A total of eight studies (38%) included the preweaning exposure protocol, of which only two used rats (10%), using doses of 5mg/kg/day in both cases (Dam et al., 2000; Levin et al., 2001).

Gestational exposure lead to impaired motor reflexes at early postnatal ages (De Felice et al., 2015; Lan et al., 2017) and motor hyperactivation during adolescence and/or adulthood (Levin et al., 2002; Icenogle et al., 2004; Ricceri et al., 2006; De Felice et al., 2015; Silva et al., 2017). However, this hyperactivation effect has not always been observed (Icenogle et al., 2004 -in Fig. 8 test-; Venerosi et al., 2006, 2009). These effects were observed following various exposure regimes ranging from 1 to 10mg/kg/day, and at different time points between gestational day (GD) 9 (e.g. Icenogle et al., 2004) and delivery (e.g. Mullen et al., 2013). In those studies, involving rats, gestational exposure generally produced hyperactivity during adolescence and/or adulthood.

Otherwise, neonatal exposure altered motor reflexes (females) and generally decreased locomotor activity during adolescence (Dam et al., 2000) as well as decreased speed in adulthood (Levin et al., 2001), although these motor alterations have not been systematically found (Ricceri et al., 2003). On the other hand, preweaning postnatal exposure has been linked to both increased (Dam et al., 2000; Levin et al., 2001; Ricceri et al., 2003; Guardia-Escote et al., 2019a), decreased (Venerosi et al., 2008; Lee et al., 2015) or unchanged (Venerosi et al., 2006) on motor rates. Concerning the preweaning exposure protocols conducted with rats (Dam et al., 2000; Levin et al., 2001), both studies revealed CPF-induced hyperactivity during adolescence and adulthood. The continuous exposure from delivery to weaning also induced a marked decrease in locomotor activity during adolescence (Carr et al., 2001).

Finally, the exposure protocols that involved continuous exposure during both gestational and postnatal stages have produced a range of outcomes, from no motor effects (Venerosi et al., 2015) to impaired motor reflexes and a decrease in motricity in males (Laporte et al., 2018), and altered motor coordination in females and increased locomotor rates in both sexes when the exposure began later during gestation (Gómez-Giménez et al., 2017).

Taken together, all of these results indicate that gestational exposure is linked to general hyperactivity, a finding that is also observed in preweaning exposed rats but is opposite to the general postnatal effects found in mice. The relatively low number of studies focused on preweaning exposure in rats are characterized by exposure to doses of 5mg/kg/day, below the threshold for systematic toxicity, but far away from realistic exposure rates.

1.4.8. Effects of developmental Chlorpyrifos exposure on sociability and social traits.

ASD is a complex and heterogeneous set of disorders characterized by alterations on communication skills, stereotyped behaviors and altered sociability (Diagnostic and statistical manual of mental disorders-5th ed., APA, American psychiatric association). The genetic base of this complex set of disorders is unquestionable (Sandin et al., 2017). Interestingly, some of the most important/common genetic risk variants associated with ASD were recently reported in Grove et al., (2019), highlighting various candidate genes, such as the Potassium Calcium-Activated Channel Subfamily N Member 2 and the Matrix Metallopeptidase 12, amongst others. This increasing knowledge of the genetic basis of ASD is also characterized by an important number of specific knock out/down and transgenic rodent models that systematically show an ASD-like phenotype in multiple behaviors, such as the fragile X mental retardation 1 and the Neuroligin 3 genes, amongst others (Lázaro and Golshani, 2015).

However, there is a general consensus that the etiology of ASD must be multifaceted, where genes work as vulnerability factors that are finally triggered or modulated by external variables. The most significant environmental factors associated with ASD development are perinatal stress and injury, family dynamics and socioeconomic status, and the exposure to environmental toxic agents such as persistent agents, pollution and metals (Chaste & Leboyer, 2012; Kalkbrenner et al., 2014). Interestingly, other chemicals generally used for clinical purposes, as in case of valproic acid, have been linked to this phenotype in both human (Christensen et al., 2013) and rodent models (Nicolini and Fahnestock, 2018). All these agents and environmental factors, along with critical changes in the diagnostic criteria (Hansen et al., 2015) and the increased use of different pesticides (Modabbernia et al., 2017; Pelch et al., 2019; Roberts et al., 2019), could partially explain why in the last two decades have been seen dramatic rise in the prevalence rise of ASD diagnosis (World Health Organization, 2019). For the purposes

of the present work, the exposure to OP agents has gained importance in the last decade (Shelton et al., 2014).

In particular, CPF exposure and its main metabolites have systematically been associated with ASD in children. Several studies involving humans have found this negative association following gestational exposure (Furlong et al., 2014; Schmidt et al., 2017; Wang et al., 2017; Philippat et al., 2018; Sagiv et al., 2018; von Ehrenstein et al. 2019). Thus, the development appears to be particularly sensitive to CPF exposure. However, this association has not always been found, and, for postnatal exposure, the association with ASD non-existent. This is particularly interesting, as some authors have proposed that the period surrounding birth could be an important developmental window regarding the sensitivity to external influences on the CNS that are associated with ASD (Getahun et al. 2017; Martinez-Morga et al. 2018).

In relation to animal studies, **Table 3** summarizes the main studies conducted in rodents concerning the effects of developmental CPF exposure on social behaviors.

Study	Strain/age/sex	Exposure dose/age/route	Exposure control	Behavioral test	Behavioral/pharmacological/physiological outcomes
Venerosi et al. 2010	Mice/>PND90; Mums postpartum/Both	6mg/kg/d GD15-18 Gavage	N.I.	Maternal aggression	- Maternal aggressive behavior. + Anxiety (Females). = Depressive-like behaviors
De Felice et al. 2014	Mice/>PND70/Both	6mg/kg/d GD14-17 Gavage	Pups, sex ratio, weight	Social Discrimination test	+ Social investigation (Females)
Venerosi et al., 2015	Mice/>PND70/Both	6mg/kg/d GD15-PND14 Diet	AChE activity, weight, litter size, sex ratio	Social Recognition test	+ Social investigation males (all phases) and females (second exposure to the same partner). + Estrogen Receptor β at Hypothalamus (Males). – Oxytocin at Amygdala (males). + Vasopressin receptor 1a at amygdala.
De Felice et al. 2015	BTBR Mice/PND4, 6, 8, 8 (USVs); >70 (Sociability)/Both	6mg/kg/d GD14-17 Gavage	Weight, litter size, sex ratio, mortality	USVs; Social interaction test	+ (trend) calls. + USVs and social investigation (sniffing) (males to females).
Lan et al. 2017	Mice/PND90/M	2.5-5mg/kg/d GD12-15 Gavage	Weight, reflexes maternal care	Three-chambers test	- Sociability (5mg/kg)
Lan et al. 2019	Mice/PND90/Both	2.5-5mg/kg/d GD12-15 Gavage	N.I.	Three-chambers test	- Social preference males vs. the rest (5mg/kg). - Social preference females vs. males (2.5mg/kg). = Oxytocin mRNA levels at hypothalamus
Mullen et al. 2013	Reeler Mice/PND7 (USVs), PND30 (Social interaction)/Both	6mg/mL CPO GD13-Delivery Pump	AChE activity	USVs; Three-chambers test	+ USVs number $^{+/-}$Reeler (males) from its vehicle. - USVs number $^{+/+}$Reeler from its vehicle. – USVs duration. + social interaction (sniffing, females, both exposed $^{+/+}$ and $^{+/-}$).
Venerosi et al., 2008	Mice/PND40-45; Mums postpartum/Both	3mg/kg/d PND11-15 s.c.	Neuromotor battery	Three-chambers test; Maternal behavior	= sociability and reaction to social novelty. + maternal care. – anxiety in mums. – maternal aggressive behavior. + maternal social investigation.
Ricceri et al., 2003	Mice/PND5-11 (USVs); PND45/Both	1-3mg/kg/d PND1-4, 11-14 s.c.	Neuromotor battery, weight and AChE activity	USVs; Social interaction test	= USVs. + aggressive behaviors in exposed animals, with larger rates for preweaning exposed.

Table 3 Continuation

Basaure et al., 2019b	APOE3 and 4 Mice/PNM5/M	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.
Venerosi et al., 2006	Mice/PNM4/F	3-6mg/kg/d GD15-18 1-3mg/kg/d PND11-14 Gavage	AChE activity, weight, cholinergic intoxication	Social recognition test; USVs	+ USVs in gestational exposed to the highest dose. This was reversed with 1mg/kg/d of postnatal exposure. + Social investigation in gestational exposed to the highest dose. Blocked by the postnatal exposure (both doses).
Ricceri et al., 2006	Mice/PND70 (Sociability); PND90 (mums' behavior)/M-FR	3-6mg/kg/d GD15-18 1-3mg/kg/d PND11-14 Gavage	AChE activity, weight, cholinergic intoxication	Social interaction test; Maternal behavior	+ aggressive behaviors in males (the highest postnatal and gestational doses). + maternal behaviors. – anxiety especially in exposed females (postnatal).
Venerosi et al., 2009	Mice/PND4-10/Both	6mg/kg/d GD15-18 Gavage	AChE activity, weight, neurobehavioral battery.	Maternal behavior; USVs	Altered maternal behavior in CPF exposed mums (increased wall rearing and decreased digging). – USVs (calls/min and duration) only in PND10.

Table 3. Main developmental studies concerning CPF exposure & sociability and communicative behaviors in rodents. PND= Postnatal day, M/F= Males/Females, B= Both sexes, PNM= Postnatal month, d= day, GD= Gestational day, s.c.= sub-cutaneous, USV= Ultrasound vocalizations, AChE= Acetylcholinesterase, NI= Not indicated.

A total of thirteen studies were reviewed. All of these used different strains of mice. Seven out of the thirteen works (54%) exposed the animals during gestation, three postnatally (23%) and the remaining three (23%) during both gestation and postnatal stages. Late gestational exposure (from GD14 onwards) generally led to a decrease in maternal aggression, enhanced social investigation in females, and an increased number of ultrasound vocalizations (USV) (Venerosi et al., 2006, 2010; De Felice et al., 2014, but see Venerosi et al., 2009) as well as increased aggression in males (Ricceri et al., 2006). However, decreased sociability and social preference were observed when the mice were exposed at an early age (GD12 onwards, Lan et al., 2017, 2019). Interestingly, gestational exposure in ASD-mouse models also enhanced social traits (Mullen et al., 2013; De Felice et al., 2015).

From these three studies that followed the postnatal-alone exposure protocol, all of them included preweaning exposure (Ricceri et al., 2003; Venerosi et al., 2008; Basaure et al., 2019). The other two studies that included both gestational and/or postnatal exposure also exposed mice during this period (Venerosi et al., 2006; Ricceri et al., 2006). Preweaning exposure enhanced maternal care and social investigation outcomes (Venerosi et al., 2008) without affecting sociability (measured by the three-chambers test), and also increased aggressive behaviors (Ricceri et al., 2003). Interestingly, a chronic dietary dose of 2mg/kg/day during adulthood enhanced social traits in Apolipoprotein (APOE) 3 mice (transgenic mice which express the isoform 3 of the human APOE) and completely reversed the negative influences of the CPF exposure at the preweaning ages, where both oxytocin and vasopressin peptides in the hypothalamus appear to play an important role (Basaure et al., 2019b).

Finally, the protocols involving both gestational and postnatal exposure also improved social investigation (Venerosi et al., 2015), whilst the postnatal exposure abolished the increase in USV rates and social investigation observed as a consequence of gestational exposure (Venerosi et al., 2006).

Taken together, the results of these studies indicate that there are variations in the effects of low doses of CPF exposure on ASD-like behaviors according to the period of development which the exposure is given, with more studies beneficial than harmful effects, and a lack of studies in the literature using rats, a closer animal model to human compared to mice, particularly concerning social behaviors (Ellenbroek and Youn, 2016).

1.4.9. Effects of developmental Chlorpyrifos exposure on attention, memory, learning and inhibitory control

The proper functioning of the attentional, memory and inhibitory control processes is essential for adequate learning and general cognitive functioning in all aspects of life. In fact, selective alterations in these superior cognitive functions appear to underlie developmental and neurodegenerative disorders (Peckham et al., 2010; Christodoulou et al., 2012; Boxhoorn et al., 2018; Picazio et al., 2018; Schmitt et al., 2018; Malhotra PA, 2019). Along with other xenobiotic compounds, OP agents have been linked to alterations in such processes (Marks et al., 2010; Suarez-López et al., 2017; Perez-Fernandez et al., 2019a).

Many studies have found an interesting link between CPF exposure and alterations in different types of memory, learning, attention, and inhibitory control behaviors in both humans (Rosenstock et al., 1991; Steenland et al., 1994; Stephens et al., 1995; Malekirad et al., 2013; Rohlman et al., 2016, 2019; Perez-Fernandez et al., 2019a) and rodents (Cohn & Macphail, 1997; Bushnell et al., 2001; Terry et al., 2003; Sánchez-Santed et al., 2004; Cañadas et al., 2005; ; Samsam et al., 2005; Cardona et al., 2006, 2011; Middlemore-Risher et al., 2010; Terry AV, 2012; López-Granero et al., 2013, 2014; Montes de Oca et al., 2013; Peris-Sampedro et al., 2016; Basaure et al., 2017; Hussein et al., 2018; Imam et al., 2018; Perez-Fernandez et al., 2019a).

Of particular interest to the present work is the fact that developmental exposure has been also associated with these behaviors, both in children (Ruckart et al., 2004; Rauh et al., 2006; Sánchez Lizardi et al., 2008; Dassanayake et al., 2009; Butler-Dawson et al., 2016; van Wendel de Joode et al., 2016; Suarez-López et al., 2017; Dalsager et al., 2019; Perez-Fernandez et al., 2019a) and rodents. **Table 4** summarizes the main studies concerning this last category.

Study	Strain/age/sex	Exposure dose/age/route	Exposure control	Behavioral test	Behavioral/pharmacological/physiological outcomes
Basaure et al., 2019a	APOE3 and 4 Mice/PNM6/M	1mg/kg/d PND10-15 Oral 2mg/kg/d PNM5-7 Diet	ChE activity	Barnes maze	+ Learning rates in adult female APOE4 mice (exposed during adulthood). Postnatal and adult exposure increased serial and spatial strategies in female APOE4 mice. Preweaning exposure altered learning transition serial-spatial strategy in APOE3. – Retention (APOE3 postnatally exposed). + Retention (APOE4 exposed females during adulthood). Gene analyses at adult hippocampus: Postnatal exposure increased ChAT (both sexes) and adult exposure increased the nicotinic receptor subunit α4 (females) mRNA expression levels in APOE4 mice. Postnatal exposure down-regulated the nicotinic receptor α7 subunit (males) and adult exposure increased it (females). Adult exposure up-regulated females APOE4 AChE-S mRNA levels.
Levin et al., 2002	SD Rats/PNW8-17/Both	1-5mg/kg/d GD17-20 s.c.	Weight, litter size	Radial-arm maze	- Working and reference memory rates in females exposed to the lowest dose. Cholinergic system hyposensitivity (Scopolamine challenge) in females exposed to the lowest dose.
Icenogle et al., 2004	SD Rats/PNW8-13/Both	1-5mg/kg/d GD9-12 s.c.	Weight, litter size	Radial-arm maze	- Working and reference memory rats in early acquisition stages in animals exposed to the largest dose. Cholinergic system hyposensitivity (Scopolamine challenge) in the largest dose group
Levin et al., 2001	SD Rats/PNW8-17/Both	1 mg/kg/d PND1-4 5mg/kg/d PND11-14 s.c.	Weight, litter size	Radial-arm maze	- Working and reference memory rates in early stages of the test in exposed males (neonatal exposure). + Working memory in exposed females (neonatal exposure). = Working and reference memory rates following preweaning exposure regime. Cholinergic system hyposensitivity (Scopolamine challenge) in females exposed during preweaning stages.
Cole et al., 2012	PON KO mice/PND62-116/Both	0.15, 0.18-0.25 mg/kg/d CPO PND4-21 s.c.	AChE activity, weight, eye opening	Fear conditioning; Morris water maze; Water radial-arm maze	= learning and memory.
Gómez-Gimenez et al., 2017	W Rats/PNM2-3/Both	0.1, 0.3-1mg/kg/d GD7-PND21 Sweet jelly	N.I.	Morris water maze; Radial-arm maze	- Learning and memory in exposed males (stronger for the lowest dose group, Morris). – Spatial memory in probe phase in exposed males (largest dose, Morris). – Reference, working memory and learning rates in exposed males (the two largest dose groups, Radial). + Reference memory and learning rates in exposed females (largest dose, Radial). Up-regulation of IL-1 Beta and down-regulation of IL-10 proteins in exposed males' hippocampus (all doses and the largest dose, respectively).: Down-regulation of Iba1 and IL-10 proteins in exposed females' hippocampus (the largest dose and the two largest doses, respectively). Increased levels of NR1, NR2A and B (lowest dose) and NR1 and GABA-A-gamma 2 subunit (largest dose) in males' hippocampus.
Jett et al., 2001	LE Rats/PND24-28/Both	0.3-7mg/kg/every 4 days PND7-15 s.c.	ChE activity, functional battery, weight	Morris water maze	- Learning rates and memory in exposed rats (highest dose). = brain muscarinic receptors number.

Table 4 Continuation

Johnson et al., 2009	SD Rats/PNM1-2/Both	1, 1-2 & 3 and 1.5, 3 and 6mg/kg/d PND1-21 Gavage	ChE activity, physical and reflex development	Radial-arm maze	- Reference and working memory and exposed males (highest dose). + Reference memory exposed females (both medium and highest dose)
Guardia-Escote et al., 2018	APOE4 Mice/PNM9/Both	1mg/kg/d PND10-15 Gavage	N.I.	Morris water maze	- Spatial memory -retention- in probe 2 (exposed APOE4 males). CPF down-regulated female control mice's nicotinic receptor α7 subunit mRNA expression at frontal cortex. CPF blocked the increased expression of muscarinic 2 receptor observed in APOE4 male mice
Alipour et al., 2019	W Rats/PNM3/Both	1mg/kg/d PND1-4 s.c.	N.I.	Radial-arm maze	= Reference memory. + Working memory rates (exposed kindled males). Hypersensitive glutamatergic system in exposed kindled males based on reference memory performance and in females based on working memory performance (MK-801 challenge).
Aldridge et al., 2005	SD Rats/PNM3-5/Both	1mg/kg/d PND1-4 s.c.	Functional battery	Radial-arm maze	- Working and reference memory in early sessions (exposed males). + Working and reference memory (exposed females). Hypersensitized serotonergic system (Ketanserin challenge), more evident in exposed males.
Wang et al., 2018	SD Rats/PND40-44, 70-74/Both	2.5mg/kg/d PND11-14 s.c.	N.I.	Morris water maze	- Spatial memory (CPF+Lipopolysaccharide)
Guardia-Escote et al., 2019	APOE3 and 4 mice//Both	1mg/kg/d PND10-15 Gavage	N.I.	Novel object recognition	- Retention memory in exposed mice (more evident in APOE4 group).

Table 4. Main developmental studies concerning CPF exposure & attention, learning and inhibitory control behaviors in rodents. CPO: CPF-Oxon, PND= Postnatal day, M/F= Males/Females, B= Both sexes, PNM= Postnatal month, d= day, GD= Gestational day, s.c.= Sub cutaneous, AChE= Acetylcholinesterase, NI= Not indicated

A total of thirteen developmental studies were included in the present analysis. Nine out of these were conducted using rats (69%). In two out of the thirteen (15%), the rodents were exposed during gestation, one (8%) during both gestation and postnatal stages and in the remaining ten studies (77%) the rodents were exposed during postnatal periods. In six of these studies the rodents were exposed during preweaning stages, seven if we include Cole et al., (2012) (70%), whose exposure protocol ranged from PND4 to 21. Finally, only three out of these seven (43%) preweaning studies were conducted with rats (Levin et al., 2001; Jett et al., 2001; Wang et al., 2018), and these exclusively focused on working, reference and spatial memory and learning; Thus, inhibitory control remained unexplored.

The studies concerning gestational exposure showed alterations in working and reference memory rates as well as a generally hyposensitized cholinergic system following drug challenges (Icenogle et al., 2004), this effect being sex-specific, favoring exposed females in some cases when the chemical was exposed at later gestational stages (Levin et al., 2002). Added to this, the work concerning continuous developmental exposure (Gómez-Gimenez et al., 2017) also found impaired learning and memory performance in exposed males, with some interesting enhanced rates in exposed females in the Radial-arm maze, along with the alteration in the expression of many proteins related to neuroinflammation, glutamatergic and GABAergic systems. The early postnatal studies found poorer working and reference memory in neonatally exposed males (Levin et al., 2001) and kindled exposed male rats (Alipour et al., 2019), but also in exposed females (Aldridge et al., 2005).

Focusing on preweaning exposure, various studies have found no effects of CPF exposure on learning, attention and/or memory (Levin et al., 2001; Cole et al., 2012) but most of these found the expected altered performance (Jett et al., 2001; Guardia-Escote et al., 2018, 2019a; Wang et al., 2018; Basaure et al., 2019a). With regard to rat models, these deficits appear to be task-specific as they have been found when using a Morris Water Maze (MWM) (Jett et al., 2001; Wang et al., 2018) but not when the Radial-arm maze protocol was used (Levin et al., 2001).

Taken together, all this information suggests that there is insufficient empirical data concerning the long-term effects of developmental exposure to low doses of CPF on attention, memory, learning and inhibitory control, which is somewhat surprising, given the vast literature on the analyses of these behaviors concerning adult CPF exposure.

More specifically, whilst studies using postnatal exposure points to a link between CPF and altered learning and memory rates, studies conducted in rats during preweaning developmental windows are sparse and inconclusive. Finally, we were unable to find a single study that analyzed the relationship between developmental CPF exposure and inhibitory control, as previously confirmed by Perez-Fernandez et al., (2019a).

1.4.10. Late postnatal exposure. Relevance of the preweaning developmental stage for the long-term neurobehavioral deficits associated with Chlorpyrifos

Based on the different empirical studies included in the previous sections, a total of thirteen studies specifically analyzed the long-term neurobehavioral effects of late postnatal, preweaning exposure to low doses of CPF, four of these using rats (Dam et al., 2000; Levin et al., 2001; Jett et al., 2001; Wang et al., 2018). These can be added to other studies that gave exposure during these developmental ages but analyzed other behavior and/or molecular outcomes (e.g., Betancourt et al., 2007; Tait et al., 2009; Buratti et al., 2011; Timchalck et al., 2012; Carr et al., 2011, 2013, 2017; Slotkin et al., 2015, amongst others). However, in comparison with the gestational and neonatal stages, this developmental period is still one of the least studied with regard to CPF exposure.

This developmental window is essential for different neurodevelopmental processes or benchmarks, and it is a good model for human perinatal damage. Briefly, the time around PND10 (>PND7) is key moment for synaptogenesis, oligodentrogenesis and the maturation of both vasopressin and oxytocin systems or the shifting of the GABAergic system in the CNS (Tait et al., 2009; Spitzer NC, 2010). Finally, the neurodevelopmental milestones are generally completed in a similar way in both human and rodent brains. However, the time at which these histological and molecular processes occur and mature is different across species. In this regard, some authors have found that the developmental state of the CNS around PND10 in rats is equivalent to the last trimester of pregnancy and the time around early post-delivery in humans (Semple et al., 2013). Consequently, its translational implications in terms of perinatal brain damage are unquestionable. **Image 6** summarizes the main developmental landmarks for humans and rats. For a more detailed description of both human and rodent brain maturation, please see Silva et al., (2020).

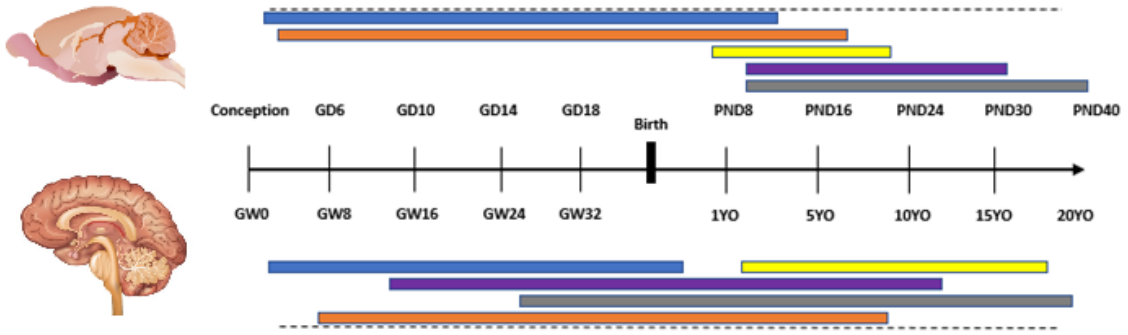

Image 6. Normal development of the CNS in both human and rats based on the most important benchmarks. This diagram was designed according to the information summarized in Semple et al., 2013, Babikian et al., 2010 and Tau and Peterson, 2010. Neurogenesis (neuronal proliferation and migration, in blue), Synaptogenesis (in purple), Myelination (in grey), Synaptic pruning (in yellow) and apoptosis (in orange) neurodevelopmental stages are displayed for both rats (upper section) and humans (lower section). GD/W: Gestational day/week. PND: Postnatal day. YO: Years old.

Since there are several neurodevelopmental, neurological, and psychiatric pathologies whose etiopathology has been linked to perinatal environmental stressors, exposure to CPF during this stage could be particularly critical for the alterations thought to be responsible for the specific disorders and behaviors previously described.

1.4.11. Effects of CPF exposure on gut microbiota composition

CNS functioning is generally considered to be the physiological substrate of behavior. Indeed, damage or disruptions to some parts of its structure trigger behavioral malfunctioning which, in the most severe cases, leads to the development of a wide range of alterations that can be categorized into a specific pathology. However, disruptions in other systems have more recently been linked to specific modulations of behavior both directly and, mainly, indirectly by inducing changes in the CNS. Aside from the well-known influences of the immune and endocrine systems (generally defined as the immune-neuroendocrine system), the gut microbiome has gained interest in the last decade (Fung et al., 2017).

Gut microbiome comprises the various bacteria populations living in the mammalian intestines. To date, it is estimated that around 3.8 x 10^{13} bacteria numbers reside in the adult colon of a human adult (using a reference male) (Sender et al., 2016), and are responsible for metabolic functions that are essential to maintaining adequate health. Although their effects on drugs, chemicals, and nutrients could be an indirect way of

affecting the CNS, the gut microbiota have a direct physiological and functional link to the CNS. This is defined by direct pathways from the intestines to the Vagus nerve via the enteric nervous system, suggesting a bidirectional inter-relationship between the two systems [microbiome-gut-brain axis (Cryan et al., 2019)]. Further, most of the serotonin molecules (around 90%) are synthesized in the gut (Yano et al., 2015), with approximately half of the dopamine being produced in the mesenteric organs (Eisenhofer et al., 1997).

Furthermore, several pathologies show a systematic alteration in gut microbiota composition (dysbiosis) in comparison with healthy subjects, indicating that the disturbances in this micro-cosmos could be at the basis of the observed symptomatology. This is the case for various neurodevelopmental (e.g. ASD and ADHD), neurodegenerative (Amyotrophic lateral sclerosis and Parkinson's disease) and psychiatric (schizophrenia, depression, stress) disorders (Strati et al., 2017; Cenit et al., 2017; Spielman et al., 2018; Nguyen et al., 2018; Cheung et al., 2019; Molina-Torres et al., 2019).

Recent years have seen the growing interest in studying the effects of pesticide exposure on gut microbiota, with OP compounds in particular being the subject of intense scrutiny (Roman et al., 2019). However, only a few studies have specifically analyzed the effects of CPF exposure on gut microbiota composition and permeability in rodents and in vitro models (Reygner et al., 2016b; Zhao et al., 2016; Joly Condette et al., 2016; Réquilé et al., 2018; Fang et al., 2018; Liang et al., 2019; Li et al., 2019). Interestingly, gut microbiota dysbiosis has been observed following developmental CPF exposure in mice (Guardia-Escote et al., 2019b) and rats (Joly Condette et al., 2013, 2014, 2015; Reygner et al., 2016a). Briefly, rat studies are characterized by continuous gestational and postnatal exposure to low doses of CPF (Joly Condette et al., 2014, 2015) and further on into early adulthood (Joly Condette et al., 2013), linking this CPF exposure to alterations in intestinal permeability, a condition generally associated with gastrointestinal pathologies (Camilleri et al., 2012), as well as modulations In the relative abundance of several bacteria populations such as Bacteroides, Lactobacillus, Bifidobacterium, and Clostridium, amongst others, some of which have been extensively linked to ASD and other disorders (e.g., Xu et al., 2019).

However, only one of these studies specifically analyzed the effects of preweaning exposure to low doses of CPF on gut microbiota in mice (Guardia-Escote et al., 2019b).

Interestingly, the authors found that CPF-induced dysbiosis depends on the APOE-type isoform. Briefly, APOE-4 mice (transgenic mice which express the human APOE 4 isoform) were more sensitive to the influences of CPF exposure, which heavily decreased the relative abundance of the Verrucomicrobia (with particular emphasis on Akkermansia municiniphila), as well as Nitrospirae and Planctomycetes bacteria. These results reflect the lack of knowledge on the medium and long-term effects of CPF exposure during the postnatal preweaning developmental stages on gut microbiota composition in rats.

1.4.12. Effects of developmental CPF exposure on metabolites

As in the case of the gut microbiome, the composition of the central, fecal, and systemic metabolite profile (metabolomics) is gaining interest for its possible relationship with brain functioning and behavior related to various disorders as well as the potential power of these profiles to serve as biomarkers for differential diagnosis. In particular, the alterations in the levels of different metabolites have been linked to ASD (Ruggeri et al. 2014; Mussap et al. 2016; Mohamadkhani A, 2018), ADHD (Chen et al., 2019), AD (Wilkins and Trushina, 2017) and PD (Picca et al., 2019; Glaab et al., 2019; Shao and Le, 2019), amongst others (Yang et al., 2020).

Of interest to our work is the observation that CPF exposure has been linked to the alteration of different metabolites and metabolic pathways in different essential amino acids and lipid metabolism (Wang et al., 2009; Xu et al., 2015). However, there only very few studies that have explored developmental CPF (Slotkin et al., 2005; Guardia-Escote et al., 2019b), observing a general alteration in lipid metabolism after neonatal exposure in rats (Slotkin et al., 2005), as well as an interesting influence of different short-chain fatty acids following preweaning exposure in mice (Guardia-Escote et al., 2019b). As in the case of the gut microbiota related studies previously described, we were unable to find a single study using rats that focused on the postnatal, preweaning developmental period concerning metabolomic outcomes.

2. Justification, objectives, and hypothesis

2.1. Justification

CPF is still one of the most widely used pesticides worldwide. Therefore, it is of critical importance to systematically study its effects on human health following a range of exposure protocols, with a special emphasis on specific developmental periods using both humans and, in this case, preclinical models. The experiments to be presented in this thesis are essential in this regard as they add relevant information on the short, medium, and long-term effects of exposure to NChEI doses of CPF during a period of development that has been relatively under-explored as observed in the scientific literature. These experiments shed some light on the possible alternative mechanism of action/toxicity of developmental CPF exposure (both at the CNS and peripherical systems -blood metabolites and gut microbiota-), and link this exposure protocol to specific alterations of several behaviors that are at the basis of multiple disorders that are currently prevalent and of global concern.

Study 1 focused on the specific analyses of both medium (adolescence) and long-term (adulthood) locomotor effects of preweaning CPF exposure, and the study of alternative mechanisms of action, regarding the most relevant neurotransmitter systems. Our rationale for studying motricity was based on the fact that alterations in locomotion are at the basis of several disorders such as ASD, ADHD, PD, and AD (Sagiv et al., 2010, 2018; Sánchez-Santed et al., 2016; Chang et al., 2018). Furthermore, to date, only two empirical preclinical studies have explored the effects of preweaning exposure on locomotor activity in rats (Dam et al., 2000; Levin et al., 2001), but using a dose 5 times higher than the one we have chosen in the present set of experiments, far away from real exposure rates. With regard to the study of the neurotransmitter systems as alternative mechanisms of action, the specific analysis of the different systems following preweaning CPF exposure has principally been studied in relation to the endocannabinoid system, and limited to components others than the CB1 receptor (Carr et al., 2011, 2013, 2014 and 2017). We also explored the long-term effects of preweaning CPF exposure on the composition of gut microbiota. Studying the bacteria population within the gut is justified, since recent decades have seen a growing interest in its importance for health and disease, whilst its direct physiological link to the CNS suggests that it could also play a central role in the biological basis of behavior (Fung et al., 2017). Moreover, to the best of our knowledge, this is the first time that the gut microbiome has been explored following

NChEI doses of preweaning CPF exposure in rats, with only one example concerning mice (Guardia-Escote et al., 2019b).

Study 2 focused on analyzing of both medium and long-term effects of preweaning CPF exposure on social domains and dominance. This is essential since, to the best of our knowledge, there is not a single published study that has explored the effects of developmental CPF exposure concerning ASD-like behaviors in a rat model. As with Study 1, we explored the effects of preweaning CPF exposure on the gut microbiota composition, but during adolescence (medium-term). Once again, our rationale for this study is that these effects have not yet been explored in rats (a better model for social traits as indicated in Ellenbroek and Youn, 2016), whilst there are few studies that have been conducted in mice (Guardia-Escote et al., 2019b). Further, we also studied the short, medium, and long-term effects of this exposure protocol on plasma metabolites levels. This is based on the fact that various metabolites have been associated with different disorders that are of our particular interest to this work such as ASD, ADHD, PD, and AD, amongst others (Ruggeri et al., 2014; Mussap et al., 2016; Wilkins and Trushina, 2017; Mohamadkhani A, 2018; Picca et al., 2019; Glalb et al., 2019; Shao and Le, 2019; Chen et al., 2019). Furthermore, there no rat studies that have analyzed the specific effects of preweaning CPF exposure on metabolites and, again, there is only one study with mice, although this focused on the analysis of the short-chain fatty acids (Guardia-Escote et al., 2019b).

With Study 3, we wanted to explore the long-term effects of CPF exposure on various executive functions such as attention, learning, motivation, and inhibitory control, assessed with a well-established as the 5-choice serial reaction time task paradigm (5C-SRTT). Although attention and learning have been (barely) explored, the effects of developmental CPF exposure on inhibitory control have not previously been analyzed using rodents' models (please see Perez-Fernandez et al., 2019a). It is also important to specifically analyze both the cholinergic and the GABAergic systems since these systems were found as altered in Study 1, thus allowing us to continue studying age (young-adults vs. adults) and function-related (motricity vs. attention/inhibitory control) effects of CPF exposure on both systems.

Regarding Study 4, we examined the effects of preweaning exposure to NChEI doses of CPF on spatial memory, anxiety and motricity during the late adulthood, a period particularly sensitive to the decline observed in both normal and prodromal aging decline,

for both physical and cognitive domains (Goodpaster et al., 2006; Harada et al., 2013). This study is justified since there are no studies using rat models to analyze the very long-term (late-adulthood and old ages) effects of developmental CPF exposure on these behavioral outcomes, which are present in several neurodegenerative disorders (Scarmeas et al., 2004; Jahn H, 2013; Aminian and Strafella, 2013; Chiaravalloti et al., 2014; Cortés et al., 2018; Armstrong and Okun, 2020).

2.2. General aim and hypothesis

The general aim of the work presented in this thesis is to study the short, medium, and long-term effects of exposure to a NChEI dose of CPF during the preweaning stage on behaviors associated with some of the most prevalent neurodevelopmental and neurodegenerative disorders. We also aimed to study the effects of this exposure on different neurotransmitter systems, and on gene expression levels in the SNC, gut microbiota and systemic metabolic systems in order to understand the physiological and molecular basis of these neurotoxic-induced alterations, in both male and female rats.

The general hypothesis is that this NChEI exposure protocol during this essential but little-explored developmental period will induce several alterations in most of these behaviors, with a certain degree of sexual-dimorphism being shown in most of them.

2.3. Specific objectives and hypotheses

The present thesis has been divided in four distinct studies composed of different behavioral and molecular experiments, each one with specific objectives and hypotheses.

2.3.1. Study 1. Long-term effects of low doses of Chlorpyrifos exposure at the preweaning developmental stage: A locomotor, pharmacological, brain gene expression, and gut microbiome analysis

This study can be divided into 5 different sections: a) confirmation of the NChEI profile of the exposure protocol, b) the long-term effects of CPF exposure on locomotor activity, c) the long-term effects of CPF exposure on the different neurotransmitter systems involved in locomotor outcomes, d) the long-term effects of CPF exposure on brain gene expression and e) the long-term effects of CPF exposure on gut microbiota composition. The specific objectives and hypotheses are displayed in the **Table 5**.

Section	Objective	Hypothesis
a	Confirmation of NChEI Profile using brain ChE activity	This exposure protocol will not significatively inhibit (<20% from control) brain ChE activity, based on previous literature
b	Long-term effects on locomotor activity (spontaneous and stress-induced)	CPF exposed animals will be more sensitive to stress derived from exposure to a novel space (spontaneous activity). The same could be expected in relation to stress induced by pain (i.p. injection), although there is not previous empirical data on this regard
c	Long-term effects on various neurotransmitter systems	CPF will induce alterations in the endocannabinoid system. It is also possible that it will affect the serotonergic and dopaminergic, and cholinergic systems based on previous literature. The effects on the remaining systems is also possible but there is less empirical support based on previous literature
d	Long-term effects on expression of different striatal and frontal genes	CPF will induce alterations in expression levels of different genes based on previous literature
e	Long-term effects on gut microbiota composition	CPF will induce dysbiosis, presumably with special sensitivity on Lactobacillus, Bacteroides, Bifidobacterium and Clostridium bacteria families, based on previous literature

Table 5. Specific objectives and hypotheses concerning the Study 1.

2.3.2. Study 2. Medium and long-term effects of low doses of Chlorpyrifos during the postnatal, preweaning developmental stage on sociability, dominance, gut microbiota, and plasma metabolites

This study can be divided into 6 sections: a) the medium (adolescence) and long-term (adulthood) effects of CPF exposure on sociability, b) the long-term effects of CPF exposure on dominance and hierarchical status, c) the effects of dominance status on sociability, d) the effects of CPF on motor behaviors, as control variables, e) the medium-term effects of CPF exposure on gut microbiota composition and f) the short, medium and, long-term effects of CPF exposure on plasma metabolic profile. **Table 6** summarizes these objectives and hypotheses.

Section	Objective	Hypothesis
a	Medium and long-term effects on sociability and reaction to social novelty	CPF exposure will alter (reduce) social interaction in both adolescents and adult rats (contrary results in previous literature)
b	Long-term effects on dominance and hierarchical status	CPF exposure will alter (reduce) dominance status
c	Influences of dominance status on sociability	Dominance status will be an important factor for other social domains
d	Influences of motor behaviors on the Crawley paradigm and performance	Alterations in motricity will not be an important factor that could explain the possible differences in sociability amongst groups
e	Long-term effects on gut microbiota composition	CPF will induce dysbiosis, presumably with special sensitivity on Lactobacillus, Bacteroides, Bifidobacterium and Clostridium bacteria families, based on previous literature
f	Short, medium, and long-term effects on plasma metabolic profile	CPF will alter different systemic metabolic pathways and specific metabolites, with a special emphasis on the lipid systems, as previously observed in the literature

Table 6. Specific objectives and hypotheses concerning the Study 2.

2.3.3. Study 3. Postnatal exposure to low doses of Chlorpyrifos induces long-term effects on 5C-SRTT learning and performance, cholinergic and GABAergic systems and brain derived neurotrophic factor (*BDNF*) expression

This study can be divided into 6 different sections: a) long-term effects of CPF exposure on locomotor activity, b) long-term effects of CPF exposure on learning rates, c) long-term effects of CPF exposure on basal performance on 5C-SRTT performance, d) long-term effects of CPF exposure on 5C-SRTT performance following changing contingencies (behavioral manipulations), e) long-term effects of CPF exposure on the cholinergic and GABAergic systems concerning 5C-SRTT performance and f) long-term effects of CPF exposure on striatal expression of different genes. **Table 7** summarizes the main objectives and hypotheses of Study 3.

Section	Objective	Hypothesis
a	Long-term effects on locomotor activity	CPF exposed animals will be more sensitive to stress derived from the exposure to a new space (spontaneous activity), as observed in previous literature
b	Long-term effects on learning	CPF exposure will lead to altered (poorer) learning rates, opposite results could be found in females, based on previous literature with adult exposure
c	Long-term effects on basal performance in attention, inhibitory control and motivation-related behaviors	CPF exposure will alter (impair) the different cognitive functions, as observed in previous literature with other exposure protocols.
d	Long-term effects on the sensitivity to changing contingences (behavioral manipulation)	CPF exposed animals will be particularly sensitive to the change of important variables in the context of a learnt task, as observed in previous literature with adult exposure
e	Long-term effects on the integrity of cholinergic and GABAergic systems	CPF exposed animals will have alterations in both neurotransmitter systems
f	Long-term effects on striatal gene expression	Various genes involved in the cholinergic system, GABAergic system, inhibitory control, and general CNS functioning are expected to be altered in the CPF animals

Table 7. Specific objectives and hypotheses concerning the Study 3.

2.3.4. Study 4. Preweaning exposure to low doses of Chlorpyrifos induces a general hypomotricity in aged rats.

Study 4 can be divided into the following sections: a) effects of CPF exposure on the learning (Acquisition stage) of the Morris Water Maze (MWM), b) effects of CPF exposure on spatial memory and influences of learning disturbance (the removal of the platform) in spatial memory performance recovery (Probe stage), c) effects of CPF exposure on compulsive-like behaviors (Reversal stage), d) effects of CPF on vision as a control variable (Visual stage), e) effects of CPF exposure on anxiety-like behaviors (Plus maze) and f) effects of CPF exposure on motricity in pre-elderly animals. All these behaviors were analyzed in aged rats. **Table 8** summarizes the main objectives and hypothesis of this study.

Section	Objective	Hypothesis
a	Long-term effects on learning	CPF will alter learning rates as observed in previous literature concerning other exposure protocols and earlier in life
b	Long-term spatial memory and disturbance to learning	CPF will alter learning spatial memory consolidation and will be sensitive to learning disturbances as observed in previous literature with adult exposure and earlier in life
c	Long-term effects on changing contingencies (perseveration)	CPF will induce a compulsive-like pattern following changes in contingencies as observed in previous literature with adult exposure
d	Long-term effects on vision	CPF will have no effect on visual performance
e	Long-term effects on anxiety	CPF will alter anxiety state, as observed in other developmental studies and earlier in life
f	Long-term effects on motricity	CPF will induce alterations in locomotor outcomes

Table 8. Specific objectives and hypotheses concerning the Study 4.

STUDY 1

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Long-term effects of low doses of Chlorpyrifos exposure at the preweaning developmental stage: A locomotor, pharmacological, brain gene expression and gut microbiome analysis

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ABSTRACT

Development is especially sensitive to Chlorpyrifos (CPF) toxicity, associated with several neurodegenerative and neurodevelopmental disorders where motor function dysfunction is a core symptom. Amongst the alternative molecular targets to cholinesterases inhibition, developmental CPF alters different components in the most important neurotransmitter systems, although this depends on the exposure period. Exposure during the late postnatal preweaning stage is the least studied by far. This period includes essential neurodevelopmental processes and has an important translational meaning. The present study analyzed the influence of low doses of CPF on this developmental window on locomotor activity and the state of the different neurotransmitter systems by pharmacological challenges. Brain gene expression and microbiome modulation following CPF were also analyzed. CPF exposure long-term increased spontaneous vertical activity, female's activity following acute stress, hyposensitized the cholinergic system and hypersensitized the GABAergic system, up-regulated both muscarinic 2 receptor and GABA-A-α2 receptor subunit in the dorsal striatum and the frontal cortex, respectively and induced gut microbiota dysbiosis at both genus and species levels. The present study supports alternative molecular targets than the ChEs following late postnatal, preweaning exposure to low doses of CPF, focusing on both cholinergic and GABAergic systems and the gut microbiome as an important factor.

1. Introduction

Organophosphate compounds (OPs) are a wide range of organic xenobiotic agents commonly used as pesticides (insecticides, fungicides, herbicides, nematicides, and acaricides) that are primarily applied for agricultural, industrial, and residential proposes. Human exposure to OPs was linked to selective alterations in most of the cognitive domains such as attention (Sánchez Lizardi et al., 2008; Ruckart et al., 2004), executive functions (Bouchard et al., 2011; Kofman et al., 2006), memory (Roldan-Tapia et al., 2006; Mackenzie Ross et al., 2010) and psychomotor abilities (Rauh et al., 2006; Malekirad et al., 2013). Furthermore, various neurodevelopmental pathologies such as autism and attentional deficit hyperactive disorder (ADHD) (Sagiv et al., 2018; Chang et al., 2018) and neurodegenerative disorders such as Parkinson's and Alzheimer's disease (Sánchez-Santed et al., 2016; Jokanović, 2018) have also been linked to OPs exposure. Although currently there is a number of OP compounds on the market, for decades Chlorpyrifos (CPF) has been the most widely used (Eaton et al., 2008).

In relation to the well-known effects of OP exposure on psychomotor function as well as ADHD symptomatology, several preclinical studies analyzed the impact of CPF and/or other OP xenobiotic

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compounds on locomotor activity or other types of motor functions following low to middle doses during gestation (Levin et al., 2002; Icenogle et al., 2004; Silva et al., 2017; Venerosi et al., 2009; De Felice et al., 2014), preweaning (Dam et al., 2000; Levin et al., 2001; Carr et al., 2017, 2001; Ricceri et al., 2003; Venerosi et al., 2008, 2006), both gestational and preweaning (Laporte et al., 2018; Cole et al., 2012; Ricceri et al., 2006; Gómez-Giménez et al., 2018), adolescence (Chen et al., 2014; Avci et al., 2018; Singh et al., 2018) and low, middle, or high doses in adulthood (Pope et al., 1992; López-Crespo et al., 2007; Savy et al., 2015; Peris-Sampedro et al., 2014; Yan et al., 2012; López-Granero et al., 2013, 2014, 2016; Carvajal et al., 2005; Adedara et al., 2018; Bockzur et al., 2010).

Like most of the OP compounds, CPF (essentially its Oxon form) exerts its toxicological profile by irreversibly inhibiting the cholinesterases (ChE), both acetylcholinesterase (AChE) in the Central Nervous System (CNS) and butyrylcholinesterase in the circulatory system (Eaton et al., 2008). This inhibition leads to the accumulation of acetylcholine neurotransmitters into the synaptic cleft, continuing with overstimulation of both muscarinic and nicotinic receptors and, finally, the mismatch of cholinergic system functioning. Nevertheless, some studies proposed alternative molecular targets of the mammalian CNS following CPF exposure at low or very low doses that do not significatively inhibit the ChEs and that are far away from the overt toxic effects (Burke et al., 2017). Other significant targets include direct effects on transcription factors, intra-cell signaling components, oxidative stress, lipid peroxidation, mitochondrial mismatch or the modulation of glial cell activity (i.e. Hussein et al., 2018; Xu et al., 2017; Saulsbury et al., 2009; Schuh et al., 2002).

Further, several studies have linked low doses of CPF during development to the modulation of other neurotransmitter systems as well as other components of the cholinergic system without ChEs mediation. The dopaminergic (Dam et al., 1999; Chen et al., 2011; Slotkin et al., 2002; Slotkin and Seidler, 2007a,c; Aldridge et al., 2005c; Savy et al., 2015), serotonergic (Slotkin and Seidler, 2005, 2007a,c; Slotkin et al., 2014, 2015; Aldridge et al., 2003, 2004, 2005a,b,c; Raines et al., 2001; Savy et al., 2015), endocannabinoid and, to a lesser extent, glutamatergic (Slotkin and Seidler, 2007b; Slotkin et al., 2010) and GABAergic (Gómez-Giménez et al., 2018) systems are the best representatives. In addition, apparent direct effects of CPF exposure on various cholinergic components such as choline acetyltransferase (ChAT), vesicular acetylcholine transporter (VAChT), muscarinic and nicotinic receptors have been found (Slotkin and Seidler, 2007b; Basaure et al., 2018, 2019; Guardia-Escote et al., 2018).

Endocannabinoid is the neurotransmitter system that has been most deeply studied with respect to the effects of low or very low doses of CPF (< 1 mg/kg/day) at the late postnatal, preweaning developmental stage. This age [> post-natal day (PND) 10] is essential in terms of synaptogenesis, neural differentiation, oxytocin, and vasopressin maturation processes. This is also a good perinatal translational model in neurodevelopmental terms because it is equivalent to a human birth date for term-newborns (Tait et al., 2009; Semple et al., 2013). Briefly, these studies found a systematic decrease in different enzymes and a subsequent temporal increase in the main endocannabinoid agonists in the mammalian CNS (Carr et al., 2011, 2013, 2014, 2017). However, the direct effects of this exposure protocol on the cannabinoid receptor 1 (CB1) functioning and related behaviors have not been analyzed. Otherwise, most of the works that have studied this CPF-CB1 receptor interaction used larger dosages, which significantly inhibited AChE (Quistad et al., 2002; Baireddy et al., 2011; Liu and Pope, 2015; Liu et al., 2015).

During the last decade, a new system has been added to the study of the biological basis of behavior along with the CNS, immune, and endocrine systems, that is, the gut microbiome (Fung et al., 2017). Alterations in this large and varied bacteria community (dysbiosis) have been linked to autism (Strati et al., 2017), depression (Cheung et al., 2019), stress (Molina-Torres et al., 2019), schizophrenia (Nguyen et al., 2018) and other pathologies with a strong motor component such as ADHD (Cenit et al., 2017), amyotrophic lateral sclerosis, and Parkinson's disease (Spielman et al., 2018). External factors such as the presence of various contaminants have also been associated with dysbiosis in both humans and experimental animals. Developmental CPF exposure is currently the subject of intense scrutiny in this field (Joly Condette et al., 2013, 2014; 2015, 2016; Reygner et al., 2016). Interestingly, some of these studies have found this influences at doses with low or null ChEs inhibition. However, to date, there has been no specific analysis of the possible dysbiotic effects of CPF exposure during the late preweaning stage.

The significance of the vast empirical work that has been dedicated to studying the effect of low doses of CPF in pre/postnatal stages is unquestionable. However, this late postnatal stage, from PND 10 to weaning, has rarely been studied in terms of alternative targets without the mediation of ChEs inhibition, with only a few studies using doses as low as 1 mg/kg/day or even less (Carr et al., 2011, 2013; 2014, 2017; Timchalk et al., 2012; Buratti et al., 2011; Tait et al., 2009; Ricceri et al., 2006; Venerosi et al., 2006), known to have little effect on ChEs activity during development. The present study, therefore, employed this exposure protocol with the aim of addressing three main objectives: 1) To study the long-term effects of CPF exposure in different locomotor activity outcomes, 2) to study the possible long-term modulations of the basal state of the main neurotransmitter systems, and 3) to extend the findings of previous studies regarding the interaction between CPF and the endocannabinoid system. In addition to this, we proceeded to analyze brain gene expression as well as the composition of microbiota bacteria in relation to both behavioral and pharmacological alterations. All of these outcomes were studied in both sexes in order to analyze possible dimorphic effects following this exposure protocol.

2. Materials and methods

2.1. Experimental animals

This study used 19 timed pregnant Wistar rats (Janvier labs) that were individually housed in our facilities 5 days prior to the estimated delivery date. 190 pups (95 females) were born on the expected day. The birth date was considered as PND0. At PND1, the pups were taken from their original mothers and randomly distributed between them, allocating 10 pups to each mother (5 females). CPF exposure had no influences on weight and ocular opening (data not shown). Rats were weaned at PND21, with 4 animals per cage (same sex). They were born and reared in our facilities, with a constant room temperature (22 ± 2 °C), humidity (50 ± 10%) and 12 light hour cycle (lights on at 8 a.m.). Rats were housed 4 per cage in Experiment 1, and 1 per cage in Experiment 2. They received both food (A04 Standard Free, Panlab) and water ad libitum. A total of 60 animals (30 females) were used in the present study. Of these, 10 males and 10 females (5 CPF exposed and 5 controls in each group) were used only for determining cholinesterase activity 24 h after the last exposure (PND16). For the behavioral and pharmacological procedures (Experiments 1 and 2), 40 young adult (postnatal month -PNM- 1,5 to 6) Wistar rats were selected (20 females and 20 males, 10 CPF exposed and 10 controls in each group). For brain gene expression analyses, samples from all the 40 animals used in the behavioral protocols were selected. For the metagenomic analysis of gut microbiota populations, the stool from a subsample (10 females and 10 males -5 CPF exposed and 5 controls in each group-) of these 40 animals was randomly selected. Image 1 summarized the whole experimental design conducted by the 60 experimental animals. The present study forms part of the project ES040130002260 and was conducted in accordance with the Spanish Royal Decree 53/2013 and the European Community Directive (2010/63/EU) for animal research and approved by the University of Almeria Animal Research Committee.

2.2. Neurotoxic agent

Chlorpyrifos (CPF) [O, O-dietil O-3,5,6-trichloropyridin-2-il fosforotioato (Pestanal, Sigma Aldrich)] was administered by oral gavage from PND10 to PND15, both inclusive. Male and female rats were randomly assigned to vehicle or CPF exposure conditions. This period was chosen for three essential reasons: 1) This stage coincides with the development of certain critical neurological mechanisms such as synaptogenesis and myelination, along with peaks in oxytocin and vasopressin levels (Venerosi et al., 2006; Semple et al., 2013; Tait et al., 2009), 2) this stage is equivalent to the day of birth in humans, thus providing an important translational model in relation to perinatal stressors (Semple et al., 2013) and 3) it is the least studied range in developmental toxicology following low doses of CPF. 1 mg/kg/ml/day of CPF was diluted in corn oil, widely used due to its facilitatory absorption properties (Timchalk et al., 2002). For the control condition, the same vehicle was used at the same dose/volume concentration. This dose was used due its well-documented non-significant inhibitory effect on ChEs (Savy et al., 2015), and it is 3 to 5-fold lower than the doses chosen for this developmental stage exposure in previous studies, and is thus closer to real exposure rates and the No-Observed-Adverse-Effect-Level following sub-chronic exposure in rats (World Health Organization, 2009).

2.3. Drugs

In Experiment 1, the locomotor activity of the animals was challenged with the following drugs in order to check the basal state of the main neurotransmitter systems: Amphetamine hydrochloride (Sigma Aldrich, lot number 101K3351, purity of ≥99%) was administered at doses of 1 and 0.5 mg/kg to check dopaminergic and serotonergic systems integrity. (−). Scopolamine Hydrobromide trihydrate (Sigma Aldrich, lot number 106H0796, purity of > 98%) was administered at doses of 1 and 0.5 mg/kg to check cholinergic system integrity. (+). MK-801 hydrogen maleate (Sigma Aldrich, lot number 105M4606V, purity of 99.5%) was administered at doses of 0.2 and 0.1 mg/kg to check glutamatergic system integrity. Buspirone (N-[4-[4-(2-Pyrimidinyl)-1-piperazinyl]butyl]-8-azaspiro[4.5]decane-7,9-dione hydrochloride) (Sigma Aldrich, lot number 065K1579, purity of ≥99%) was administered at doses of 1.5 and 0.5 mg/kg to check serotonergic system integrity. Alprazolam (8-cloro-1-metil-6-fenil-4H-[1,2,4] triazolo [4,3-a]) [1,4] benzodiazepine) (Pfizer, lot number J3873, purity of ≥99%) was administered at doses of 0.5 and 0.1 mg/kg to check GABAergic system integrity. For all compounds, the injection was via intraperitoneal (i.p.). For Amphetamine, Scopolamine, MK-801, and Buspirone, the Physiological serum 0.9% NaCl was chosen as a solvent, which was used as a vehicle in the control condition. For Alprazolam, the solvent was composed of Ethanol (15%), Propanediol (72%) and Physiological serum 0.9% NaCl (13%), which was used as a vehicle in the control condition (the total compound without the drug molecule). In Experiment 2, the food consumption, weight, and locomotor activity were studied before, during, and after challenging the endocannabinoid system of the rats. WIN 55,212-2 (WIN -Sigma Aldrich, lot number 062M4603V, purity of ≥98%-) was sub-chronically administered at a daily dose of 1 mg/kg for 4 days. The solvent was composed of 10% Dimethyl sulfoxide, 0.1% Tween 80, and Physiological serum 0.9% NaCl. This mix was also used as a vehicle in the control condition. The volume of administration was 1 mL/kg for all drugs except for WIN, where 2 mL/kg was chosen due to its lipophilic features. No heat application was needed for compound preparations, but ultrasonic baths were used for WIN dilution.

2.4. Behavioral procedures

2.4.1. Experiment 1

Locomotor activity was studied in standard open field boxes in order to establish whether pre weaning CPF exposure influences a) spontaneous locomotor activity when placed in a novel environment, b) locomotor habituation to cages and experimental contexts, c) stress/pain-induced changes in locomotor activity, d) stress/pain "habituation" and e) the basal state of the main neurochemical systems and their implied effects on locomotor activity.

2.4.1.1. Description of apparatus. A total of 8 Plexiglas activity cages (39 × 39 × 15cm) were used in order to evaluate locomotor activity by photocell beams interconnected to a PC. Both VersaMax® and VersaData® Software (PLC Control System SL) were respectively used for automatic behavioral setup and data collection. Total distance and vertical activity variables were chosen as measures of locomotor activity. Once the animals had been placed into their previously assigned cages, locomotor activity was recorded for 60 min.

2.4.1.2. Procedure. The animals were driven to an experimental room for environmental habituation 1 h before starting the experiment. This protocol was implemented every day, from Monday to Friday, until the end of the experiment. Room temperature and humidity were set at 22 ± 2 °C and 50 ± 10%, respectively. The light condition was set as Dim-Light. Behavioral analyses were conducted between 8.00h and 15.00h. Males completed the odd series and female rats the even series, each one with 4 animals of each treatment condition and distributed between cages in a randomized pattern in order to avoid cage and hour of the day bias. For the cleaning protocol, 70% ethanol was used prior to starting the first series and between series.

Firstly, the animals received 5 consecutive days of habituation (1 h per day) starting at the PND40. The first 20 min of the first day were considered for the assessment of spontaneous locomotor activity. For habituation, only the last day was considered for statistical analyses, in order to establish whether repetitive exposure to the open field differentially affects the animals according to sex or exposure conditions. Once the rats had habituated, on 4 consecutive days they were administered with saline (0.9% NaCl, Physiological Serum) via i.p. The first day (c) was used for pain/stress-induced locomotion, while the fourth day was considered for "habituation" to the i.p. injection, finishing this habituation period around the PND50. For drug challenges, the experimental protocol was conducted following a complete-randomized Latin-square design, with two different doses and the vehicle condition per drug. Animals were directly placed into the open field cages just after the drug was administered in order to observe the development of drug effects. The injection days were Mondays and Thursdays with at least 72h between administrations of the same drug and at least one week between different drugs (Wash-out periods). Animals were around 55 days of age (≈PNM2) when the drug challenge started and finished it with 4 months of age.

2.4.2. Experiment 2

For this experiment the animals were housed in individual cages in order to ensure the reliability of the consumption data. A period of 3 weeks of habituation to the new conditions was set prior to the start of the experiment, together with 3 days per week of 2 min of handling in order to reduce isolation stress. After this habituation period, 17 days were enough to complete the whole experiment, in which body weight and consumption were measured daily in order to assess interactions between WIN and CPF exposure. Rats started this experiment with around 160 days of life (≈PNM5, counting from the first day of baseline recording) and finished it in their PND177 (≈PNM6, counting the last day of weighting and locomotor activity assessment).

2.4.2.1. Description of the apparatus. The weighing of remaining food and assessment of body weight were carried out in the homeroom, and the i.p. injections were given in an adjacent room. Locomotor activity was measured in the same open field apparatus and controlled by the same software as that described for Experiment 1.

Table 1
Primers selected for the RT-qPCR study. From left to right, the name of the Gene, ID, forward primer, reverse primer and source.

Gene	Gene ID (Rattus)	Forward Primer	Reverse Primer	Source
GAPDH (Intron)	Gapdh	ctgggtggctcaaggaata	cacacgcatcacaaaaaggt	Own design
GAPDH (Exon)	Gapdh	cttcaccaccatggagaag	catggactgtggtcatgag	Own design
Nicotinic α7	Chrna7	tatcaccaccatgaccctga	cagaaaccatgcacaccagt	Chamoun et al. (2016)
M1 Receptor	Chrm1	catggagtccctcacatcct	gggcatcttgatcaccactt	Own design
M2 Receptor	Chrm2	caagacccagtatctccaagtctg	cgacgacccaactagttctacagt	Chamoun et al. (2016)
ChAT	Chat	atggccattgacaaccatcttctg	aacaaggctcgctcccacagcttc	Lips et al. (2007)
VAChT	Slc18a3	gccacatcgttcactctcttg	cggttcatcaagcaacacatc	Lips et al. (2007)
AChE-S	Ache	gtgagcctgaacctgaagcc	tcctgcttgctatagtggtc	Jameson et al. (2007)
GABA-A α1	Gabra1	gcccaataaactcctgcgtatc	attcggctctcacagtcaacct	Fujimura et al. (2005)
GABA-A α2	Gabra2	ccaggatgacggaacattgc	ggaaagtcctccaagtgcattg	Fujimura et al. (2005)
GAD1	Gad1	gtgagtgccttcagggagag	cgtcttgcggacatagttga	Own design
GAD2	Gad2	ctgagaagccagcagagagc	agagtgggcctttctccttc	Own design

2.4.2.2. Procedure. A period of 8 consecutive days was chosen for establishing a baseline (BL) level. Once the BL data had been obtained, the WIN challenge started for 4 consecutive days. After this period, weight and consumption on 5 consecutive days were again analyzed in order to see post exposure development of the various outcomes. Averaged variables were created for all three periods (BL, WIN challenge, and post-treatment data) in order to simplify analysis. A further variable referred to as "ratio C/W (consumption/weight)" was created in order to study feeding efficiency in greater detail. The daily protocol was as follows: 1) After being weighed, each rat was allowed free access to100g of standard food in their own home-cage, 2) Both body weight and consumption (100g - remaining food) were obtained 24h ± 5 min after depositing the food, 3) Any remaining food was completed until 100g again, 4) During the WIN challenge phase, each animal was injected immediately after being weighed in an adjacent room. Locomotor activity (1 h) was measured at BL as a control measurement, during WIN injection phase (between days 2–3 of injection) and the last post-treatment day. The contents of the home cages were checked daily in order to detect possible food storage. Room temperature and humidity were set according to the same criteria described previously.

2.5. Biochemical procedures

2.5.1. Sacrifice protocol

Rats used for ChEs activity (n = 20) were sacrificed 24 h after the last day of exposure (PND16). The animals used in behavioral and pharmacological analyses (n = 40, Experiments 1 and 2) were sacrificed two weeks after the last procedure was finished (with 192 days of age, PNM6.5). All rats were fast decapitated. For brain tissue samples, the whole brain was removed and dissected (frontal cortex, dorsal striatum, hippocampus, hypothalamus and cerebellum). Only the dorsal striatum and frontal cortex were analyzed in this experiment with samples from the 40 animals that conducted the behavioral and pharmacological procedures. For stool samples, extraction was conducted by carefully taking all the fecal material from the whole large intestine. For this analysis, only a subsample of 10 males and 10 females (5 CPF exposed per group) was taken from the original 40 samples. All samples, both brain structures, and stool samples were flash frozen in dry ice as soon as possible in order to avoid RNA and DNA degradation, and finally stored at −80 °C until use. All the materials and laboratory facilities were autoclaved (Class-B P Selecta) and treated with RNAase ZAP (Sigma Aldrich) in order to avoid RNA degradation.

2.5.2. Analysis of cholinesterase activity

Brain frontal cortex activity was assessed 24 h following the last exposure (PND16) in a total of 20 animals (10 males and 10 females, 5 CPF exposed per groups). Briefly, tissues were homogenized with 1% Triton X-100 in 0.1M Na phosphate buffer (pH 8) at a ratio of 1/10 (w/v). The homogenate was centrifuged at 15,000 × *g* for 15 min. ChE activity was measured following Ellman's method (Ellman et al., 1961) with slight modifications using a 96-well microplate reader (DTX 880, Multimode Detector, Beckman Coulter). The supernatant was diluted with 0.1M Na phosphate buffer (pH 8.0) at a ratio of 1/10 (v/v). Ten microliters of this dilution (all determinations in triplicates) were mixed with 5,5-dithiobis-2-nitrobenzoic acid (60 μL, in 0.1M Na phosphate buffer; pH 8.0; final concentration = 0.33 mM) and 221 μL of sodium phosphate buffer (0.1 M; pH 8.0). After 300 s of incubation at 37 °C, the reaction was initiated by the addition of 9 μL of acetylthiocholine iodide (diluted in 0.1 M Na phosphate buffer; pH 8.0; final concentration = 0.5 mM). The reaction rate was monitored at 37 °C for 22 min. Absorbance was measured at 405 nm, in 30s intervals and agitated for 3-s prior to each reading, for a total of 45 cycles. Once the slopes had been analyzed, the proper two cycles (60 s) from all samples were chosen for statistical management. Enzyme activity was calculated as the increase in absorbance over time according to the formula given by Ellman et al. (1961) using the molar absorption coefficient of the yellow reaction product at 412 nm. Protein concentration was measured by the Bradford method (Bradford, 1976) in order to normalize samples.

2.5.3. Gene expression analysis: reverse transcription quantitative Polymerase chain reaction (RT-qPCR)

Total RNA was purified from the frontal cortex and dorsal striatum samples (6 months old) using Trizol reagent (Invitrogen) following the manufacturer's instructions. RNA was quantified by fluorescence signaling with Qubit® fluorometer (Life Technologies). RNA quality was assessed by agarose gel electrophoresis. Contamination of genomic DNA (gDNA) was removed by using the TURBO ® DNAse-I kit (Ambion). Complementary DNA (cDNA) was synthesized from the DNA-free total RNA by means of Maxima First Strand cDNA Synthesis Kit® (Thermo Scientific) using as primers a mixture of random hexamer and 18-mer oligo(dT). Expression analysis was conducted with RT-qPCR assays using the SYBR Green PCR Master Mix kit in a Step-One Real-Time PCR System (Applied Biosystems) and a specific primer pair for each gene analyzed (Table 1). The proper efficiency of the primers was controlled by serial dilutions (1:10 dilution factor). The housekeeping GAPDH gene was used as an internal reference for the gene expression analyses. The absence of gDNA contamination in the RNA sample analyzed by RT-qPCR was demonstrated using a specific amplicon from an intron section of the GAPDH gene as a control. Melting curves were analyzed in order to ensure the specificity of the amplification.

2.5.4. Metagenomic: gut microbiota composition

The total stool material stored at −80 °C was mixed (Heidolph RZR1) and 100 mg was taken from the mixture in order to obtain a general representation of the whole intestinal tract, in order to examine significant variations in bacteria concentration depending on the different parts of the gut (Joly Condette et al., 2013, 2014, 2015). gDNA was isolated from stool samples with PureLink™ Microbiome DNA

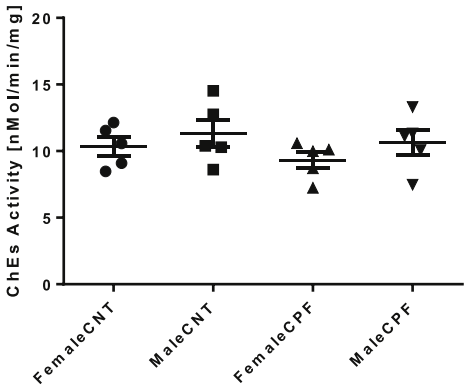

Fig. 1. ChEs activity in Frontal Cortex 24h (PND16) after the last exposure to CPF. Data are expressed by means, SEM and the individual plots.

Purification Kit (Invitrogen), following the manufacturer's instructions. gDNA samples were stored until use at −80 °C. Handling and analysis of the samples were carried out externally by STABvida laboratories (Caparica, Portugal), thus creating a single-blind design. Briefly, the samples were first analyzed for quality control of the DNA samples for integrity (both 1% agarose gel electrophoresis and Phred quality score at each amplification cycle) and quantity (fluorometry by Qubit) checking for optimal amplification. Once the samples showed acceptable quality parameters, bacteria DNA enrichment (amplification of V3 and V4 regions of 16S rRNA gene), library construction (Illumina 16S Metagenomic Library preparation protocol) and sequencing of these DNA libraries using 250bp paired-end sequencing reads (MiSeq Reagent Kit v2 in the Illumina MiSeq platform) were conducted. The initial number of Pass Filtered sequence reads the following amplification were then classified with Illumina 16S metagenomics workflow at different taxonomic levels, and Shannon Species Diversity Classification was carried out in order to check the diversity index throughout the samples. Although we analyzed the total number of detected bacteria at every taxonomic level, as well as species diversity, in the present study the specific analysis of the relative abundance of each type of bacteria was conducted only at the taxonomic levels of genus and species and the five most abundant phylum.

2.6. Statistical analyses

For Experiment 1, a two-way analysis of the variance (ANOVA) was used to analyze the different baseline measures (spontaneous activity, paradigm habituation, stress influences and habituation to stress) for total distance and vertical activity, with two factors SEX (male and female) and TREATMENT (control and CPF) - from here, we refer to both factors as “mentioned factors”-. Drug challenges were analyzed with repeated measures ANOVA, individually for each compound, with the within-subject variable DOSE (saline, low dose, and high dose) and the mentioned factors as between-subject variables. For Experiment 2, averaged weight, consumption and feeding efficiency (consumption/weight ratio) were also analyzed with individual repeated measures ANOVA with the within-subject variable DAY (BL, during WIN and post WIN) and the mentioned factors as between-subject variables. To discard motor influences on these behaviors, individual two-way ANOVAs were also conducted for total distance variable in every single stage of the protocol. For the ChEs assay, the Δ absorbance/min was set into the Beer-Lambert equation and the final ChEs enzymatic activity was corrected by the total protein concentration (nMol/min/mg). This was analyzed with a two-way ANOVA, with the mentioned factors. For RT-qPCR, data were obtained from StepOne Software (v2.2.2). Ct means were transformed to relative expression 2^ΔΔCt (fold relation after normalization to housekeeping and one reference sample), expressed in arbitrary units. A two-way ANOVA was done for every single target gene, using the mentioned factors. For gut microbiota analyses, the percentage of successful reads (pass filtered) and the Shannon index for species diversity were also analyzed as a general quality analysis with individual two-way ANOVAs, using the mentioned factors. The % of each bacterial population was calculated [(number of hits/total number of hits)*100] and analyzed for both genus and species taxonomic levels, as well as the 5 most important bacteria from the phylum category, using the mentioned factors. First, a Two-way ANOVA was conducted for every single bacteria at the genus level. When significant, multivariate analysis of all the different species which composed the genus category was done. When significant, univariate two-way ANOVAs were carried out on the specific species, always using the mentioned factors. For all the analyses, significant ANOVAs drove to post hoc pairwise comparisons analyses. When complex interactions (≥ 3 variables) were found as significant at the main analysis, this was simplified by blocking one factor to obtain simple interactions (analysis decomposition). Significant outlier extreme values were discarded following Grubb's test/ESD method. Data were considered statistically significant at $p < 0.05$. Means and SEMs are showed at figures and tables. Individual plots are displayed also for the molecular outcomes' figures, while p values do the proper in the text. SPSS v19 was used for statistical analyses. For figures design, GraphPad Prism v6.0 was chosen. Tables were designed with Microsoft Excel office 365®.

3. Results

There were no significant differences between animals assigned to CPF exposure and controls in terms of weight (before, during nor after the exposure period) nor ocular opening.

3.1. Brain cholinesterase activity. ChEs inhibition in the frontal cortex was not observed 24h following the last CPF exposure day

Analyses of ChEs activity were conducted for the Frontal Cortex samples of 20 animals (5 per group) 24h after the last exposure. Fig. 1 shows the effects of CPF exposure on ChEs activity. CPF exposure resulted in little ChEs inhibition in relation to the control animals 24h after the last exposure, with larger rates being shown by females (≈12%) in comparison with males (≈6%). Nevertheless, no significant effect was observed for either TREATMENT or the SEXxTREATMENT interaction.

3.2. Experiment 1. Locomotor activity

Female rats showed higher rates of locomotor activity than males on most of the variables and phases analyzed, as expected. Thus, a description of simple SEX influences throughout the text is omitted in order to focus on TREATMENT, SEXxTREATMENT as well as complex interactions.

3.2.1. Spontaneous, habituation to novel environment, stress-induced and habituation on stress-induced effects on locomotor activity

For spontaneous locomotor activity behavior, we analyzed the first 20 min of exploration of the new environment in the open field paradigm. CPF exposure increased locomotor activity, which was statistically significant for vertical activity. For total distance, no significant effect was found for TREATMENT, whilst the SEXxTREATMENT interaction also failed to reach significance (Supplementary Fig. 1). For vertical activity, CPF exposure significantly increased rearing behavior according to TREATMENT [$F(1,36) = 4.857$, $p = 0.034$], but the interaction SEXxTREATMENT was not significant (Fig. 2a).

For habituation to a novel environment, all animals showed similar rates of activity, with no significant effect of exposure condition, whilst strong sex differences were found in both studies (Supplementary Figs. 2 and 3). There was no significant effect of TREATMENT or an interaction SEXxTREATMENT for either distance or vertical activity.

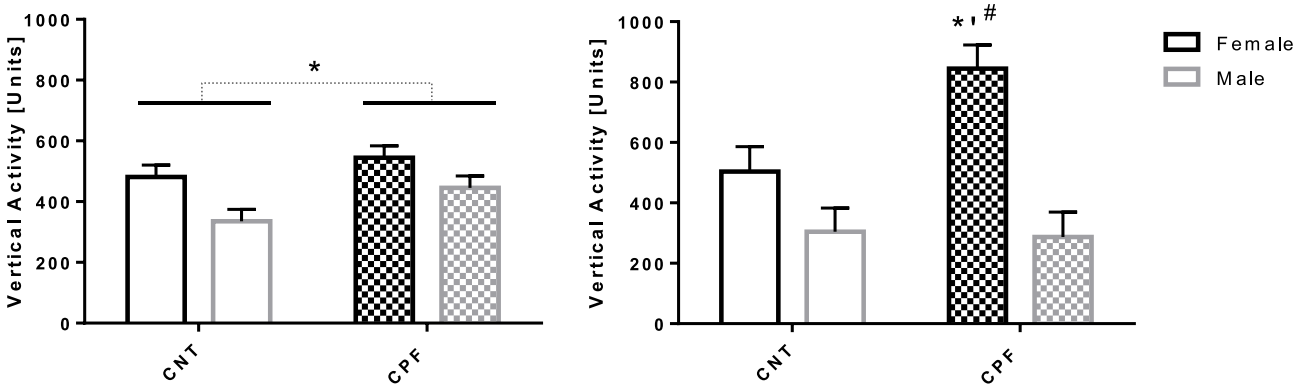

Fig. 2. Locomotor activity. a) Spontaneous locomotor activity in the open field for vertical activity (Left). b) Locomotor activity alteration following acute stress (saline i.p. injection) for vertical activity (Right). Data are expressed by means and SEM. * indicates a significant ($p < 0.05$) difference between treatment conditions. *' indicates significant ($p < 0.05$) differences between both female's groups. # indicates significant ($p < 0.05$) differences between both CPF exposed groups.

Thus, repeated exposure to the paradigm produced a stabilization of altered basal motor behavior in the spontaneous analysis. For stress-induced influences on locomotor activity, there was an increase in activity for female rats. For total distance, only exposed female rats suffered changes in behavior, albeit non-significant, with the effect of TREATMENT and the interaction SEXxTREATMENT both failing to reach significance (Supplementary Fig. 4). However, vertical activity was differentially affected by stress, as revealed by the significant interaction SEXxTREATMENT [$F(1,34) = 4.999$, $p = 0.032$] (Fig. 2b). Post hoc analysis revealed that stress increased rearing behavior primarily in exposed females, who showed higher rates of this behavior than control females ($p = 0.005$) and CPF males ($p < 0.001$).

Finally, for habituation to stress-induced effects, repeated i.p. injections appear to produce clear habituation and normalization of rats' locomotor activity, by slightly reducing this behavior in females whilst increasing this behavior in males, particularly in the case of vertical activity (Supplementary Figs. 5 and 6). In terms of total distance, no significant differences were found for TREATMENT or the interaction SEXxTREATMENT. A similar pattern of results was observed for vertical activity.

3.2.2. Drug challenges

Once the basal motor performance of animals had been studied, habituated rats were challenged with different drugs whilst recording their motor behavior.

3.2.2.1. Buspirone, MK-801 and Amphetamine: no effects of CPF exposure on Glutamatergic, Serotonergic, and Dopaminergic systems. We observed no significant effects of CPF exposure on locomotor activity following drug challenges with Buspirone (Serotonin), MK-801 (Glutamate) and Amphetamine (Dopaminergic and Serotonergic systems), regardless of the specific behavior analyzed (data not shown).

3.2.2.2. Scopolamine: hyposensitive cholinergic system in CPF-exposed animals. Scopolamine administration increased locomotor activity, primarily in control females and, to a lesser extent, CPF females. For total distance, significant differences were found for the simple interaction TREATMENTxDOSE [$F(2, 35) = 5.804$, $p = 0.007$], whilst the interaction SEXxTREATMENTxDOSE failed to reach significance. Post hoc analyses revealed that control animals showed higher rates of activity in comparison with those given CPF exposure at the lowest dose ($p = 0.022$). Further, control animals showed a significant increase in their activity in a dose-dependent manner (from saline, $p < 0.001$ for both doses), whilst CPF animals only showed a significant increase in their rates following the highest dose (from saline, $p = 0.06$ and 0.021 for low and high doses, respectively) (Fig. 3a). This effect was not observed in males ($p = 0.717$). Finally, although this same pattern of findings was observed for vertical activity (Fig. 3b), differences between groups were not significant, with no significant interaction TREATMENTxSEX or SEXxTREATMENTxDOSE.

3.2.2.3. Alprazolam: Hypersensitive GABAergic system in females exposed to CPF. Similarly, Alprazolam altered the behavior of females more than that of males. Thus, while control males did not vary their behavior according to the doses, control females showed an inverted U-shaped pattern in which the lowest dose increased both total distance and vertical activity and the largest dose heavily decreased behavior according to both measures. However, females exposed to CPF did show an expected motor decrease even at 0.1 mg/kg, with a strong decrease at 0.5 mg/kg, as in the case of exposed males (Fig. 4a and b). For total distance, a significant interaction SEXxTREATMENTxDOSE was found [$F(2,33) = 3.807$, $p = 0.033$]. Further analysis of the data broken down by dose revealed no significant effects in either the saline condition or the lowest dose, although a significant effect was found at the largest dose for TREATMENT [$F(1,34) = 6.327$, $p = 0.017$], with a stronger sedative effect of Alprazolam exposure (lower activity rate) on CPF animals compared with control. For vertical activity, a significant three-way interaction SEXxTREATMENTxDOSE was found [$F(2,33) = 6.335$, $p = 0.005$]. Once again, further analysis was conducted on the data broken down by DOSE. In addition to the expected null effects in the saline condition for TREATMENT and TREATMENTxSEX, for both low and high doses the interaction SEXxTREATMENT [$F(1,34) = 6.655$, $p = 0.014$] and the effect of TREATMENT [$F(1,34) = 4.817$, $p = 0.035$] were found to be significant. Post hoc analyses revealed that the significantly greater rates of vertical activity shown by control females compared with control males ($p = 0.001$) following the lowest Alprazolam dose were blocked in the CPF condition ($p = 0.871$). At the highest dose, a greater decrease in motor activity following Alprazolam was observed for CPF animals in comparison with controls. Taken together, these data seem to indicate the existence of a hypersensitive GABAergic system in CPF animals, particular females, which require a lower dose of benzodiazepine in order to show sedative effects (reduced behavior).

3.3. Gene expression: Long-term up-regulation of both the frontal GABA-A-α2 subunit and the dorsal striatal muscarinic 2 (M2) receptor

Exposure to CPF did not modulate RNA expression in most of studied genes in both frontal (M1r, M2r, Nicotinic α7r, AChE-S, GABA-A-α1 subunit, GAD1 and GAD2) and striatal (M1r, Nicotinic α7r, ChAT, VAChT, GABA-A- α1 & α2 subunits regions. However, we found up-regulation of both the frontal GABA-A-α2 subunit and M2 receptor

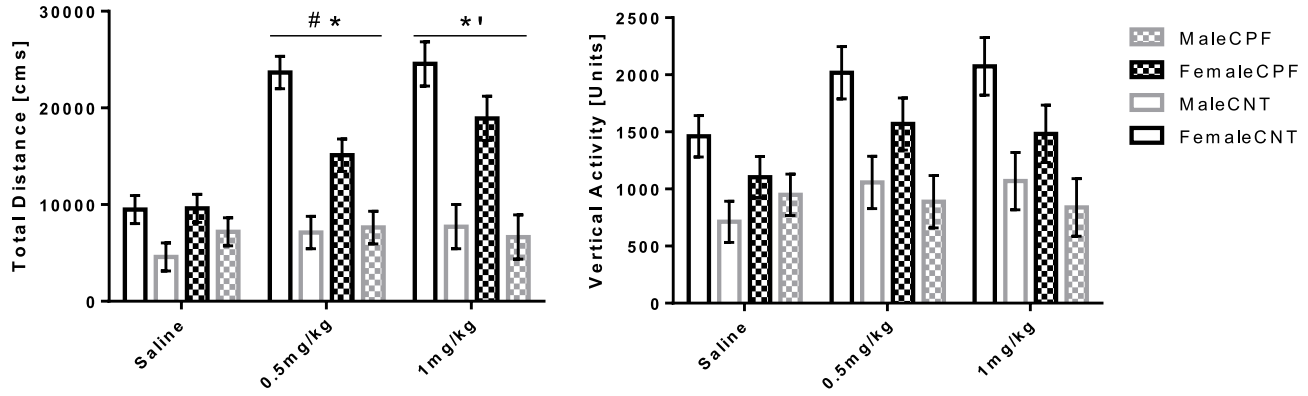

Fig. 3. Scopolamine (i.p.) effects on locomotor activity. a) Total distance and b) Vertical activity. Data are expressed by means and SEM. # indicates significant ($p < 0.05$) differences between groups at the lowest dose. * indicates significant differences for CNT animals at the lowest dose from saline. *' indicates significant differences for CNT and CPF groups at the highest dose from saline.

expression in the dorsal striatum in CPF exposed animals in relation to the TREATMENT condition [$F(1,34) = 4.512$, $p = 0.041$; $F(1,36) = 6.519$, $p = 0.015$, respectively] (Fig. 5a and b). The remaining non-significant genes are displayed in the Supplementary materials (Figs. 7–20).

3.4. Experiment 2. WIN challenge for weight and consumption

3.4.1. Low doses of postnatal, preweaning CPF exposure do not alter feeding rates, body weight or feeding/weight ratio when the cannabinoid system is challenged

4 consecutive days of 1 mg/kg of WIN 55, 212-2 did not affect body weight but reduced consumption to baseline recovery level a few days after exposure (Fig. 6a and b). The main analyses revealed no significant effects of either the TREATMENTxWEIGHT or TREATMENTxCONSUMPTION interactions. Similarly, the complex interactions of SEXxTREATMENTxWEIGHT and SEXxTREATMENTxCONSUMPTION also failed to reach significance. However, a significant interaction of SEXxCONSUMPTION was found [$F(2,34) = 4.508$, $p = 0.018$]. Post hoc analyses revealed that male rats ate more food pellets compared with females at every time point, whilst female rats also showed a marked decrease in consumption from baseline following WIN exposure ($p < 0.001$), with a clear recovery at the post-exposure stage ($p = 0.577$). Interestingly, males also showed a marked decrease in consumption following WIN exposure ($p < 0.001$), but almost non-existent recovery in the post-exposure stage ($p = 0.021$). Finally, the feeding efficiency ratio revealed a similar pattern to that observed for consumption, with a marked decrease following exposure, after which recovery was observed during the post-exposure phase. Both the interactions of SEXxTREATMENTxRATIO and TREATMENTxRATIO failed to reach significance (Fig. 6c). Although not significant, visual analysis shown that exposed females were, by far, the rats with higher feeding efficiency and the least during the WIN exposure.

3.4.2. Locomotor activity: endocannabinoid system in female rats given early CPF exposure

Finally, locomotor activity was studied in order to assess the motor effects of WIN exposure. In general, females showed higher rates of total distance covered in comparison with males, although these rates were reduced in both groups following WIN administration, except for exposed females that showed hyporeactivity to the endocannabinoid system challenge (Fig. 7). In the final phase, all animals recovered to their previous baseline levels. At baseline, analysis of total distance data revealed only a significant effect of SEX [$F(1,36) = 7.262$, $p = 0.011$] whilst both the interaction SEXxTREATMENT and the simple effect of TREATMENT failed to reach significance. Following WIN administration, a significant effect of SEX [$F(1,36) = 14.447$, $p = 0.001$] was found, with females showing higher rates than males, whilst both the interaction SEXxTREATMENT and the simple effect of TREATMENT failed to reach significance. Finally, 5 days after the last exposure, all animals returned to baseline activity levels, where the effect of SEX was found once again, with female rats showing higher rates of activity than males [$F(1,36) = 10.435$, $p = 0.002$], whilst a significant interaction of SEXxTREATMENT was also found [$F(1,36) = 4.646$, $p = 0.038$]. Post hoc analyses revealed higher locomotor rates for female controls than those that received exposure to CPF at an early stage of postnatal development ($p = 0.037$). At the same time, the natural significant differences between both sexes in favor of female rats (control group females-males $p < 0.001$) had disappeared by this final phase ($p = 0.452$).

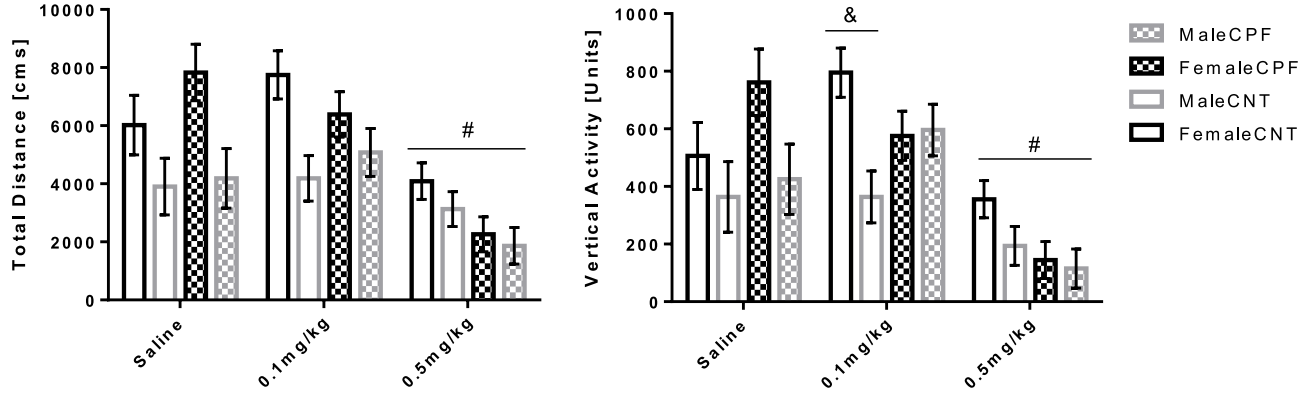

Fig. 4. Alprazolam (i.p.) effects on locomotor activity. a) Total distance and b) Vertical activity. Data are expressed by means and SEM. # indicates significant ($p < 0.05$) differences between CPF-CNT groups at the largest doses. & indicates significant differences ($p < 0.05$) between both control groups at the lowest dose.

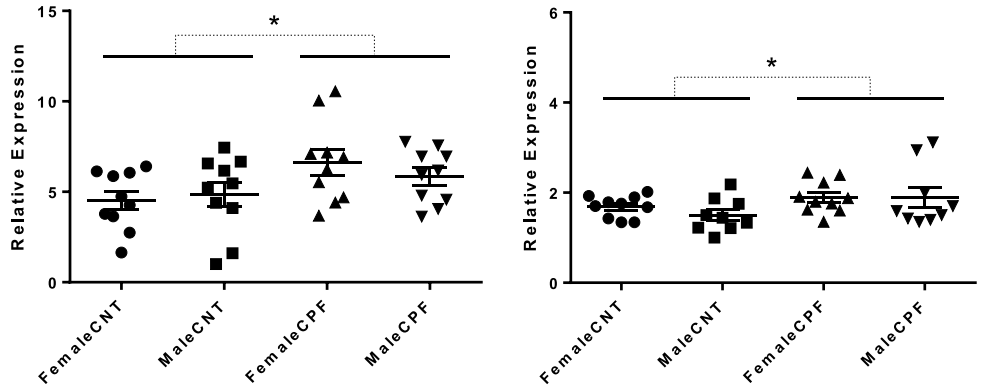

Fig. 5. Relative expression of different genes. a) dorsal striatum (Muscarinic 2 receptor) and b) frontal cortex (GABA-A-α2) at PNM6. Data are expressed by means, SEM and the individual plots. * indicates significant ($p < 0.05$) differences between CPF-CNT groups.

3.5. Metagenomics: Long-term gut microbiome dysbiosis following CPF exposure at the level of both genus and species

After quality control checking, using total Pass Filtered detections and the Shannon Species Diversity Classification (SSDC), all samples showed acceptable results (Pass Filtered detections ranged from 99.8% of the Kingdom to 56.7% of the species and the SSDC index ranged from 1.65 to 2.73). Prior to specific bacteria analysis at both genus and species levels, a general check was carried out on the percentage of reads successfully classified into each taxonomic level. There was no significant effect of TREATMENT or an interaction SEXxTREATMENT were found for the kingdom, phylum, class, order, family, genus or species. In addition, for the species diversity index there was no effect of TREATMENT or a SEXxTREATMENT interaction. Prior to analyze genus and species levels, we checked relative abundance of the 5 most important Phylum bacteria (Fig. 8). No significant differences were found neither TREATMENT nor SEXxTREATMENT interaction.

Analysis of the relative abundance at the genus level is summarized in Table 2. CPF leads to both a significant increase (Anaerobranca, Borrelia, Brevundimonas, Butyrivibrio, Candidatus Endobugula, Mogibacterium, and Pelagicoccus) and decrease (Candidatus Contubernalis, Hyphomicrobium, Nitrincola, Paracoccus, Rhizobium and Vogesella) in the relative abundance of different bacteria in relation to total genus composition. Furthermore, CPF leads to sexual dimorphic effects, with both an increase (Actinokineospora, Borrelia, Candidatus Microthrix, Microcoleus, and Vogesella) and decrease (Aliivibrio, Hyphomicrobium, Longilinea, Luteimonas, Nitrincola) in bacteria populations in exposed females. However, exposed males were also affected by the

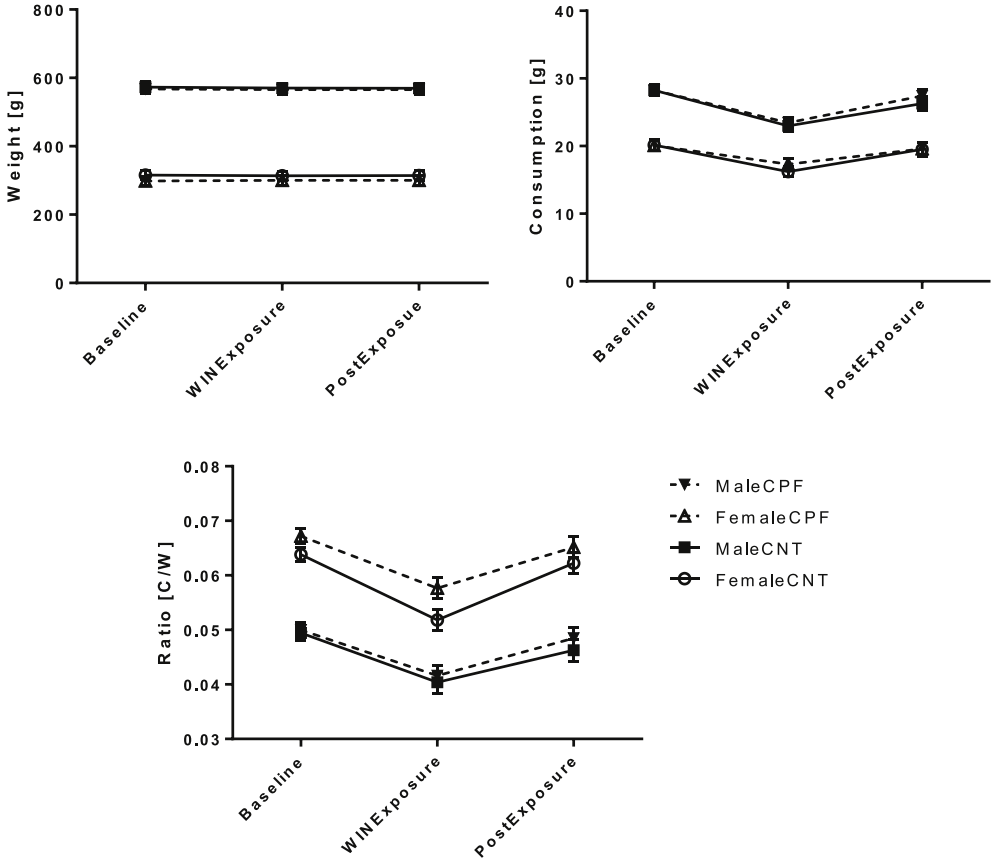

Fig. 6. Effects of 4 consecutive days (i.p.) of WIN 55–212,2 administration. a) weight, b) food consumption, and c) feeding efficiency ratio. Data are expressed by means and SEM.

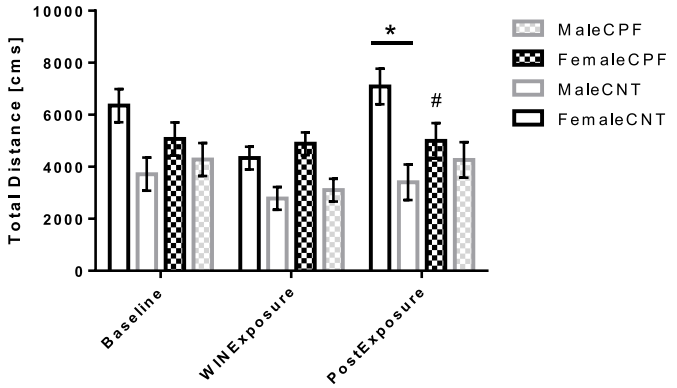

Fig. 7. Effects of 4 consecutive days (i.p.) of WIN 55–212,2 administration on total distance. Data are expressed by means and SEM.* indicates significant ($p < 0.05$) differences from the other stages. # indicates significant differences between groups.

increased relative abundance of Petrotoga and the decreased percentage of Brevundimonas and Vibrio bacteria. Of these, Pelagicoccus, Butyrivibrio, and Anaerobranca are the most important bacteria in terms of their abundance in relation to total bacteria composition. On the basis of this information, there generally appears to have been an increase in these bacteria following CPF exposure.

Analysis at the species level revealed that CPF exposure increased the relative abundance of Anaerobranca Zavarzinii and decreased that of Candidatus Contubernalis Alkalaceticum, Nitrincola Lacisaponensis, and Vorgesella Perlucida. Once again, females were more affected by CPF-induced dysbiosis than males with both significant increases (Actinokineospora Inagensis, Candidatus Microthrix Parvicella, Microcoleus Antarcticus, and Vorgesella Perlucida) and decreases (Hyphomicrobium Vulgare, Longilinea Arvoryzae, Nitrincola Lacisaponensis) in the representation of several species in relation to the total species in the gut (Table 3). With regard to their relevance for the bacterial composition of the gut, Anaerobranca Zarvazinii, Candidatus Cotuernalis Alkalaceticum and Candidatus Microthrix Parvicella were the most marked out of the 17 significant results, without referring to unspecific populations at this level, with 32 times more relative abundance of the first bacteria in relation to the second. Thus, and similar to the three most important candidates at the genus level, CPF exposure leads to specific increases in the most important species, Anaerobranca Zarvazinii.

4. Discussion

1 mg/kg/day of CPF administered at the second postnatal week (PND10-15) induced long-term spontaneous hyper motricity, stress-induced motor hyperreactivity (in female rats), hyposensitized cholinergic, and hypersensitized GABAergic systems. We also observed CPF-induced up-regulation of both the M2 and GABA-A α2 receptor subunits in the dorsal striatum and frontal cortex (respectively), as well as dysbiosis of gut microbiota in a multitude of bacteria at both genus and species levels. All these effects were found in the absence of any evidence for the mediation of ChE enzyme activity. To the best of our knowledge, this is the first demonstration of such effects following this dosage and developmental stage, whilst some of these effects have not previously been published for any dose, developmental stage, or exposure regimen.

CPF exposure increased adult's spontaneous vertical activity in a novel environment. Only a few studies have examined the motor effects of exposure to CPF in low doses during a similar developmental window (Dam et al., 2000; Levin et al., 2001; Ricceri et al., 2003, 2006; Venerosi et al., 2008). The earlier study by Ricceri showed a general increase in total distance traveled but not in rearing frequencies in exposed adolescent mice following both 1 and 3 mg/kg, whilst the later study revealed no significant effects of 1 mg/kg postnatal exposure on motor activity but increased locomotor rates in exposed animals following 3 mg/kg/day exposure in this late postnatal window, strengthened by the preexposure to CPF during gestation. All of these data are also in agreement with previous findings on habituation to activity (Levin et al., 2001). Dam et al. (2000) also found that 5 mg/kg/day of CPF exposure from PND11-14 lead to significantly higher rates of rearing but not total distance covered, an effect that was found only in males. Both Dam et al. (2000) and Ricceri et al. (2006) found similar results to those reported here, except that their dosages were between 3 and 5-fold higher than ours. Taken together, these findings have implications for both biochemical and mechanistic explanations and, more importantly, show replicability in different rodent models and are of translational significance.

Interestingly, a single saline injection (i.p.) just before the first open field session altered the locomotor activity behavior of female exposed rats by increasing both total distance and, above all, vertical activity outcomes. We have been unable to find any other results similar to those in the current literature. However, this overt reaction to stress could be linked to altered basal regulation of the hypothalamic-pituitary-adrenal axis. Previous studies found that developmental CPF exposure do alter anxiety outcomes in murines, with both increased (Silva et al., 2017; Braquenier et al., 2010) and decreased anxious response (Carr et al., 2017; Ricceri et al., 2006), these two latter studies using similar doses and developmental stage as we did here. However, we found neither altered center and/or margin time activity in the exposed compared to control animals at any phase of following any drug challenge in the present study nor abnormal behavior in a plus-maze from other experiments parallelly conducted in our laboratory (data not shown).

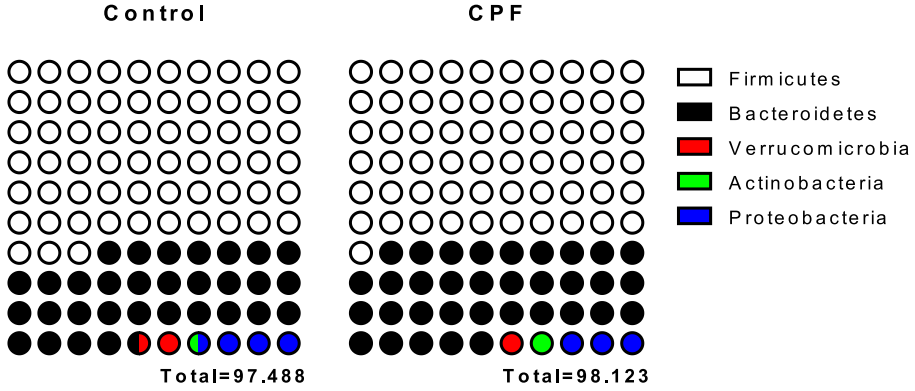

Fig. 8. The relative abundance of the 5 most important bacteria in the Phylum taxonomic category in both control and CPF animals. The percentage from the total gut microbiota of the 5 bacteria is displayed (bottom-right).

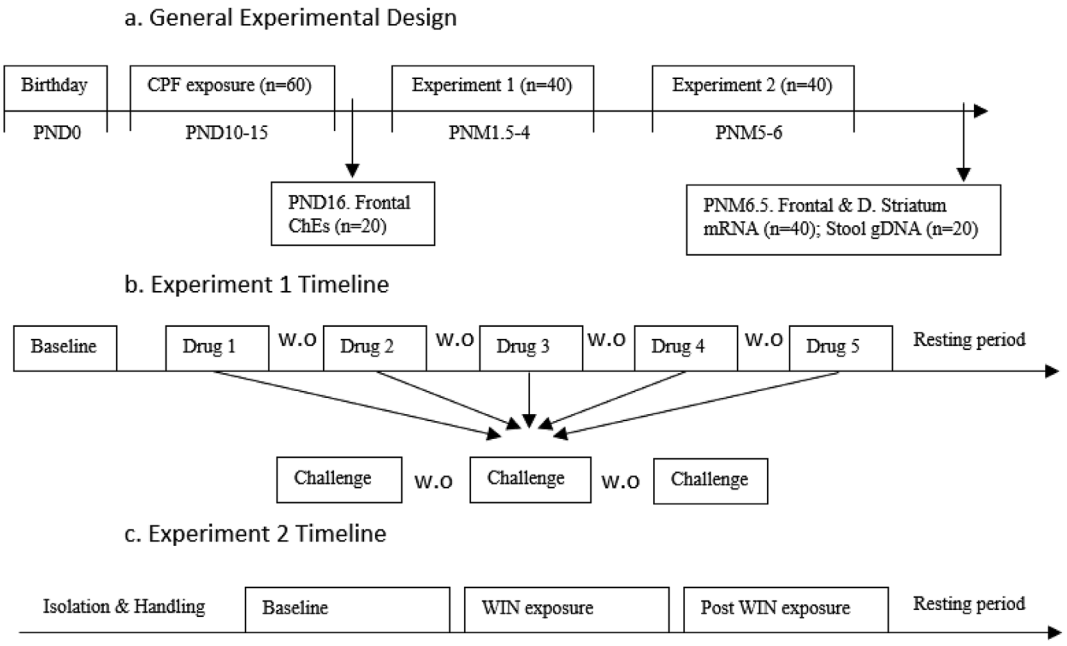

Image 1. Experimental design. a) General experimental design. Half of the male and female rats were orally exposed (gavage) to CPF from PND10 to 15. Frontal cortices from 20 rats (half females, 5 CPF exposed on per sex) were taken 24 h after last exposure for ChEs analyses. Other 40 rats (half females, 10 exposed per sex) completed both the Experiment 1 and 2. Two weeks after the end of Experiment 2 all the 40 rats were sacrificed. The frontal cortex and dorsal striatum samples from all the 40 rats were then used for RT-qPCR analyses. A random selection of the stool from the half of the animals (20 rats, 10 females, 5 CPF exposed from each sex) was used for gut microbiota analyses. b) Experiment 1 started around PNM1.5 and finished at PNM4 with 5 consecutive days of baseline (BL) and 4 of saline exposure (both considered as BL period), followed by the drug challenges (5 different drugs). The drug challenges followed a completely randomized Latin squared design, with 72 h of wash-out (W.O.) between different doses of the same drug ("Challenge" in the timeline) and 1 week between different drugs. After this, animals were let to rest and isolated to prepare them for Experiment 2 (c). Isolation was done for 3 weeks before Experiment 2 and rats were daily handled to minimize stress. Once animals were habituated (around PNM5), food consumption, weight, and locomotor activity were monitored before, during and after four consecutive days of WIN55, 212-2 (WIN) exposure.

We were unable to find influences of CPF on most of the main neurotransmitter systems analyzed. Especially surprising was the case of the endocannabinoid system, as the recent literature on the exposure to even lower doses of CPF than used here have systematically altered different components of this system following exposure during this preweaning period (Carr et al., 2011, 2013, 2014, 2017). Furthermore, direct interaction between the xenobiotic and the CB1 receptor has been proved (Quistad et al., 2002; Baireddy et al., 2011; Liu et al., 2015; Liu and Pope, 2015). However, the general lack of effects had two exceptions: The cholinergic and the GABAergic systems.

Low doses of scopolamine hydrobromide in adulthood increased the rates of locomotor activity to a greater extent in controls than CPF exposed animals at both doses, but particularly at the lowest dose, this effect being strong in females and subtle in males. Similar hyposensitivity to muscarinic challenge following developmental exposure to CPF was found in earlier studies (Levin et al., 2001, 2002; Icenogle et al., 2004). In Levin et al. (2001), the authors exposed the rats to 5-fold our dosage from PND11-14 and observed a lack of responsiveness to scopolamine in a working memory paradigm, but only for the exposed female rats. Thus, low doses of CPF at the late preweaning stage affect certain cholinergic components other than/independently of ChE. Striatal auto/hetero receptors have been linked to the regulation of locomotor activity (Myslivecek et al., 2017). Interestingly, some early works put forward the hypothesis that muscarinic autoreceptors play a role in scopolamine-induced hyperactivity (Mathur et al., 1997).

This exposure protocol up-regulated the M2 receptor mRNA in the dorsal striatum, an essential area for the regulation of motor activity and coordination, amongst other functions (Kravitz and Kreitzer, 2012). To the best of our knowledge, this is the first demonstration of this long-term effect on the dorsal striatum, observed at the level of mRNA following this exposure protocol. Interestingly, previous studies also found the general up-regulation of M2 receptors following similar doses of CPF but at PND1-4 and shortly after exposure (Slotkin and Seidler, 2007b). The increase of both M2 and 4 autoreceptors following CPF exposure has long been proposed to be the result of the significant inhibition of Adenylyl cyclase functioning and cAMP formation as an indirect effect of presumably increased autoreceptor regulation (Ward and Mundy, 1996; Huff et al., 1994; Zhang et al., 2002). Of special relevance for our work, Rhodes et al. (2004) found significant inhibition of M2 receptor binding 24 h after exposure that ended at the late postnatal preweaning stage (5 mg/kg), but a general subtle long-term increase at young adulthood, with higher rates in medial structures (hippocampus) for female rats. The results of this binding study along with the present gene expression data provide support for the notion that developmental exposure to CPF can generate the up-regulation of muscarinic autoreceptors months after the exposure.

M2 receptors seem to play an important role in neural differentiation and cell proliferation, both neurons and glial cells (Abreu-Villaça et al., 2011). Furthermore, M2 receptors gradually increase between PND5 to early adolescence in the mice's CNS (Hohmann et al., 1995). Added to this, a study on the neuromuscular junction in mice found that M2 receptors play an important role in the synaptic pruning, eminently by increasing axonal elimination from PND7 enhancing this in conjunction to M1 receptors at PND9 and stabilizing the process around PND15 (Nadal et al., 2016). If the muscarinic receptors show this similar role in the brain, this could partially indicate why the exposure to CPF during this preweaning period is more likely to alter these receptors than gestational and early postnatal windows.

The exposure to the OP affected the integrity of the GABAergic system, which was revealed following low doses of Alprazolam. Although the effects on males were modest, control females showed a biphasic inverted u-shaped dosing profile -a feature of Alprazolam that was reported in earlier work by Lopez et al. (1988)-. Here, the increase in activity with the lower dose contrasts with the significant sedative

Table 2
The relative abundance of the bacteria (genus level) with significant influence of CPF exposure. The significant bacteria are scheduled based on their relative abundance average (‰). Statistics and final outcomes in relation to TREATMENT and SEXxTREATMENT factors for significant bacteria. T = Treatment, S*T = SEXxTREATMENT, +/− = CPF exposure increased/decreased the relative abundance of the referred bacteria, M/F = Male/Female, N.S. = Not significant.

Genus	Factor	Two-way ANOVA	Outcome (Post Hoc)	Relative Abundance
Pelagicoccus	T	F(1,16)= 5.004, p= 0.040	+	52,221087
Butyrivribio	T	F(1,16)= 5.687, P= 0.03	+	10,197049
Anaerobranca	T	F(1,16)= 4.495, p=0.05	+	7,935816
Vibrio	S*T	F(1,16)= 5.344, p= 0.034	CPF/M + CPF/F; CNT/M - CPF/M	1,406024
Alivibrio	S*T	F(1,16)= 5.778, p= 0.029	CPF/M + CPF/F	0,25912
C. Contubernalis	T	F(1,16)= 6.364, p= 0.023	-	0,241885
Mogibacterium	T	F(1,16)= 9.608, p= 0.007	+	0,230838
Borrelia	T	F(1,16)= 4.621, p= 0.047	+	0,221347
	S*T	F(1,16)= 5.347, p= 0.034	CPF/M - CPF/F; CNT/F - CPF/F	0,221347
Hyphomicrobium	T	F(1,16)= 7.312, p= 0.016	-	0,148989
	S*T	F(1,16)= 10.938, p= 0.004	CNT/M - CNT/F; CNT/F + CPF/F	0,148989
C. Microthix	S*T	F(1,16)= 6.980, p=0.018	CNT/M - CNT/F	0,110804
Paracoccus	T	F(1,16)= 4.700, p= 0.046	-	0,105909
Longilinea	S*T	F(1,16)= 5.352, p= 0.034	CNT/M - CNT/F; CNT/F + CPF/F	0,079644
Hydrogenophaga	S*T	F(1,16)= 6.067, p= 0.025	N.S.	0,070742
Luteimonas	S*T	F(1,16)= 5.912, p= 0.027	CNT/M - CNT/F; CNT/F + CPF/F	0,069353
Actinokineospora	S*T	F(1,16)=7.374, p= 0.015	CPF/M - CPF/F; CNT/F - CPF/F	0,064061
C. Endobugula	T	F(1,16)= 5.638, p= 0.03	+	0,062201
Vogesella	T	F(1,16)= 7.170, p= 0.017	-	0,056282
	S*T	F(1,16)= 7.170, p= 0.017	CNT/M - CNT/F; CNT/F - CPF/F	0,056282
Petrotoga	S*T	F(1,16)= 7.395, p= 0.015	CNT/M + CPF/M	0,051465
Brevundinomas	T	F(1,16)= 4.576, p= 0.048	+	0,048346
	S*T	F(1,16)= 6.256, p= 0.024	CPF/M + CPF/F; CNT/M - CPF/M	0,048346
Nitrospira	S*T	F(1,16)= 5.029, p= 0.039	N.S.	0,034644
Rhizobium	T	F(1,16)= 4.931, p= 0.041	-	0,028568
Ancylobacter	S*T	F(1,16)= 7.475, p= 0.015	N.S.	0,024109
Cycloclasticus	S*T	F(1,16)= 4.695, p=0.046	N.S.	0,020637
Pseudidiomarina	S*T	F(1,16)= 4.586, p= 0.048	N.S.	0,018606
Microcoleus	S*T	F(1,16)= 5.268, p= 0.036	CPF/M - CPF/F; CNT/F - CPF/F	0,017617
Nitrincola	T	F(1,16)= 4.534, p= 0.049	-	0,014124
	S*T	F(1,16)= 4.534, p= 0.049	CNT/M - CNT/F; CNT/F + CPF/F	0,014124

effect that was observed following the highest dose. However, CPF exposed females did not behave in the same way as controls since they started to show a decline in activity even at the lowest dosage. This is suggestive of a hyper-sensitized GABAergic system. Furthermore, CPF exposed animals also showed greater sedative effects (less activity) at the higher dose in comparison with control rats, supporting the hypersensitivity hypothesis. From the few studies that have linked CPF to the GABA system, the majority used high doses of CPF (Montes de Oca et al., 2013; Sánchez-Amate et al., 2002; Cardona et al., 2006) or low but chronic exposure protocols in adulthood (López-Granero et al., 2016), and thus it is not appropriate to make comparisons between these works and the present experiment. However, we found only one developmental, low-dosage exposure study that analyzed GABAergic components concerning CPF exposure (Gómez-Giménez et al., 2018). The authors found increased extracellular GABA levels in the cerebellum only in male rats following very low exposure (0.3 mg/kg/day) during the whole developmental period. Interestingly, these data correlated negatively with motor coordination.

CPF exposed animals showed an up-regulation of the GABA-A-α2 subunit at the frontal cortex 6 months after the exposure protocol ended. This is, to the best of our knowledge, the first time that CPF exposure has been linked to the specific modulation of this GABAergic subunit. GABA-A-α2 subunits are present at 15–20% of all GABA-A receptors (Engin et al., 2012). These units are thought to play important roles in CNS development such as control of spinal pain, anxiety, and depression, as well as dependence on certain drugs of abuse such as cocaine, ethanol, or cannabis (Gonzalez-Nunez, 2015; Engin et al., 2012). With regard to this last point, recent research has focused on the

Table 3
The relative abundance of the bacteria (species level) with significant influence of CPF exposure. The significant bacteria are scheduled based on their relative abundance average (‰). Statistics and final outcomes in relation to TREATMENT and SEXxTREATMENT factors for significant bacteria. T = Treatment, S*T = SEXxTREATMENT, +/− = CPF exposure increased/decreased the relative abundance of the referred bacteria, M/F = Male/Female, Uns. = Unspecific bacteria, N.A. = Not applicable (genus composed by only one specie), N.S. = Not significant.

Genus	Specie	Factor	Multivariate ANOVA	One/Two-way ANOVA	Outcome (Post Hoc)	Relative Abundance
Anaerobranca	**Zavarzinii**	T	N.A.	F(1,16)= 4.495, p=0.05	+	7,935816
C. Contubernalis	**Alkalaceticum**	T	N.A.	F(1,16)= 6.364, p= 0.023	-	0,241885
Mogibacterium	**Uns.**	T	F(2,15)= 5.006, p= 0.022	F(1,16)= 10.423, p= 0.005	+	0,178438
C. Microthix	**Parvicella**	S*T	N.A.	F(1,16)= 6.980, p= 0.018	CNT/M - CNT/F	0,110804
Longilinea	**Arvoryzae**	S*T	F(2,15)= 3.797, p= 0.046	F(1,16)= 7.100, p= 0.017	CNT/M - CNT/F; CNT/F + CPF/F	0,073507
Actinokineospora	**Inagensis**	S*T	N.A.	F(1,16)=7.374, p= 0.015	CPF/M - CPF/F; CNT/F - CPF/F	0,064061
Vogesella	**Perlucida**	T	N.A.	F(1,16)= 7.170, p= 0.017	-	0,056282
Vogesella	**Perlucida**	S*T	N.A.	F(1,16)= 7.170, p= 0.017	CNT/M - CNT/F; CNT/F - CPF/F	0,056282
Petrotoga	**Uns.**	S*T	N.A.	F(1,16)= 7.395, p=0.015	CNT/M + CPF/M	0,051465
Hyphomicrobium	**Vulgare**	T	F(4,13)= 4.464, p= 0.017	N.S.	N.S.	0,041678
Hyphomicrobium	**Vulgare**	S*T	F(4,13)= 7.460, p= 0.002	F(1,16)= 5.298, p= 0.035	CNT/M - CNT/F; CNT/F + CPF/F	0,041678
Zobellia	**Laminariae**	S*T	N.A.	F(1,16)= 4.492, P= 0.05	N.S.	0,030782
Cycloclasticus	**Oligotrophus**	S*T	N.A.	F(1,16)= 4.695, p= 0.046	N.S.	0,020637
Pseudidiomarina	**Uns.**	S*T	N.A.	F(1,16)= 4.586, p= 0.048	N.S.	0,018606
Microcoleus	**Antarcticus**	S*T	N.A.	F(1,16)= 5.268, p= 0.036	CPF/M - CPF/F; CNT/F - CPF/F	0,017617
Nitrincola	**Lacisaponensis**	T	N.A.	F(1,16)= 4.534, p= 0.049	-	0,014124
Nitrincola	**Lacisaponensis**	S*T	N.A.	F(1,16)= 4.534, p= 0.049	CNT/M - CNT/F; CNT/F + CPF/F	0,014124

influence of this subunit on the GABAergic regulation of a preclinical model of alcohol disorders, and the results of several studies point to the possibility that the up-regulation of this subunit could guide the positively reinforcing properties of alcohol (the main empirical findings are summarized in Olsen and Liang, 2017; Engin et al., 2012).

The GABAergic system is eminently excitatory as depolarizes the neuronal signaling during the first postnatal stage when most of the neurons are still immature. The shift to the final hyperpolarizing profile of the GABAergic receptors generally occurs around the beginning of the second postnatal week in the rat CNS (although this seems to be area-dependent -i.e. Ehrlich et al., 2013-), when the expression and activity of the potassium-chloride cotransporter 2 (KCC2) increases, thus leading to a more efficient efflux of Cl-out of the neuron (Spitzer, 2010). Interestingly, previous studies found that there is a transition from the most expressed α2 to similar α1/α2 expression during postnatal development, where α2 subunits are more abundant in the mammalian CNS until around the 1 month of life (Ehrlich et al., 2013). This could also partially explain the specificity of our CPF exposure protocol on this GABAergic subunit and not the α1. Authors also observed that this shifting from α2 to α1 occurred along with the transition from the high activated sodium-potassium-chloride cotransporter 1 and the decrease of this along with the enhanced KCC2 activity. Taking all together, CPF exposure during essential developmental preweaning ages could have altered the dynamics of the GABA-A receptor composition and, for instance, mismatched the whole maturation pattern of this system with both long-term behavioral and molecular consequences.

It is known that both acetylcholine and GABA neurotransmitters corelease within the cholinergic system in the forebrain (Saunders et al., 2015) and co-transmit at the hippocampus (Takács et al., 2018). Interestingly, antidepressant-like behaviors induced by scopolamine on M1 receptors seem to be mediated by the frontal GABA interneurons (Wohleb et al., 2016). To our special interest, an early study conducted by Sarter et al. (1988) found that the alterations in working memory following 1 mg/kg of scopolamine were blocked when the GABA-A receptor was antagonized. Furthermore, a study with cats found that the acetylcholine release is modulated by muscarinic autoreceptors in certain brain areas and inhibited by GABA-A receptors functioning (Vazquez and Baghdoyan, 2003). Added to this, around one third of the cortical GABAergic neurons also express M2 receptors (Disney and Aoki, 2008), thus it is possible that increased M2 heteroreceptors could drive to decreased GABA release and, finally, an up-regulation of different components of the GABAergic receptors as a compensatory mechanism following the decreased GABAergic tone in the cortex. Taking this information all together, it is plausible that the effects of CPF exposure on both cholinergic and GABAergic systems could share a common molecular mechanism, although the present manuscript does not give empirical support to this notion.

Apart from this long-term behavioral and brain gene modulations, CPF exposure at the pre-weaning stage leads to gut microbiota dysbiosis by altering the relative abundance of several bacteria at both genus and species taxonomic levels. In terms of the most relevant bacteria from the composition of the entire gut, CPF increased the relative abundance of Pelagicoccus, Butyrivibrio, and Anaerobranca, the latter being specific to the species Zarvazinii. This is the first time that these specific bacteria have been linked to CPF exposure, independently of the dosage or developmental window. In fact, of all the bacteria described, we have found only one gut microbiota study, linking CPF exposure to the bacterium Brevundimonas (Fang et al., 2018), but with opposite results probably due to the exposure stage (development vs. adulthood), length of exposure (6 days vs. chronic), and the time-frame of the effects studied after exposure (long-term vs. short-term).

Previous reports found that CPF exposure increase (Enterococcus, Clostridium, Staphylococcus, Bacteroidaceae, Bacteroides and Bacteroidetes) and decrease (Bifidobacterium, Lactobacillus, Lactobacillaceae, Aerococcus, Brevundimonas, Thricococcus, Olsenella, Clostridium ss1, Amphibacillus, Enterorhabdus, Alloprevotella, Firmicutes and Firmicutes/Bacteroides ratio) the relative abundance and/or total counts of a multitude of bacteria families, genus, and species in the human gut, simulated human gut, and murine microbiome, in some cases with a 1 mg/kg/day dose of CPF (Joly Condette et al., 2013, 2015; Fang et al., 2018; Zhao et al., 2016; Reygner et al.,

2016). Furthermore, CPF exposure has been linked to increased gut permeability, a condition generally associated with various gastrointestinal pathologies (Joly Condette et al., 2014; Tirelli et al., 2007).

In spite of the lack of empirical knowledge on the implications of both Pelagicoccus and Anaerobranca Zarvazinii for biochemical functions and behavioral outcomes, Butyrivibrio bacteria have been linked to butyrate production for the facilitation of the processes involved in the digestion of different fibers, proteins, sugars, cellulose, and lipids in ruminants. Regarding this latter effect, the link between CPF exposure and Butyrivibrio is unsurprising, since both molecules interact and modify fatty acids. Furthermore, Butyrivibrio has been associated with anti-inflammatory responses via the production of linoleic acids (Zhu et al., 2014), linking lower rates of relative abundance of these bacteria to different inflammatory pathologies such as Behcet's disease (Shimizu et al., 2019).

Finally, the GABAergic system has been recently linked to specific bacteria populations into the human gut microbiome (Strandwitz et al., 2019). Furthermore, GABA supplementation also regulated the population and diversity of gut microbiota in Piglets (Chen et al., 2019). Interestingly, other studies found that the improvements in anxiety and depression-like outcomes following the administration of Lactobacillus rhamnosus also altered GABA-Aα2 mRNA expression levels in murines, a process mediated by the Vagus nerve which regulates the communication between the CNS and the gut microbiota (Bravo et al., 2011). Unfortunately, the specific bacteria linked to the GABAergic system or GABA production observed in these studies were not altered in the present work and the bacterias found as modulated by CPF here have not been previously related to the GABAergic system.

5. Conclusion and future guidelines

1 mg/kg/day of CPF for 6 consecutive days at pre-weaning developmental stages increased spontaneous activity, increased motor reaction to stress (in females), hypersensitized animals to both antimuscarinic and GABAergic challenges (predominantly in females), upregulated transcription of both M2 receptor and GABA-A-α2 subunit genes in the dorsal striatum and frontal cortex, respectively, and induced gut microbiota dysbiosis at both genus and species taxonomic levels. All these effects were observed months after the exposure protocol was ended. However, only subtle effects were observed on the endocannabinoid system, which was surprising given previously reported data. These novel empirical data, taken together with the findings of certain earlier studies, provide support for the notion that even low doses of CPF that induce little to no inhibition of the ChEs during essential stages of the postnatal neurodevelopment can exert a multitude of alterations. Further studies are needed in order to specifically analyze both the molecular and behavioral basis of the present findings.

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.

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Transparency document

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Appendix A. Supplementary data

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Supplemental Figure 1. Spontaneous locomotor activity in open field for total distance. Data is expressed by means and SEM.

Supplemental Figures 2-3. Habituation-to-paradigm locomotor activity for: 2) Total distance and 3) Vertical activity. Data is expressed by means and SEM.

Supplemental Figure 4. Locomotor activity following acute stress (saline i.p. injection) for total distance. Data is expressed by means and SEM.

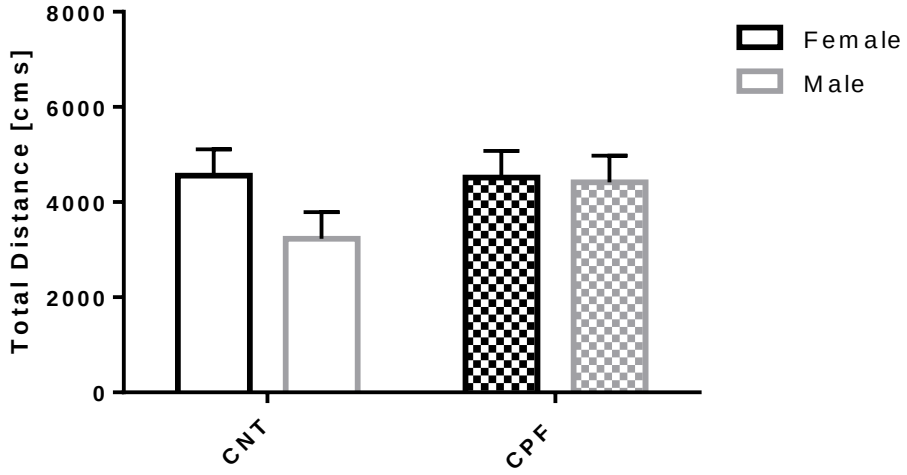
Supplemental Figures 5-6. Locomotor activity after habituation to stress for: 5) total distance and 6) vertical activity. Data is expressed by means and SEM.

Supplemental Figures 7-13. Relative expression of different genes in dorsal striatum at PNM6. The genes are: 7) M1r, 8) Nα7, 9) ChAT, 10) VAChT, 11) GABA-A-α1, 12) GABA-A-α2 and 13) GAD1. Data is expressed by means, SEM and the individual plots.

Supplemental Figures 14-20. Relative expression of different genes in frontal cortex at PNM6. The genes are: 14) M1r, 15) M2r, 16) Nα7, 17) AChE-S, 18) GABA-A-α1, 19) GAD1 and 20) GAD2. Data is expressed by means, SEM and the individual plots.

Supplementary figures

S1.



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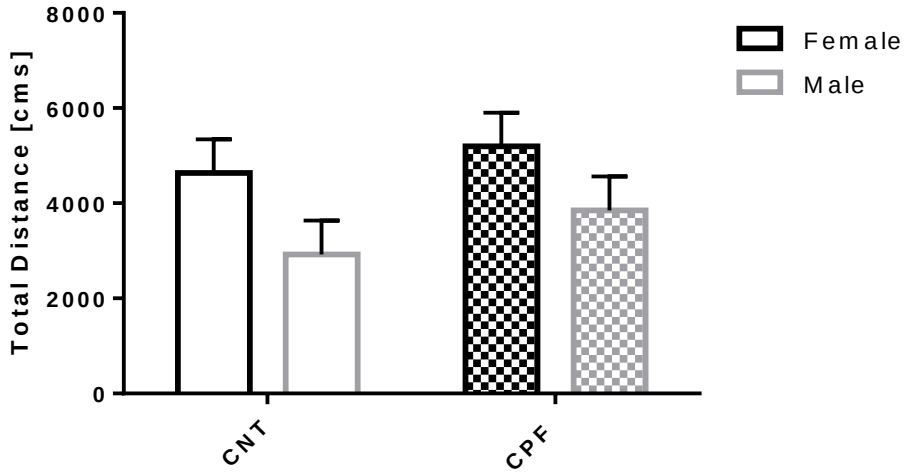

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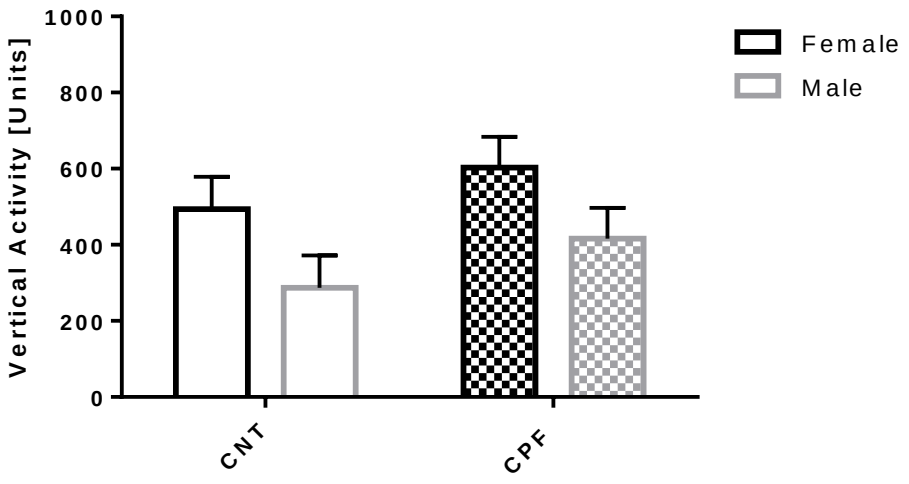
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CPF
Female
Male

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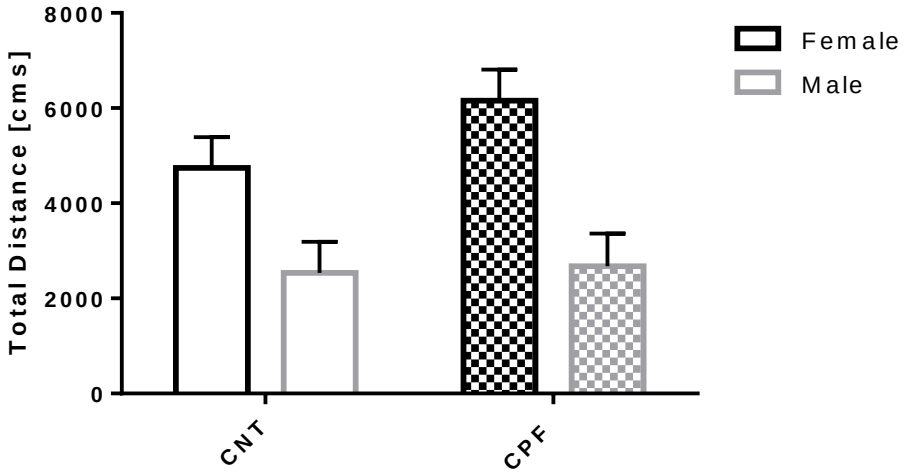
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Male

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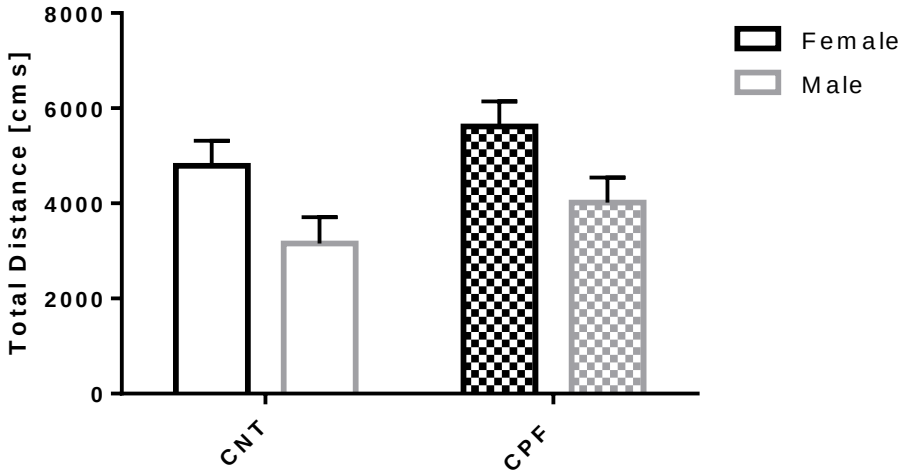
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CNT
CPF
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Male

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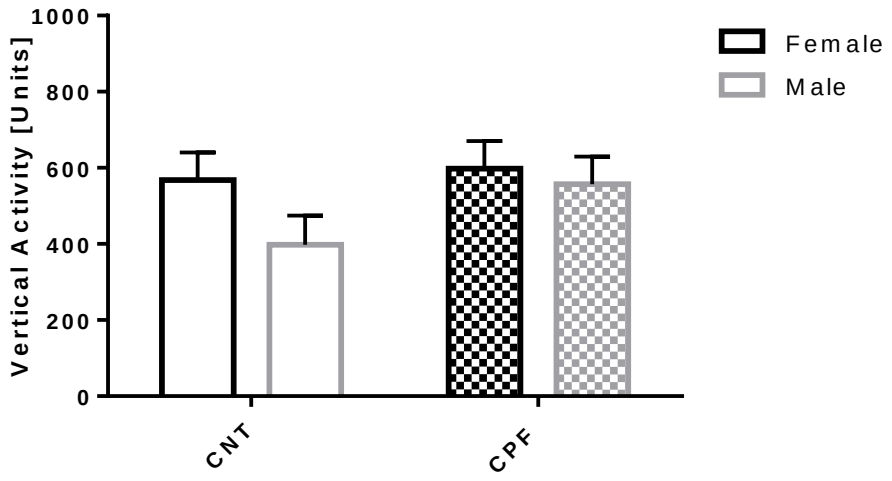
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CPF
Female
Male

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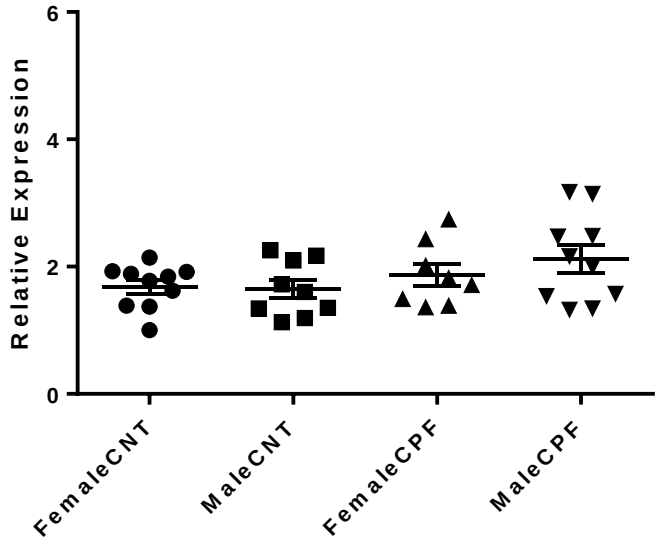
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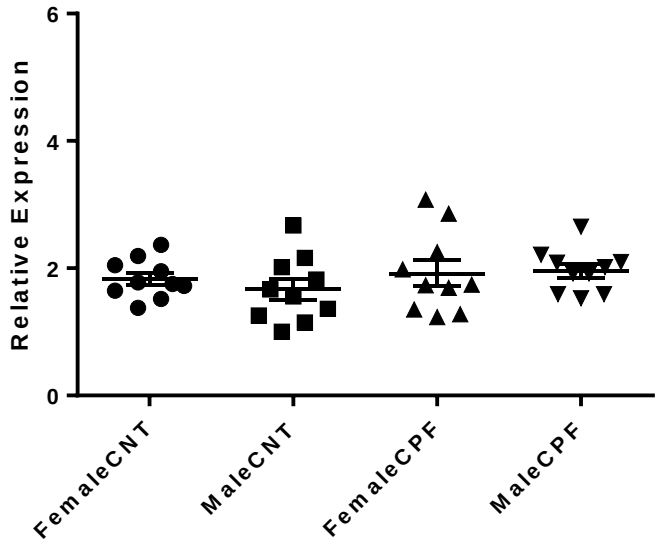
MaleCNT
FemaleCPF
MaleCPF

S8.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MaleCNT
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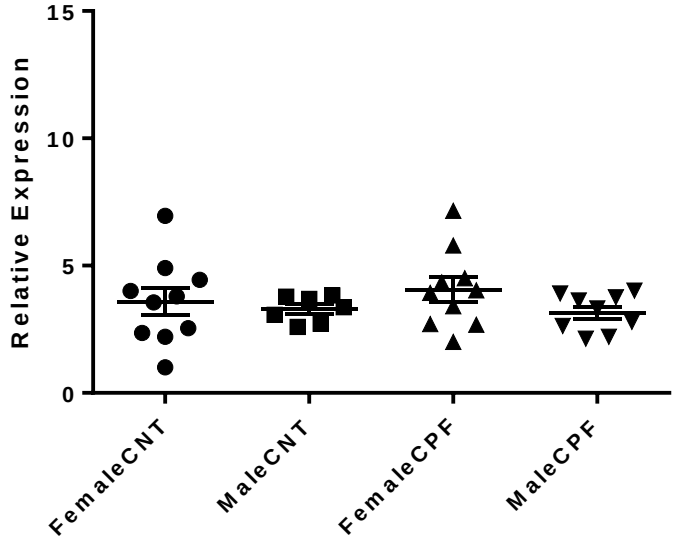
S9.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MaleCNT
FemaleCPF
MaleCPF

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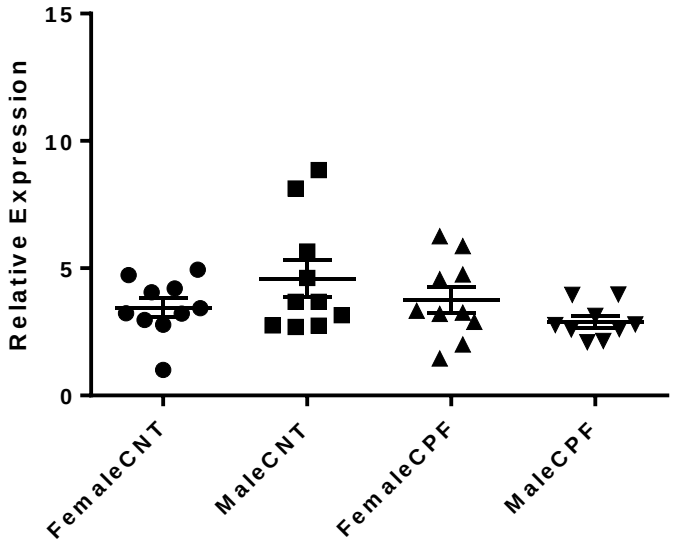
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MaleCNT
FemaleCPF
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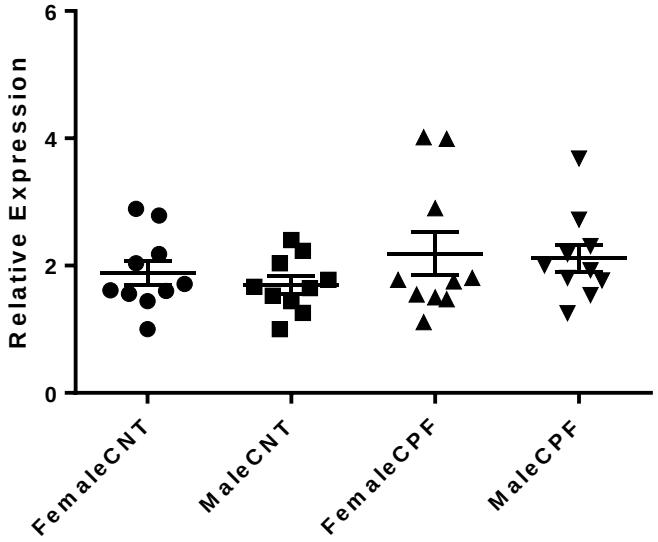
S11.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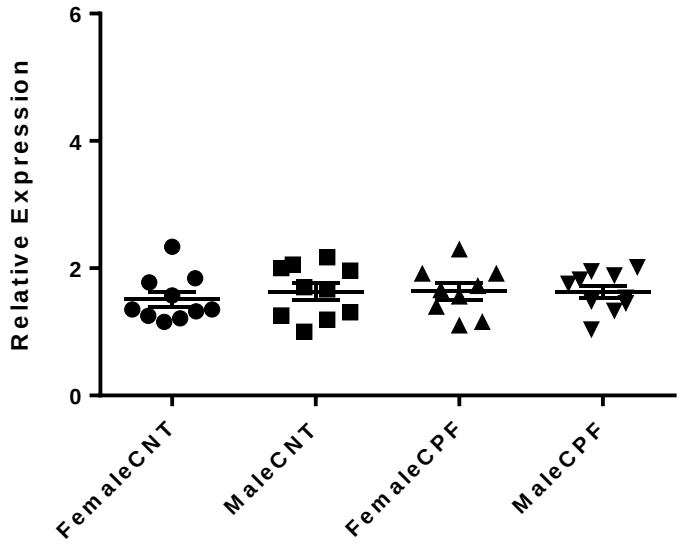
S12.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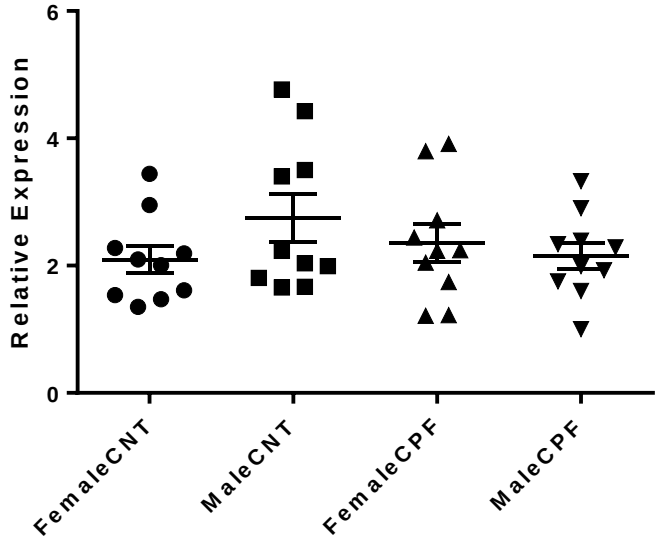
FemaleCPF
MaleCPF

S13.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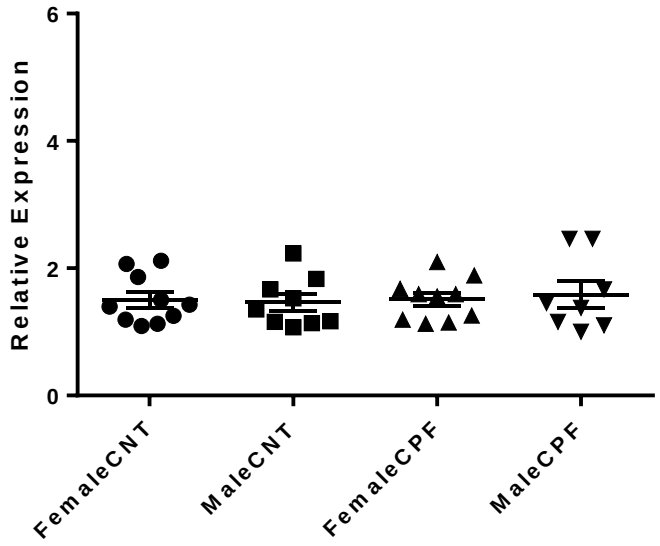
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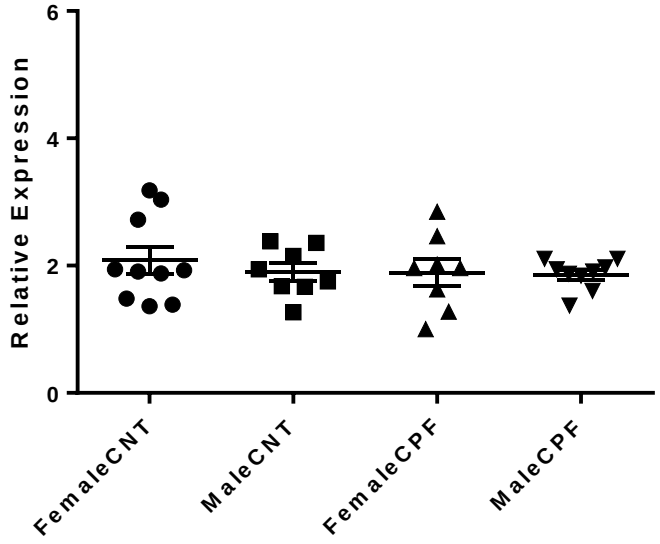
MaleCNT
FemaleCPF
MaleCPF

S15.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FemaleCPF
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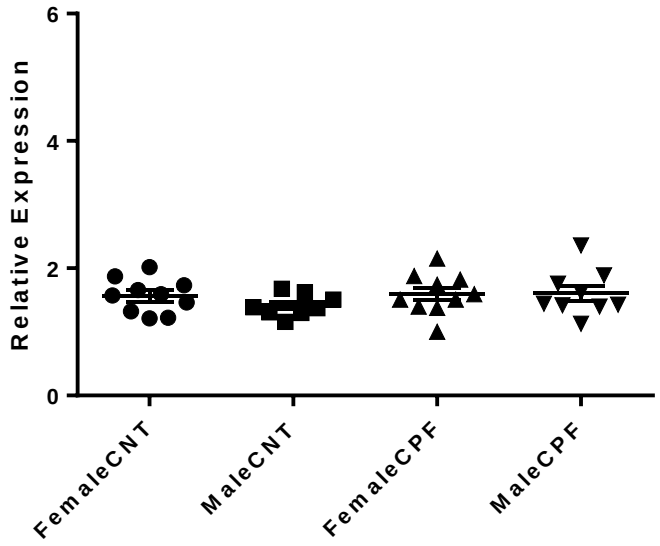
S16.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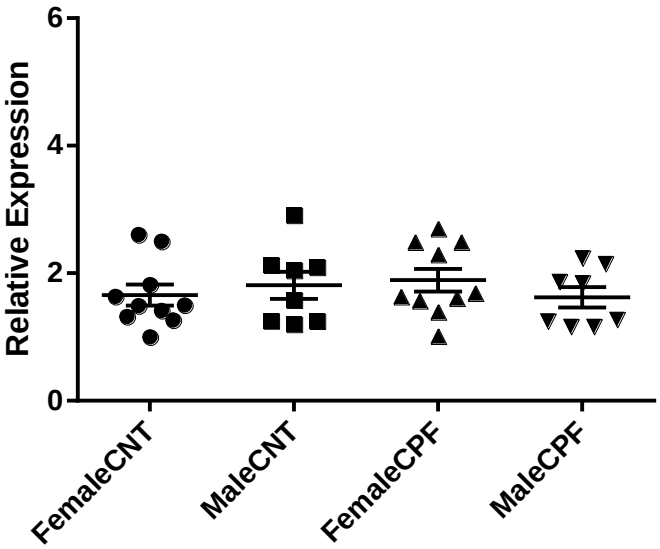
S17.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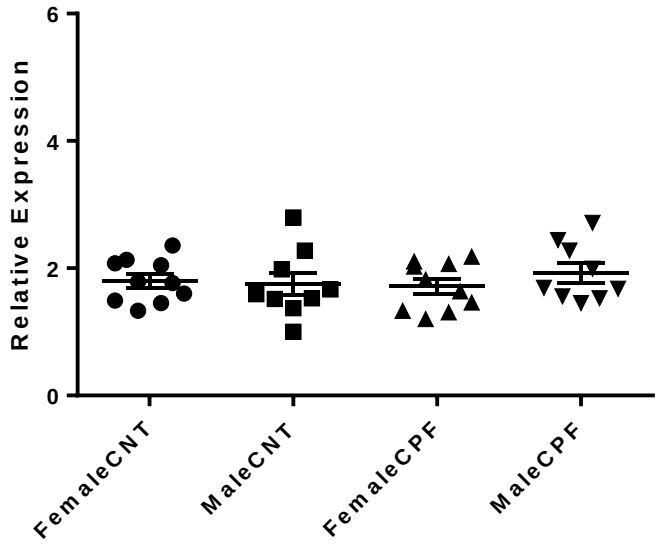
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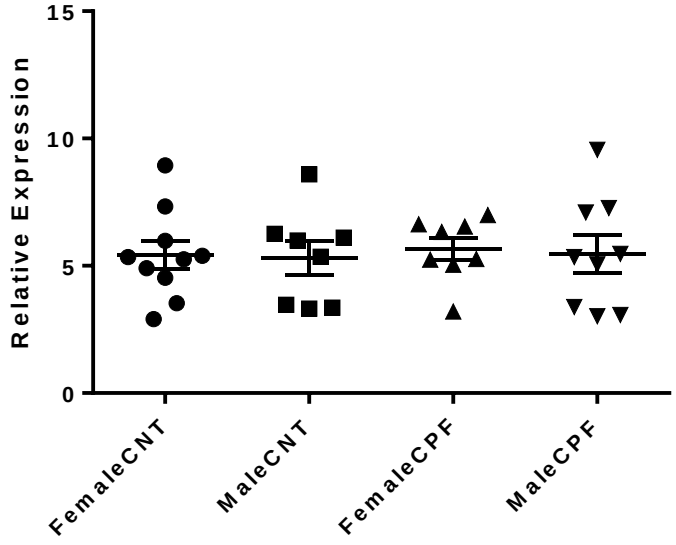
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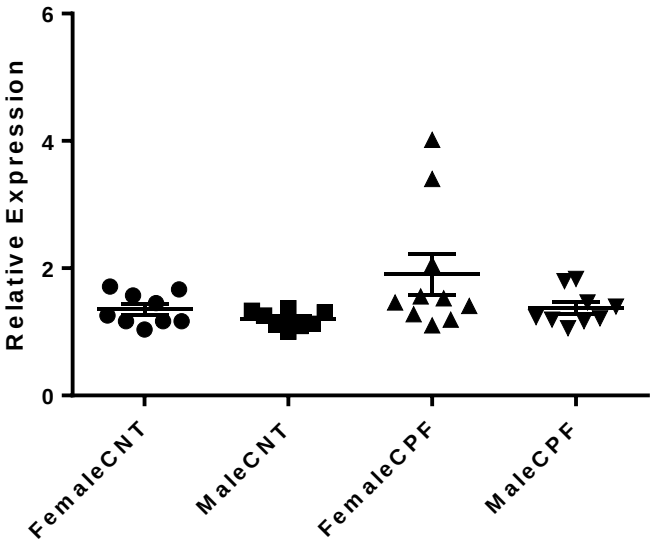
S20.


Relative Expression
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FemaleCNT
MaleCNT
FemaleCPF
MaleCPF

STUDY 2

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Medium and long-term effects of low doses of Chlorpyrifos during the postnatal, preweaning developmental stage on sociability, dominance, gut microbiota and plasma metabolites

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ABSTRACT

Autism spectrum disorder (ASD) is a complex neurodevelopmental pathology characterized by altered verbalizations, reduced social interaction behavior, and stereotypies. Environmental factors have been associated with its development. Some researchers have focused on pesticide exposure. Chlorpyrifos (CPF) is the most used Organophosphate. Previous developmental studies with CPF showed decreased, enhanced or no effect on social outcomes eminently in mice. The study of CPF exposure during preweaning stages on social behavior is sparse in mice and non-existent in rats. d stressors could be at the basis of ASD development, and around postnatal day 10 in the rat is equivalent to the human birthday in neurodevelopmental terms. We explored the effects of exposure to low doses (1mg/kg/mL/day) of CPF during this stage regarding: sociability, dominance gut microbiome and plasma metabolomic profile, since alterations in these systems have also been linked to ASD. There was a modest influence of CPF on social behavior in adulthood, with null effects during adolescence. Dominance and hierarchical status were not affected by exposure. Dominance status explained the significant reduction in reaction to social novelty observed on the sociability test. CPF induced a significant gut microbiome dysbiosis and triggered a hyperlipidemic, hypoglycemic/hypogluconeogenesis and a general altered cell energy production in females. These behavioral results in rats extend and complement previous studies with mice and show novel influences on gut metagenomics and plasma lipid profile and metabolomics, but do not stablish a relation between the exposure to CPF and the ASD phenotype. The effects of dominance status on reaction to social novelty have an important methodological meaning for future research on sociability.

1. Introduction

Autism spectrum disorder (ASD) is a complex neurodevelopmental pathology defined by reduced verbalizations and communication abilities, increased stereotypies and ritualistic/repetitive behaviors, and altered sociability skills (Diagnostic and statistical manual of mental disorders-5th ed., APA, American psychiatric association, 2013). 1 out of 166 children meet the diagnostic criteria for ASD (WHO, World Health Organization, 2018; DiCicco-Bloom et al., 2006).

Empirical studies regard ASD as having a high degree of heritability, and specific analyses of a multitude of genetic factors provide support for its polygenic nature (Grove et al., 2019). However, the impact of environmental factors on ASD development, progression, and severity has attracted increasing interest in recent decades with emphasis on

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socio-economic status, perinatal stress events and drug/xenobiotic exposure (Chaste and Leboyer, 2012). Regarding the latter category, developmental exposure to various pesticides such as Carbamates and Organophosphates (OP) has been the focus of several experimental studies (Herbert 2010; Shelton et al., 2014). Chlorpyrifos (CPF) is the most widely used OP in recent decades. CPF is used as an insecticide, fungicide and herbicide for agricultural and industrial purposes. CPF exerts its main neurotoxicological profile by inhibiting the different Cholinesterases (ChEs) at both the Central Nervous (CNS) and systemic level (Eaton et al., 2008). However, various preclinical studies have also proposed alternative molecular targets for the neurotoxic profile of developmental CPF exposure (Burke et al., 2017).

Studies in mice have analyzed the effects of gestational (Lan et al., 2017, 2019; De Felice et al., 2014, 2015; Venerosi et al., 2010; Mullen et al., 2013), postnatal (Venerosi et al., 2008; Ricceri et al., 2003; Basaure et al., 2019) and both gestational and postnatal (Venerosi et al., 2006, 2015; Ricceri et al., 2006) exposure to CPF on social and/or ultrasound vocalization outcomes. Briefly, developmental exposure doses ranged from 1 to 6 mg/kg/day, and only a few of these studies found decreased social rates in exposed animals (Lan et al., 2017, 2019; De Felice et al., 2014; Venerosi et al., 2010), with enhanced social skills being found in other cases (Ricceri et al., 2006; Venerosi et al., 2006, 2015). The effects of CPF exposure on social and communication skills are highly dependent on the basal state of the organism, as found in KO Reeler mice with basal abnormal social traits (Mullen et al., 2013), other ASD-like strains (De Felice et al., 2015) and/or the APOE variant (Basaure et al., 2019).

Studies on late postnatal, preweaning exposure to CPF and ASD's symptomatology are sparse and focused on mice (Basaure et al., 2019; Venerosi et al., 2006, 2008; Ricceri et al., 2003, 2006). Other authors have also proposed that the human perinatal window could be an essential stage in ASD development (Getahun et al., 2017; Martinez-Morga et al., 2018). This stage has its murine equivalence at around postnatal day (PND) 10 in neurodevelopmental terms. Moreover, some essential cellular and molecular mechanisms characterize this period, such as synaptogenesis and myelinization development as well as the peak period of maturation of vasopressin and oxytocin systems (Venerosi et al., 2006; Semple et al., 2013; Tait et al., 2009).

The relation between CNS and gut microbiome composition and the metabolomic profile of ASD patients and preclinical models is under intense research. The gut microbiota dysbiosis -the alteration of the relative abundance of different bacteria populations-associated with ASD is shown in the reviews recently published (i.e. Srikantha & Mohanjeri 2019; Fattorusso et al., 2019; Mohamadkhani, 2018; Fowlie et al., 2018). Alternatively, both fecal and systemic metabolomic studies have also revealed altered patterns in ASD patients and preclinical models (Mohamadkhani, 2018; Mussap et al., 2016; Ruggeri et al., 2014). On this way, ASD diagnosed children have been linked to both decreased (Gamma-aminobutyrate and Butyric acid) and increased (Isopropano, Glutamate, Propionic acid, amongst others) fecal metabolites (Mohamadkhani, 2018) as well as the they present alterations on different metabolites associated with mitochondrial dysfunction or amino acid metabolism in different biofluids (Mussap et al., 2016).

As observed in the ASD-associated research, CPF exposure has been also associated with alterations in both microbiota and metabolome profiles. Following this, extensive research using low doses of CPF administered at development stages induce gut dysbiosis in rodents (Joly Condette et al., 2013, 2014; 2015, 2016; Reygner et al., 2016). Albeit with less intensity than the microbiome, developmental CPF exposure has also been linked to the specific alteration of various hepatic, brain, and systemic metabolites and metabolic pathways. From all the different metabolic pathways and components, CPF exposure has been linked to important alterations in metabolites that intermediate cell energy production, amino acid metabolism (Xu et al., 2015; Wang et al., 2009), as well as glucose and lipid metabolism (Wang et al., 2009), also following low doses during critical developmental stages (Slotkin et al., 2005). However, studies on the influences of exposure to CPF during postnatal, preweaning stages developmental stages on both gut microbiota and metabolic profile is essentially inexistent, with some very recent exceptions (Perez-Fernandez et al., 2019).

The aim of the present study is to explore the effects of late postnatal, preweaning exposure to low doses of CPF on 1) Social outcomes at both the medium (adolescence) and long-term (adulthood), 2) The constitution of dominance and social hierarchies, and 3) gut microbiota and systemic metabolites, including the lipid profile. The present study also included both Sexes for possible dimorphic specificities. We hypothesize that this dosage regime could decrease social rates and induce different alterations both microbiota populations and metabolites.

2. Materials and methods

2.1. Experimental animals

60 (30 females, half of each exposed to CPF) adolescents (PND 32–33) and 85 (41 females −19 exposed to CPF- and 44 males −22 exposed to CPF-) adult (postnatal month -PNM- 6 to 7) Wistar rats were used. The rats were born in our facilities. Briefly, full-term pregnant mothers (n = 19) arrived at our facilities and were individually caged. After 5 days of acclimation, the animals gave birth to 6–15 pups per mother (190 pups). At PND1 (birthday was set as PND0), all pups were separated from their original mothers, mixed, and randomly distributed 10 (5 females) to each mother ensuring a representative population and avoiding dam-related bias. Weaning (4 animals per cage of the same Sex) was done at PND21. For the present experiments, animals from all the 19 dams were selected to avoid the litter bias. The room was set up with a constant temperature of 22 ± 2 °C and humidity of 50 ± 10%, and a 12-h cycle with lights on at 8:00h. Young animals were fed ad libitum (A04 Standard Free, Panlab), whilst adults followed a maintenance diet of 20g for males and 17g for females from PND74, in order to control weights. Water was provided ad libitum. Experimental timeline is displayed in the Image 1. The experimental units were, in all cases, every single animal behavioral outcome or biological samples (blood plasma or stool). The present study is included in the project ES040130002260. The various experiments were conducted in accordance with the Spanish Royal Decree 53/2013 and the European Community Directive (2010/63/EU) for animal research. The Animal Research Committee of the University of Almería gave their approval for the experiments.

2.2. Neurotoxic agent

CPF (Fluka Analytical, purity of 99.9%) was administered by forced oral administration from PND10 to PND15 inclusive, between 12h and 13.30h. Half of the animals of each Sex from each dam were randomly assigned to CPF or vehicle (corn oil) exposure. This period was chosen because some critical neurological mechanisms take place during this time, such as the peak of oxytocin and vasopressin hormones, and the development of myelinization; PND10 is approximately the day of birth in humans, and is thus considered a good model for perinatal influences on health for translational purposes (Venerosi et al., 2006; Semple et al., 2013; Tait et al., 2009). 1 mg/kg/ml/day of CPF was chosen and diluted in Corn Oil, which is widely used due to its facilitatory absorption properties (Timchalk et al., 2002). For the control condition, vehicle was used at the same volume.

2.3. Behavioral tasks

2.3.1. Crawley's sociability test both adolescence (18–19 days after exposure) and adulthood (5 and a half months after exposure)

Description of the paradigm. In order to avoid some possible limitations of the traditional three-chambered Crawley's test paradigm, we designed the procedure without walls (Supplementary Image 1).

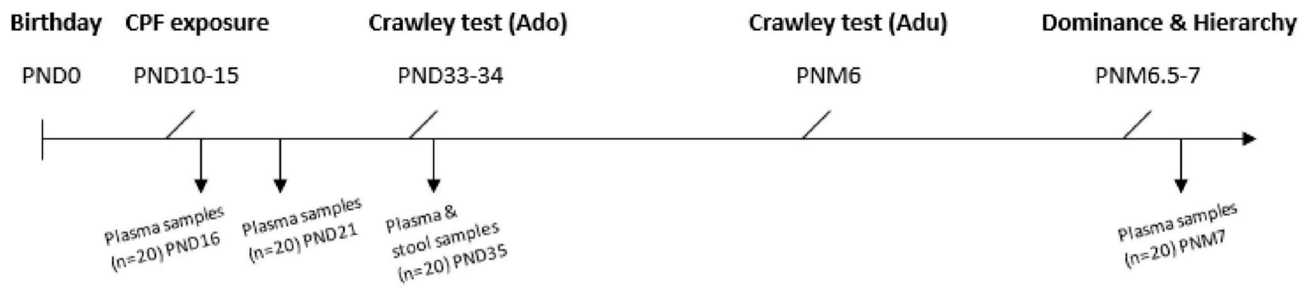

Image 1. Experimental design. A total of 185 rats were used. Half of them females. Half of each sex were randomly allocated to CPF exposure, and the remaining to vehicle condition from PND10 to 15. The social and social novelty behavior of both adolescent -Ado- (PND33-34, n = 60) and adult -Adu- (PNM 6) in a modified Crawley test. Dominance (n = 85) and established hierarchies (n = 72) of the adult rats were also evaluated. Plasma samples were obtained at PND16, 21, 35 and PNM7 (n = 20 at each time, half females, half of each sex exposed to CPF) for metabolomic analyses. Stool samples (n = 20, half females, half of each sex exposed to CPF) were also taken at PND35 for metagenomic analyses.

Sociability testing was conducted in an open field (75 × 75cm) and the chambers were digitally created without physical barriers. We made these 2 changes (size and lack of physical barriers) as these conditions created a more unrestrained exploratory environment for the animals. Distances between the center of the strangers' walls and the limits of "contact" and "approximation" (equivalent to the chamber in traditional paradigms) zones were set following pilot studies in both adolescent and adult rats separately. The dependent variables analyzed were defined as two categories: 1) Motor control, with total distance (cm), time in movement (seconds), mean velocity (cm/seconds), and rearing (frequencies) and 2) Sociability and reaction to social novelty indexes, using the total time [for social (Time S1 - Time empty)/(Time S1 + Time empty) and reaction to novelty (Time S2 - Time S1)/(Time S2 + Time S1)], as described in previous reports (Baronio et al., 2015), in approximation and contact zones, as well as the time of sniffing behavior for the active exploration of the animal. The design of the digital arena and the recording of the outcome were both conducted using Ethovision 3.1. (Noldus).

Behavioral procedure. As with the classical three-chambered Crawley's test, the protocol was divided into three different phases: 1) Habituation. The experimental animal had 5 min of free exploration, 2) Sociability. Stranger 1 was placed at a corner of the apparatus, isolated from the experimental rat but being allowed both visual and odor contact for 10 min, and 3) Reaction to Social Novelty. Stranger 2 was placed on the opposite corner, maintaining the Stranger 1 in its location, and forcing a social choice situation for a further 10 min.

Animals (all 60 adolescents and 85 adults) were driven to the experimental room one day before the procedure for a 1-h session of room acclimation. On the experimental days, the animals were driven to the room 1 h before the procedure. The classical phases previously described were then completed for each animal. The cleaning protocol was carried out with ethanol (70%) between animals and Clidox (1:5:1) between cages. Odd series were developed by males and even by females. Treatment condition was also balanced throughout the day for time of day control. Room temperature and humidity were set as the normal housing parameters and under dim-light conditions between 9h and 14h. Both Strangers 1 and 2 were completely unknown to the experimental animal.

2.3.2. The tube test: social dominance and hierarchical status (6 – and a half months after exposure)

Description of the paradigm. Two classical tube tests were conducted using opaque PVC tubes. For males, the tube was 100 cm in length and 7 cm in diameter. For females, the tube was 85 cm length and 5.5 cm in diameter. These measures were chosen for two reasons: 1) The tube should be of sufficient length to force the "dominant" rat to push for an acceptable time/distance criteria, whilst the "submissive" animal should have time to react, and 2) The diameter should be wide enough to allow the animals to move back and forth but narrow enough to prevent them from turning on their own axis. A small longitudinal aperture was made to the upper part of the tubes in order to control the localization of the animals. Three gates (2 at the tube external segments and another in the center) were designed to limit free movement and proper disposition before the "fights". The criteria for winning a match was defined as the opponent placing 4 paws out of the tube in its initial external box. The dependent variable analyzed was the percentage of wins of each animal. The tube test was used in order to study both the direct dominance (unknown animals) and the well-established hierarchies (animals from the same home-cage).

Behavioral procedure. The animals were moved to the experimental room 6 days before the start for paradigm habituation and training. At the beginning, some rats started to get into the tube and move back and forth following some gentle pressure by the experimenter. This was followed by 5 consecutive days of reinforced straight-run behavior. Briefly, a few pellets were placed in the final segment of the tube and the opposite external box. Most of the animals quickly learned to go straight to the opposite direction as this reinforcement schedule was counterbalanced (all animals were reinforced for moving in both directions).

On experimental days, animals were driven to the experimental room 1 h before the start. Firstly, we assessed dominance (direct dominance experiment). Each rat was faced with 3 different unknown rats of the same Sex, similar weight, but from a different dam, different home cage (completely unknown) and opposite Treatment condition. Experimental animals' order was counter-balanced 1 exposed followed by one control and so on. Each animal "fought" 3 consecutive times against the same animal. Following this, a rest period g was set for the next fight (between 30 and 45 min). This created a total of 9 matches for each rat. All of the 85 adult rats completed this protocol.

Although the first dominance test was designed to study the direct influences of CPF on dominance, it does not provide us with information on well-established hierarchies, transitivity, and paradigm validation. Given that this information is critical for studying the validation of the paradigm throughout transitivity (when animal A beats B, and B beats C, A must beat C), we proceeded to analyze pre-established hierarchies (dominance and situation in the hierarchy of the rats from the same home cage) (well-established hierarchies experiment). Because of this, we only used animals from n = 4 home cages. The total sample in this case was 72 rats (36 females −16 exposed to CPF- and 36 males −19 exposed to CPF). Each animal "fought" against the other three co-habitants 3 times each, thus creating a final number of 9 matches for each animal. Room temperature, humidity, light conditions, and experimental hours were as described previously.

2.4. Molecular analyses

2.4.1. Sacrifice protocol

Two days after completion of the behavioral procedures, a

representative subsample of 5 animals per group (randomly selected) were sacrificed, with 16, 21, 35 days old and 7 months of age. Briefly, rats were sacrificed by fast decapitation and blood was collected into a PYREX tube covered with 2,2′,2″,2‴-(Ethane-1,2-diyldinitrilo) tetraacetic acid (EDTA). While one of the experimenters processed the blood samples for plasma extraction (3500 rpm for 20 min at 4 °C), another took stool samples from the whole gut, and these were then flash frozen. All the samples were then stored at −80 °C until use.

2.4.2. Gut microbiota composition (20 days after exposure)

The total stool was removed from −80 °C and quickly mixed (Heidolph RZR1) in order to obtain a proper representation of the whole microbiome. 100 mg was taken and the genomic DNA (gDNA) isolation was conducted following the company's instructions (PureLinkTM Microbiome DNA purification kit). Samples were stored at −80 °C and later analyzed in an external laboratory by blind -to both sex and treatment-technicians (STABvida, Portugal). The quality of the samples was checked with gel electrophoresis (1% agarose gel) and a Phred quality score was recorded at each amplification cycle. gDNA was quantified by fluorometry (Qubit). Following this, the 16S rRNA V3 and V4 regions were amplified, the library was completed following the Illumina 16S Metagenomic Library preparation, and sequencing (250bp paired-end sequencing reads) was conducted in the MISeq reagent Kit v2 in the Illumina MiSeq platform (for a deep revision on Illumina systems, please see Garrido-Cárdenas et al. 2017). Finally, initial Pass Filtered sequence reads were classified with Illumina 16S metagenomics workflow at the different taxonomic levels. The secondary dependent variables analyzed in the present study were: 1. Species diversity, analyzed by Shannon Species Diversity Classification (Index used to characterize species diversity in a specific community) and 2. Total number of detected species. The main dependent variable was the relative abundance (percentage of bacteria from the whole microbiome that belongs to a specific family or category) at genus and species taxonomic category. The relative abundance of some of the most important bacteria from the phylum level were also analyzed as some of them (i.e. Firmicutes and Bacteroidetes) have been systematically linked to ASD and other developmental pathologies. A total of 20 animals (10 females, half of each sex exposed to CPF) were randomly selected for this analysis, with 35 days of age.

2.4.3. Plasma NMR metabolomomics (24h, 6 days, 35 days and 6 and a half months after exposure)

All chemical reagents used were of analytical grade. D_2O (99.9%) was purchased from Eurisotop and NaCl was purchased from Sigma Aldrich. The samples were prepared according to Beckonert et al. (2007) with some modifications. Briefly, 200 μL of blood plasma was mixed with 400 μL of D_2O containing 0.9% NaCl. The resulting mixture was centrifuged for 5 min at 13500 rpm. 500 μL of supernatant was transferred into an oven-dried 5 mm NMR tube for the analysis.

^{1}H NMR spectra of serum samples were obtained at 600 MHz on a Bruker Avance III HD 600 spectrometer, equipped with a 5 mm QCI quadruple resonance pulse field gradient cryoprobe and a thermostat-controlled sample case with 24 positions. The water-suppressed Carr-Purcell-Meibom-Gill (CPMG) pulse sequence with a total spin echo delay of 96 ms was used to attenuate broad signals from lipoprotein or protein signals. All samples were measured at 293 ± 0.1 K, without rotation and using 16 dummy scans prior to the 180 acquired scans. The spectrometer transmitter was locked to D_2O frequency using a mixture of H_2O-D_2O (9:1). Acquisition parameters were set as follows: size of fid = 32K, spectral width = 22.0 ppm, acquisition time = 1.24 s, relaxation delay = 3 s, number of loops = 120, spin-echo delay = 400 μs, line broadening = 0.3 Hz, receiver gain = 50.8. All spectra were automatically phased, baseline-corrected, and calibrated to the anomeric proton signal of glucose at δ_H 5.23 ppm. Acquisition and processing of NMR spectra were carried out by the TOPSPIN software (version 3.6).

All NMR spectra were phased, baseline corrected, and then data reduced to 250 integrated regions of equal width of 0.04 ppm corresponding to the region of δ_H 0.5 to 10.5 using the Amix 3.9.4 software (Bruker Biospin GmbH). The regions of δ_H 5.18 to 4.34 ppm, of δ_H 3.70 to 3.46 and of δ_H 3.34 to 3.02 ppm were excluded from the bucketing process to remove artifacts of residual water and EDTA resonances. The area for each segmented region of chemical shift (bucket) was calculated, and the integral values contributed to an intensity distribution of the whole spectrum. Scaling the intensity of individual peaks to the total intensity recorded in the defined regions reduced any significant concentration differences from individual animals. Bucket tables were imported into the SIMCA-P software version 14.0 (Umetrics) for multivariate statistical analysis. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) models were scaled to pareto and unit variance, respectively. 80 rat plasma samples from four sampling time points (20 rats per time point, 10 females, half of each sex exposed to CPF) PND16, 21, 35 and 209 -PNM7-were analyzed by ^{1}H NMR spectroscopy.

2.5. Statistical analyses

For social behavior, repeated measures analysis of variance (ANOVA) for both contact and approximation zones were conducted with the within-subject factor of *Index* (two levels, social Index, and novelty Index) and the between-subject factors of *Sex* and *Treatment* (CPF and control). For locomotor control, individual two-way ANOVAs at each phase were conducted with these factors. For dominance and hierarchy status, the average number of won fights per animal (as a percentage) was analyzed using a further two-way ANOVA. For the influence of dominance on social behavior, the previously indicated ANOVA was conducted but adding the third-factor *Dominance* derived from the dominance experiment (unknown rivals). For gut microbiota composition, Shannon species diversity Index, percentage of successful reads at each taxonomic level, the relative abundance of the 5 most important bacteria at phylum level as well as the relative abundance of each bacteria at genus were analyzed with individual two-way ANOVAs. When significant at the genus level, a Multivariate ANOVA was also conducted on the significant genus by taking all the species that comprised the specific genus. When significant, down-stream univariate analyses were carried out in order to identify which specific species accounted for the significant effect of genus. For all of these analyses, *post hoc* pair-wise comparisons were chosen. All the analyses were conducted with SPSS v25. Statistical significance was set at $p \leq 0.05$. The data are represented in terms of means and SEM in the various figures and tables. For metabolomics analysis, Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) modeling, these were carried out using SIMCA-P.

3. Results

A subsample of 5 animals per group from this cohort was used for ChEs and AChE activity analysis. CPF exposure did not significatively inhibit total ChE (Perez-Fernandez et al., 2019) and AChE (unpublished results) activity at the frontal cortex 24 h following the final exposure. There were no significant differences between animals in terms of ocular opening or weight during development. Furthermore, weight was not affected by CPF exposure throughout the life span (data not shown). The data of all the 60 adolescent and 85 adult rats were included in the final statistical analyses for sociability, locomotor, and direct dominance (vs. unknown rivals of the opposite treatment group). In the case of gut microbiome, 19/20 animals' stool samples were finally included in the statistical analysis because the gDNA sample of one female exposed to CPF had not enough quality to be processed by the external laboratory. In the case of the metabolomic analyses, plasma sample from all the **80** animals were included.

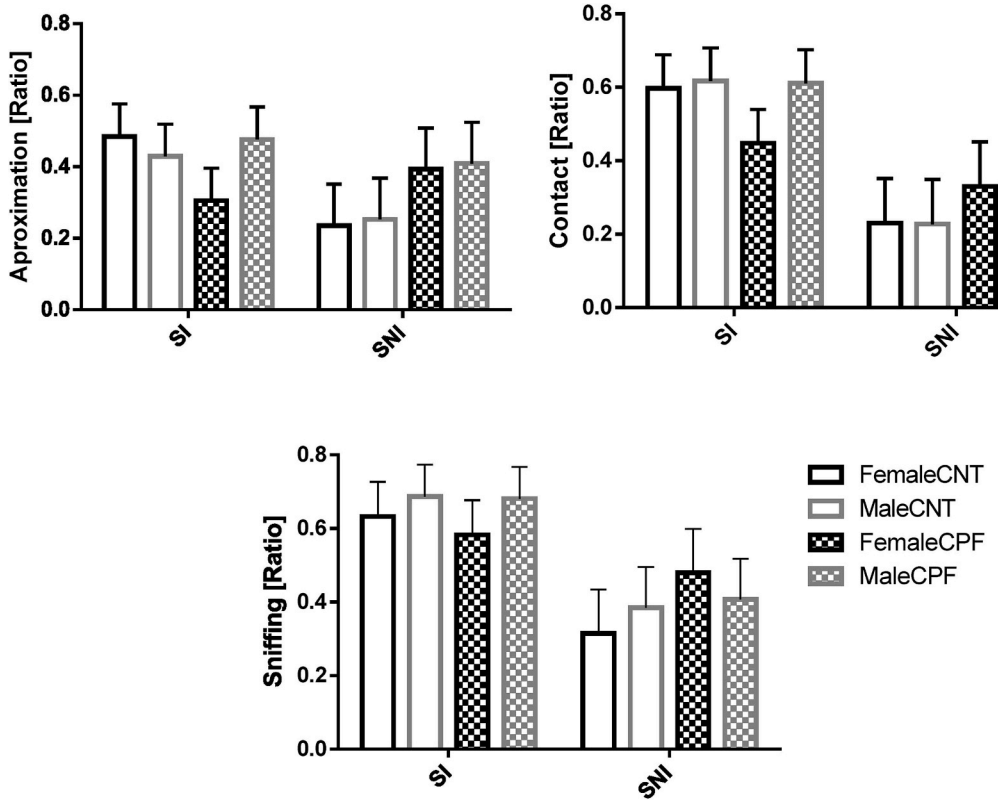

Fig. 1. CPF influences on sociability during adolescence. Sociability (SI) and reaction to social novelty (SNI) indexes in approximation zone (a, up-left), contact zone (b, up-right) and sniffing behavior (down) in adolescent rats. Data are expressed by means and SEM.

3.1. Behavioral outcomes

3.1.1. CPF & sociability

Adult rats generally decreased their sociability in the reaction to social novelty phase, showing a significant *Index x Sex* × *Treatment* interaction in the approximation zone [F (1,81) = 5.564, p = 0.021] (Fig. 2a). Post hoc analysis revealed that both control females and exposed males strongly decreased their reaction to novelty exploration in relation to their own rates at the social stage (p = 0.004 and 0.005, respectively), something that was not observed in their exposed (females) and control (males) counterparts (p = 0.937 and 0.351, respectively). There were not further significant effects concerning *Sex*, *Treatment* or their interaction neither in adults nor adolescent rats in approximation, contact or sniffing behavior (Fig. 1a, b and c and Fig. 2b and c).

3.1.2. CPF & dominance

When given a tube test with direct matches between CPF and unknown control animals, CPF rats had a similar percentage of victories compared to control animals (Fig. 3). No significant effects were found for either *Treatment* or the *Sex* × *Treatment* interaction. A parallel analysis of well-established social hierarchies to study the validity of the test revealed high rates of transitivity of females (92%) but only moderate rates for males (64%). Similarly, a specific analysis of these well-established hierarchies also revealed no significant effects of either *Treatment* or the *Sex* × *Treatment* interaction (Supplementary Fig. 1). Interestingly, when comparing both models (dominance with unknown and hierarchy with well-known animals) CPF exposed males showed enhanced dominance when faced with a known rat (Supplementary Fig. 2). However, the ANOVA revealed only a marginally significant *Sex* × *Treatment* interaction. Since not all the animals assessed by Crawley's test were included in the hierarchy analysis (only cages of 4 animals were included), the final dominance status was extracted from the direct dominance test.

3.1.3. Reaction to social novelty & dominance

Adult rats were labeled as dominant or non-dominant (submissive) when their percentage of victories against unknown animals was > (dominant) or < (non-dominant) 50%. This factor was introduced in the reaction to social novelty analysis. The significant decrease of the reaction to social novelty in adulthood was completely explained by dominance status, where dominant animals did not react to the novelty (Fig. 4a, b and c). the rmANOVA showed significant main effects of *Dominance* [F (1,77) = 7.040, p = 0.010; F (1,77) = 6.308, p = 0.014; F (1,73) = 10.239, p = 0.002 for approximation and contact zones and sniffing behavior, respectively] and the *Index* × *Dominance* interaction [F (1,77) = 8.025, p = 0.006; F (1,77) = 7.983, p = 0.006; F (1,73 = 13.781, p < 0.001)]. Post hoc analysis revealed that both dominant and submissive rats had similar sociability indexes during the social phase. However, the dominant rats drastically reduced their reaction to social novelty Index compared with the non-dominant rats (p = 0.001; p = 0.002; p < 0.001 for contact and approximation zones and sniffing behavior, respectively) as well as their own index profile during the social phase (p < 0.001 both zones and sniffing behavior). No further significant differences were found concerning the exposure condition, *Sex* or any other interaction.

3.1.4. CPF & locomotor activity

Four variables were studied in order to rule out the possibility that "social" differences could be due to motor alterations following CPF exposure. In this regard, total time of movement, distance traveled, mean velocity and rearing frequencies did not produce significant main effects of *Treatment* or a *Sex* × *Treatment* interaction at any phase (habituation, social, and novelty phases) for either adolescent or adult rats (Tables 1 and 2).

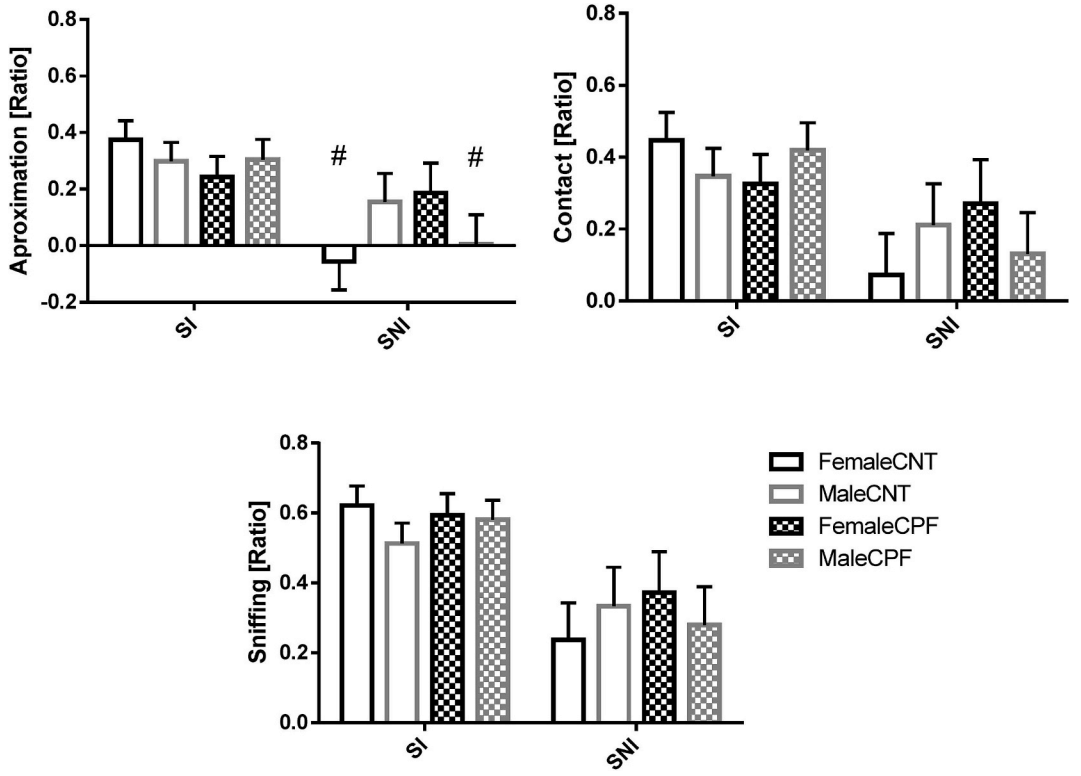

Fig. 2. CPF influences on sociability during adulthood. Sociability (SI) and reaction to social novelty (SNI) indexes in approximation zone (a, up-left), contact zone (b, up-right) and sniffing behavior (down) in adult rats. Data are expressed by means and SEM. # means significant differences ($p < 0.05$) in SNI from the respective SI values.

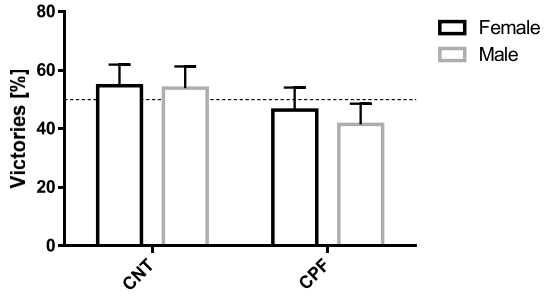

Fig. 3. Dominance Test. Percentage of victories after 9 matches versus unknown animals. Data are expressed by means and SEM.

3.2. Molecular outcomes

3.2.1. CPF & gut microbiota composition

The analysis of total number of species and the Shannon species diversity did not reveal significant effects of CPF exposure for *Treatment* or a *Sex* × *Treatment* interaction. A significant effect of *Sex* was found in Shannon's species diversity with higher rates in male rats [$F(1,15) = 4.861$, $p = 0.043$]. The analysis of the percentage of the passed filters successful reads at each taxonomic category revealed no significant effects of *Treatment* or a *Sex* × *Treatment* interaction. No significant differences were found for any bacteria at phylum (Fig. 5).

Otherwise, CPF induced an important dysbiosis at the taxonomic categories of genus and species (Tables 3 and 4). At the genus level, CPF exposure generally reduced the relative abundance of most of the significant bacteria (13 out of 16). Of these, Moryella, Slackia, Aggregibacter, and Caldicellulisiruptor were the most abundant and thus the most important in terms of their influence on general microbiome functioning. Although Moryella showed no significant differences at post hoc analysis, the relative abundance of Slackia and Aggregibacter was found to increase as a result of CPF exposure. Regarding the *Sex* × *Treatment* interaction, most of the significant effects were derived from the decreased relative abundance of the various bacteria in exposed males (Rhodosphirilum, Actinobaculum, and Phascolarctobacterium) and females (Amaricocus, Chondromyces, and Zhouia) compared with their respective controls.

The relative abundance of 9 bacteria was significantly altered by CPF exposure and 8 were affected in a sex-dimorphic manner at the species level. The relative abundance of all except Carboxydocella Ferrireducens was reduced in the CPF group. In terms of Sex influences on CPF exposure, we found decreased Actinobacculum Suis and Phascolarctobacterium Suiccinatutens in CPF exposed males and Chondromyces Pediculatus and Zhouia Amyolitica in exposed females. Interestingly, CPF exposure increased the relative abundance of unspecific Mycoplasma in males compared with controls.

3.2.2. CPF & metabolomic and lipid profile

Chemical shifts, signal multiplicities and coupling constants of the metabolites identified in plasma samples are detailed in Supplementary Table 1. Metabolites mainly belong to the following classes: amino acids, lipids, nucleic acids derivatives, organic acids, and carbohydrates. Metabolite identification was achieved thanks to the BBIOREFCODE 2 database from Bruker, public NMR databases such as COLMAR (Bingol et al., 2014)) and HMDB (Wishart et al., 2018) and the literature (MacIntrye et al. 2010; Wang et al., 2009, 2012; Stringer et al., 2011).

The PCA was conducted on the ^{1}H NMR data for visualizing major trends in high-throughput datasets. PCA scores plot that groups similar samples based on the input data, and PCA loadings plot that indicates which spectral areas (or buckets) contribute more to the variation between groups were generated. The PCA scores and loadings plots for the NMR spectra at the four sampling time points are shown in Supplementary Fig. 3a and b, respectively. Supplementary Fig. 3a

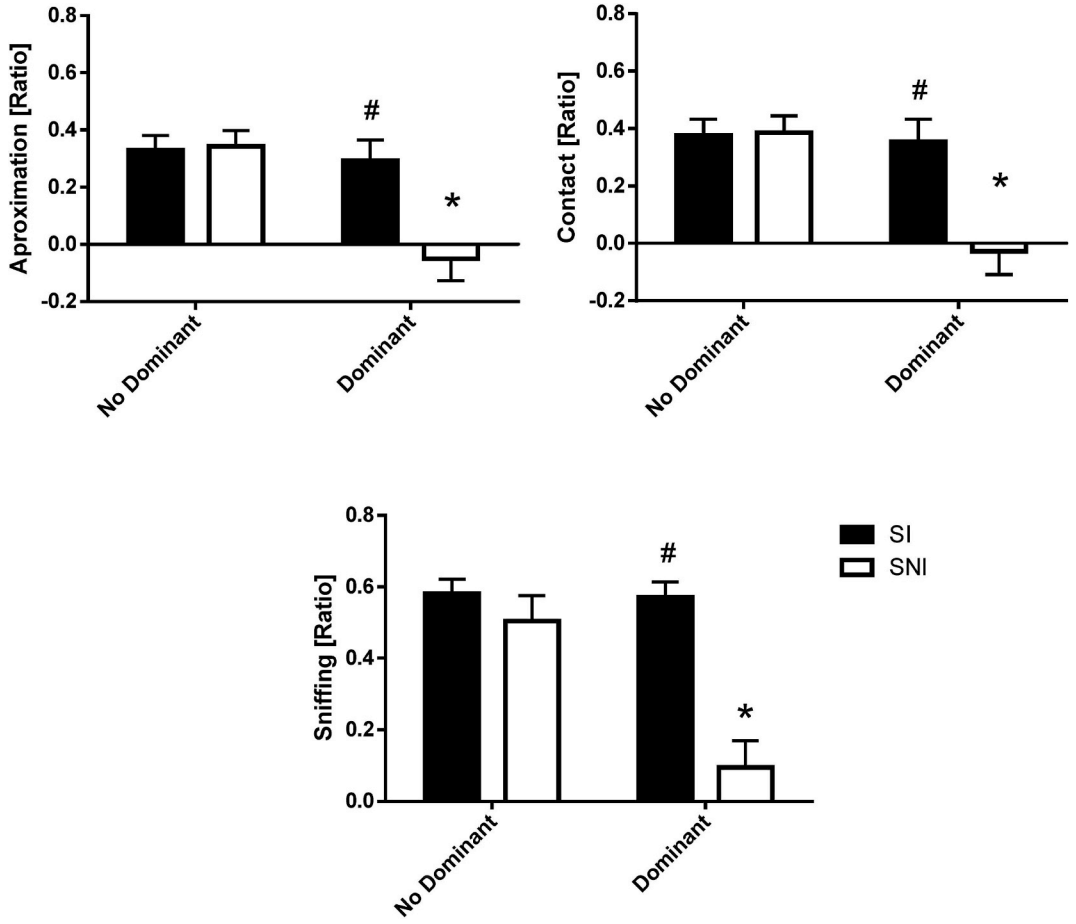

Fig. 4. Influences of dominance on the social traits. Influences of dominance status on sociability (SI) and reaction to social novelty (SNI) indexes in approximation zone (a, up-left) and contact zone (b, up-right) **and sniffing behavior (c, down)** in adult rats. Data are expressed by means and SEM. * means significant differences ($p < 0.05$) between dominant and no dominant rats in SNI. # means significant differences ($p < 0.05$) between both indexes in dominant animals.

displays a clear discrimination of rat plasma samples into four groups based on the aging process, regardless of the administration of the CPF, with PC1 and PC2 explaining 58.3% and 11.3% of the total variance, respectively. Supplementary Fig. 3b shows the discriminant buckets that correlate with the aging process in the whole set of rat plasma samples analyzed. Rat plasma samples from PNM7, because of the aging process, presented higher amounts of lactic acid, fatty acids (FA), unsaturated fatty acids (UFA), glucose, low and very low-density lipoproteins (LDL and VLDL).

PCA was then applied to the NMR data for each sampling time point. Additionally, the effect of CPF exposure was assessed for male or female rats (Supplementary Figs. 4a–d). Analyzing the PCA score plots, no difference was observed between exposed and control groups, except for female rats at PNM7. Variable importance in projection (VIP) scores from a partial least squares discriminant analysis (PLS-DA) was obtained to select the most discriminant variables responsible for the differences among the exposed and control samples from female rats at PNM7. The PLS-DA scores plot and VIP-scores are shown in Fig. 6a and

Table 1
CPF exposure on locomotor activity (adolescence). Locomotor activity of 4 different outcomes in every phase of the Crawley's test in adolescent rats. Data are expressed by means and SEM. Two-way ANOVA results from *Treatment* and *Sex x Treatment* are defined.

	Female control	Male control	Female CPF	Male CPF	Two-way ANOVA *Treatment*	Two-way ANOVA *Sex x Treatment*
			Phase 1. Habituation			
Time in movement	108.1 ± 3.84	129.6 ± 3.87	114.7 ± 4.76	127.2 ± 4.5	$F(1,56) = 0.242$, $p = 0.624$	$F(1,56) = 1.126$, $p = 0.293$
Distance traveled	3232.8 ± 236.85	2849.4 ± 67.73	3200.5 ± 162.84	3078.6 ± 107.71	$F(1,56) = 0.393$, $p = 0.534$	$F(1,56) = 0.692$, $p = 0.409$
Mean velocity	11.5 ± 0.77	10 ± 0.31	11.6 ± 0.52	11.1 ± 0.41	$F(1,56) = 1.288$, $p = 0.261$	$F(1,56) = 0.803$, $p = 0.374$
Rearing frequency	58.3 ± 3.78	58.3 ± 4.38	61.9 ± 3.82	67.3 ± 3.43	$F(1,56) = 2.655$, $p = 0.109$	$F(1,56) = 0.488$, $p = 0.488$
			Phase 2. Social interaction			
Time in movement	201.2 ± 11.67	208.1 ± 14.10	207.3 ± 17	202.2 ± 8.59	$F(1,56) < 0.001$, $p = 0.995$	$F(1,56) = 0.210$, $p = 0.649$
Distance Traveled	3613.5 ± 671.25	2571.5 ± 99.86	3168.2 ± 303.12	2689.3 ± 165.89	$F(1,56) = 0.185$, $p = 0.669$	$F(1,56) = 0.547$, $p = 0.463$
Mean velocity	6.3 ± 1.10	4.4 ± 0.18	5.6 ± 0.57	4.6 ± 0.29	$F(1,56) = 0.133$, $p = 0.717$	$F(1,56) = 0.486$, $p = 0.489$
Rearing frequency	77.6 ± 6.81	71.9 ± 6.30	77.2 ± 9.12	64.9 ± 5.33	$F(1,56) = 0.282$, $p = 0.597$	$F(1,56) = 0.225$, $p = 0.637$
			Phase 3. Reaction to social novelty			
Time in movement	201.2 ± 9.42	209.2 ± 26.87	170.6 ± 16.67	221 ± 23.67	$F(1,56) = 0.215$, $p = 0.645$	$F(1,56) = 1.090$, $p = 0.301$
Distance Traveled	2914.7 ± 566.17	2081.8 ± 170.31	2182.8 ± 257.96	2346.8 ± 241.14	$F(1,56) = 0.460$, $p = 0.501$	$F(1,56) = 2.095$, $p = 0.153$
Mean velocity	5.2 ± 0.92	3.7 ± 0.31	4 ± 0.53	4.3 ± 0.43	$F(1,56) = 0.176$, $p = 0.676$	$F(1,56) = 2.393$, $p = 0.127$
Rearing frequency	72.9 ± 6.92	64.3 ± 9.17	65 ± 8.58	69.4 ± 9.72	$F(1,56) = 0.027$, $p = 0.869$	$F(1,56) = 0.563$, $p = 0.456$

Table 2
CPF exposure on locomotor activity (adulthood). Locomotor activity of 4 different outcomes in every phase of the Crawley's test in adult rats. Data are expressed by means and SEM. Two-way ANOVA results from *Treatment* and *Sex x Treatment* are defined.

	Female control	Male control	Female CPF	Male CPF	Two-way ANOVA *Treatment*	Two-way ANOVA *Sex x Treatment*
			Phase 1. Habituation			
Time in movement	160.3 ± 3.76	154.5 ± 4.63	167.3 ± 4.81	154.9 ± 5.93	F (1,81) = 0.560, p = 0.456	F (1,81) = 0.462, p = 0.499
Distance Traveled	2691.8 ± 87.7	2318.8 ± 80.74	3131.7 ± 279.64	2347.2 ± 68.85	F (1,81) = 2.650, p = 0.107	F (1,81) = 2.046, p = 0.156
Mean velocity	9 ± 0.30	7.7 ± 0.27	10.5 ± 0.93	7.8 ± 0.23	F (1,81) = 2.672, p = 0.106	F (1,81) = 2.040, p = 0.157
Rearing frequency	52.5 ± 3.58	41.2 ± 2.24	53.7 ± 3.46	42 ± 2.74	F (1,81) = 0.108, p = 0.744	F (1,81) = 0.009, p = 0.927
			Phase 2. Social interaction			
Time in movement	215.1 ± 15.47	183 ± 13.93	220.8 ± 10.91	160.2 ± 16.63	F (1,81) = 0.333, p = 0.566	F (1,81) = 0.929, p = 0.338
Distance Traveled	3156.4 ± 220.36	2391.1 ± 138.24	3775.1 ± 424.35	2212.8 ± 181	F (1,81) = 0.765, p = 0.384	F (1,81) = 2.504, p = 0.117
Mean velocity	5.3 ± 0.37	4 ± 0.23	6.3 ± 0.71	3.7 ± 0.30	F (1,81) = 0.753, p = 0.388	F (1,81) = 2.506, p = 0.117
Rearing frequency	59 ± 5.90	42.1 ± 3.90	60.6 ± 4.6	34.5 ± 3.81	F (1,81) = 0.425, p = 0.516	F (1,81) = 0.981, p = 0.325
			Phase 3. Reaction to social novelty			
Time in movement	148.8 ± 16.70	128.2 ± 20.06	160.6 ± 12.65	140 ± 19.85	F (1,81) = 0.434, p = 0.512	F (1,81) < 0.001, p = 0.997
Distance Traveled	2356.2 ± 272.34	1528.6 ± 140.62	3050.7 ± 665.10	1978.3 ± 279.41	F (1,81) = 2.402, p = 0.125	F (1,81) = 0.110, p = 0.741
Mean velocity	4 ± 0.46	2.6 ± 0.24	5.1 ± 1.11	3.3 ± 0.47	F (1,81) = 2,348, p = 0.107	F (1,81) = 0.100, p = 0.753
Rearing frequency	41.7 ± 6.39	26.2 ± 4.20	41 ± 4.49	31 ± 4.85	F (1,81) = 0.151, p = 0.699	F (1,81) = 0.292, p = 0.590

b, respectively. Fig. 6a shows that plasma samples from female rats at PNM7 can be distinguished into two groups due to the administration of CPF. The PLS-DA model was validated by the permutation test. VIP's value expresses the contribution of the individual variables in the definition of the F-latent vector model. Due to the normalization applied in the definition of the VIP, discriminant buckets showing values above 1 were considered to contribute significantly to the discrimination (Fig. 6b).

The metabolites whose NMR signals are contained in these buckets of largest VIP coefficients, and therefore contributing more significantly to the discrimination, are shown in Table 5. In terms of the female rat metabolome, CPF exposure produced an increase of LDL/VDL, N-acetylglycoprotein (NAc), FA and UFA at PNM7. However, the administration of CPF reduced the levels of glucose, the organic acids citrate and lactate, and the amino acids glutamine, alanine, leucine, and serine.

4. Discussion

1 mg/kg of CPF for 6 consecutive days at the late postnatal preweaning developmental stage induced a modest alteration in the reaction to social novelty in adulthood by both enhancing (females) and decreasing (males) novelty exploring indices in relation to their respective social scores. It induced medium-term effects on gut dysbiosis and a hyperlipidemic, hypoglycemic/hypogluconeogenesis profile in adult females. This is the first time that these behavioral and molecular findings have been linked to this exposure protocol. We also found an important implication of dominance status on reaction to social novelty behavior, with important implications in future uses of the Crawley test. All these effects were found at doses without systemic toxicity and unrelated to ChEs inhibition in the CNS (Perez-Fernandez et al., 2019).

4.1. Influences of CPF exposure on behavioral outcomes

Exposure did not affect sociability or reaction to social novelty skills in adolescent rats, but exposed animals increased (females) and decreased (males) their reaction to social novelty indexes in relation to the social scores in adulthood. Both control females and exposed males significantly decreased their novelty rates from the social phase. CPF exposure blocked this in females, producing a similar behavior in both phases. The subtle reduction at the novelty phase found in male controls was stronger in exposed males, although given that the differences between groups at each phase did not reach significance, this conclusion must be treated with caution. In fact, the weakness of this data is confirmed by the lack of significant effects of the exposure condition in the closer interactions (time in contact zone), active exploratory behavior (sniffing) included.

Adult control females did not react to social novelty when the approximation zone was analyzed. This unexpected behavior could reflect some degree of attachment to the familiar rat, a general lack of reaction to novelty, or both. However, the influence of the modulations done to the Crawley paradigm (open field version) in the present study could

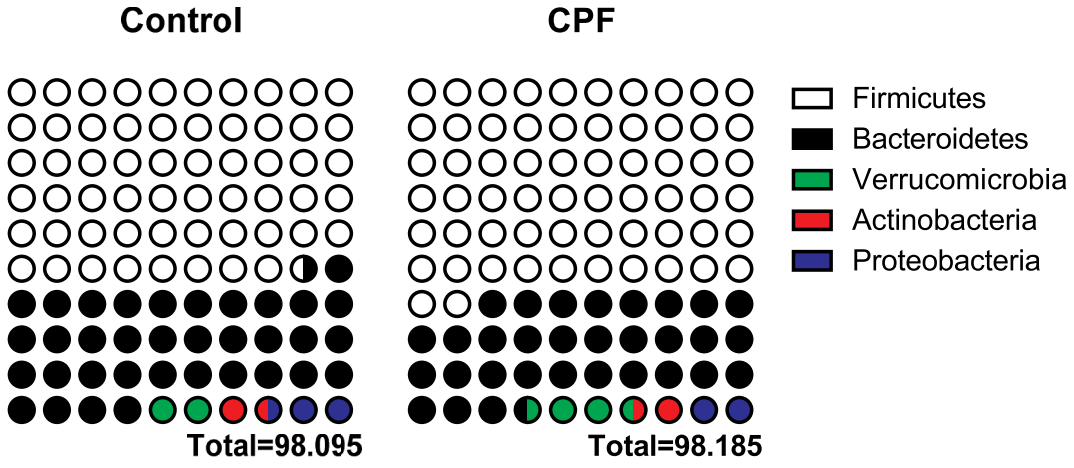

Fig. 5. Influences of CPF exposure on gut microbiome (phylum). Relative abundance of the 5 most important bacteria in the phylum taxonomic category in both control and CPF animals.

Table 3
CPF influences on gut microbiome (Genus). Significantly altered bacteria at the genus taxonomic category both *Treatment* and *Sex* × *Treatment* interaction. + and – means higher or lower relative abundance in CPF exposed rats. CNT/M = control male, CNT/F = control female, CPF/M = exposed male and CPF/F = exposed female. N.S. means no significant differences at post hoc. Bacteria is scheduled based on the relative abundance average (‰).

Genus	Factor	Two-way ANOVA	Outcome (Post Hoc)	Relative Abundance (‰)
Moryella	S*T	F (1,15) = 5.401, p = 0.035	N.S.	28.02662711
Slackia	T	F (1,15) = 4.644, p = 0.048	+	23.70090868
Aggregatibacter	T	F (1,15) = 4.592, p = 0.049	+	1.37064989
Caldicellulosiruptor	T	F (1,15) = 12.676, p = 0.003	–	1.06289442
Rodhosphirillum	T	F (1,15) = 9.446, p = 0.008	–	0.98866895
	S*T	F (1,15) = 6.257, p = 0.024	CNT/M + CNT/F; CNT/M + CPF/M	0.98866895
Neorickettsia	T	F (1,15) = 10.576, p = 0.005	–	0.70328963
Mycoplasma	T	F (1,15) = 5.971, p = 0.027	–	0.63817684
Thiomonas	T	F (1,15) = 5.227, p = 0.037	–	0.32625821
Helicobacter	T	F (1,15) = 12.413, p = 0.003	–	0.206281
Methylobacterium	T	F (1,15) = 7.908, p = 0.013	–	0.18126758
Ehrlichia	T	F (1,15) = 7.450, p = 0.016	–	0.15871684
Rhodobacter	T	F (1,15) = ,5.011 p = 0.041	–	0.15044579
Saccaropolyspora	T	F (1,15) = 10.667, p = 0.005	–	0.14010784
Carboxydocella	T	F (1,15) = 4.871, p = 0.043	+	0.13514595
Chlorobaculum	T	F (1,15) = 4.722, p = 0.046	–	0.10745932
Amaricoccus	S*T	F (1,15) = 8.098, p = 0.012	CNT/M – CNT/F; CNT/F + CPF/F	0.10648653
Zhouia	S*T	F (1,15) = 5.796, p = 0.029	CNT/M – CNT/F; CNT/F + CPF/F	0.08071653
Chondromyces	S*T	F (1,15) = 9.394, p = 0.008	CPF/M + CPF/F; CNT/F + CPF/F	0.04413932
Jeotgalicoccus	T	F (1,15) = 8.994, p = 0.009	–	0.02541116
Phascolarctobacterium	S*T	F (1,15) = 9.533, p = 0.008	CNT/M + CNT/F; CNT/M + CPF/M	0.01606011
Leptothrix	T	F (1,15) = 7.194, p = 0.017	–	0.01539174
Actinobaculum	S*T	F (1,15) = 6.557, p = 0.022	CNT/M + CNT/F; CNT/M + CPF/M	0.01164579
Ferrimicrobium	S*T	F (1,15) = 4.521, p = 0.05	N.S.	0.00972205

also be responsible of this behavioral pattern, although the normal behavior shown during adolescence could point towards other explanations. In fact, the active exploratory sniffing of the control females was closer to the expectations, although they still being the group with the lower reaction rate. Interestingly, CPF exposure blocked this effect in females. Previous studies have found enhanced sociability or reaction to social novelty following gestational CPF exposure in females using different paradigms of social investigation as well as ultrasound vocalizations (De Felice et al., 2014; Venerosi et al., 2006). Venerosi et al. (2006) found that this effect following gestational exposure was blocked by re-exposing the animals during the preweaning stage. To our special interest, Ricceri et al. (2003) found that low doses of CPF from PND11 to 14 enhanced social behaviors in both sexes and aggressive responses in males in late adolescence. These authors found that CPF exposure also increased the rate of reaction to novelty. This decreased "fear" in response to novelty could be partially explained by anxiety modulation following CPF exposure during this period (Ricceri et al., 2006). This latter study also found that preweaning exposure to low doses of CPF increased both maternal behaviors in females and aggression in males and CPF postnatal exposure influence depends on previous gestation administration. Increased maternal responses in female rats following preweaning CPF exposure were also found, along with decreased anxiety in females (Venerosi et al., 2008).

Both direct dominance and hierarchy status were unaffected by CPF exposure, but the influence of dominance status on reaction to social novelty rates was relevant, as the animals that showed a dominant profile showed a reduced reaction to social novelty, non-observed in submissive rats. This is the first time that dominance status has been demonstrated to be an essential factor in the regulation of the reaction to social novelty. However, Jupp et al. (2016) found the opposite effect but in terms of reaction to a novel environment, where dominant rats did show higher rates of motricity. Nevertheless, this observation of a decrease in social skills in dominant animals could be linked to the findings of a two other studies with monkeys (Czoty et al., 2010; Riddick et al., 2009). From our perspective, the opposite results found in Jupp et al. (2016) versus these two studies with monkeys and the present work with rats could be the nature of the stimulus and are not necessary contradictory. That is, from an ethological perspective, it could have sense that dominant animals are prone to extensively explore novel environments but avoid interaction with novel stimuli. Thus, the present study would extend this last category to the social dimension. However, the dominance status in the present study was obtained exclusively from the tube test, which also suppose a limitation for the final conclusions.

4.2. Influences of CPF exposure on molecular outcomes

CPF induced an important dysbiosis in both genus and species levels, showing the influence of CPF exposure on multitude bacteria, which has never before been linked to this OP. Exposure to CPF has been linked to an increase in different bacteria at family, genus and species levels (Joly Condette et al., 2013, 2015; Fang et al., 2018; Zhao et al., 2016; Reygner et al., 2016). However, we failed to confirm these modulations following the preweaning exposure protocol described here. The specific bacteria population affected following this exposure protocol differently evolved by age, as showed in recent reports using samples extracted 6 months after exposure (Perez-Fernandez et al., 2019). Both Slackia (increased) at the genus and unspecific Caldicellulosiruptor (decreased) at species levels were the most important affected bacteria. Slackia bacteria have recently been associated with hyperlipidemia in colorectal cancer patients (Han et al., 2019), along with this type of cancer in dogs (Herstad et al., 2018). However, this association has not been systematically found (Kasai et al., 2016). Furthermore, enriched Slackia exigua species was also found in other types of cancer (Coker et al., 2018). Caldicellulosiruptor is an anaerobic, Gram-positive bacterium known for its ability to degrade complex carbohydrates such as cellulose (Ozdemir et al., 2012). However, its implication for health and behavior regulation is unknown as well as its interaction with CPF, OPs or any other inexistent xenobiotics. Regarding the remaining significant, less abundant bacteria found in the present study, there are no known implications for health, and evidence for their associations with CPF exposure is sparse and, in most the cases, non-existent.

CPF also induced hyperlipidemic (increased plasma LDL, VLDL, fatty acids and unsaturated fatty acids levels), hypoglycemic/hypogluconeogenesis (decreased plasma glucose levels), and altered amino acid

Table 4
CPF influences on gut microbiome (Species). Significantly altered bacteria at species taxonomic category both *Treatment* and *Sex* × *Treatment* interaction. An initial multivariate ANOVA, when significant, lead to a two-way ANOVA and, when significant, post hoc analyses were carried out. + and – means higher or lower relative abundance in CPF exposed rats. CNT/M = control male, CNT/F = control female, CPF/M = exposed male and CPF/F = exposed female. N.S. means no significant differences at post hoc. N.A. means non-applicable. Bacteria is scheduled based on the relative abundance average (‰).

Genus	Specie	Factor	Multivariate ANOVA	Two-way ANOVA	Outcome (Post Hoc)	Relative Abundance (‰)
Moryella	Indoligenes	S*T	F (2,14) = 4.530, p = 0.030	F (1,15) = 5.411, p = 0.034	N.S.	28.0179673
Caldicellulosiruptor	Uns.	T	F (2,14) = 5.924, p = 0.014	F (1,15) = 12.688, p = 0.003	–	1.050833
Neorickettsia	Helminthoeca	T	N.A.	F (1,15) = 10.576, p = 0.005	–	0.70328963
Methylobacterium	Uns.	T	F (2,14) = 7.442, p = 0.006	F (1,15) = 11.046, p = 0.005	–	0.17451895
Ehrlichia	Ovina	T	N.A.	F (1,15) = 7.450, p = 0.016	–	0.15871684
Mycoplasma	Edwardii	T	F (9,7) = 3.788, p = 0.046	F (1,15) = 15.887, p = 0.001	–	0.15467358
Carboxydocella	Ferrireducens	T	N.A.	F (1,15) = 4.871, p = 0.043	+	0.13514595
Helicobacter	Suncus	T	F (2,14) = 6.849, p = 0.008	F (1,15) = 8.244, p = 0.012	–	0.10467958
Saccaropolyspora	Uns.	T	F (3,13) = 7.320, p = 0.004	F (1,15) = 8.671, p = 0.010	–	0.08702563
Zhouia	Amylolitica	S*T	N.A.	F (1,15) = 5.796, p = 0.029	CNT/M – CNT/F; CNT/F + CPF/F	0.08071653
	Uns.	S*T	F (9,7) = 4.809, p = 0.025	F (1,15) = 4.798, p = 0.045	CNT/M – CPF/M	0.06806442
Chondromyces	Pediculatus	S*T	N.A.	F (1,15) = 9.394, p = 0.008	CPF/M + CPF/F; CNT/F + CPF/F	0.04413932
	Haemominutum	S*T	N.A.	F (1,15) = 5.641, p = 0.031	N.S.	0.02638758
Phascolarctobacterium	Succinatutens	S*T	N.A.	F (1,15) = 9.533, p = 0.008	CNT/M + CNT/F; CNT/M + CPF/M	0.01606011
Leptothrix	Discophora	T	N.A.	F (1,15) = 7.194, p = 0.017	–	0.01539174
Actinobaculum	Suis	S*T	N.A.	F (1,15) = 6.557, p = 0.022	CNT/M + CNT/F; CNT/M + CPF/M	0.01164579
Ferrimicrobium	Acidiflum	S*T	N.A.	F (1,15) = 4.521, p = 0.05	N.S.	0.00972205

profiles in adult (PNM7) female rats, indicating a clear sex-dimorphic effect with females as the most vulnerable population target in terms of metabolites and the lipid system. Hyperlipidemia is a commonly found metabolic profile following OP exposure (Elsharkawy et al., 2013). As previously indicated, we only found one developmental study that showed this profile (Slotkin et al., 2005). The authors exposed animals during the early postnatal window (PND1 to 4) at the same dose that we used here but they did not find altered glucose at the serum level, and lipid alteration was focused on males. Thus, our study is the first developmental model that describes long-term lipidic and glucose alterations following early CPF exposure in female rats. In relation to CPF exposure during adulthood, middle-high doses of acute CPF in adult rats increased general levels of triglycerides, low-density lipoprotein, as well as decreased high-density lipoproteins (Acker and Nogueira, 2012). Interestingly, ASD patients have also been linked to abnormal lipid metabolism (Tierney et al., 2006; Shedlock et al., 2016).

Lipid metabolism is influenced by glucose levels and vice versa (Parhofer, 2015). Our hypoglycemia/hypogluconeogenesis pattern found in females contrasts with the most common hyperglycemic/gluconeogenesis profile following hepatic alterations (i.e. glycogen synthase modulation) following OP exposure (Rahimi and Abdollahi, 2007; Acker and Nogueira, 2012). Interestingly, a temporal decrease in serum levels of glucose and total triglycerides has recently been found in young adults following chronic exposure to low doses of CPF (0.3mg/kg/day) in fat-enriched diets (Fang et al., 2018). This could also be the consequence of a hypocorticosteronemia process, since the opposite has been linked to a hyperglycemic profile in previous studies (i.e. Acker and Nogueira, 2012). Other authors found that a ketogenic diet (rich in lipids but poor in carbohydrates) enhanced various ASD-like behaviors in asocial mice, including sociability (Ruskin et al., 2017). Only females that followed the ketogenic feeding regime exhibited a significant preference for Stranger 2. Ruskin's study and the present work support the notion that lower levels of glucose and higher levels of lipids are linked to an enhanced reaction to social novelty in female rats. Indeed, low glycemic diets have also been linked to an enhancement of autistic behaviors in ASD mice models (Currais et al., 2016).

This hypoglycemia/hypogluconeogenesis is congruent with the general alteration of energy production by basic elements such as lactate, alanine, and citrate (Cori, Cahill and Krebs cycles, respectively). Decreases in alanine can also generate a general reduction in hepatic production of glucose (Felig, 1973). Citrate is a product derived from the first reaction in the Krebs cycle, which converts oxaloacetate and acetyl CoA into citrate and CoA. Interestingly, the downregulation of ATP citrate synthase (which regulates this process) was previously found in a mixture of CPF and nickel (Boatti et al., 2012). Thus, the reduction of lactate, glucose, and citrate in exposed females is compatible with a general decrease in energy production. Different enzymes associated with the Krebs cycle were hypoactive following CPF exposure and cold stress application, supporting the notion of a decreased cellular metabolic rate at the CNS (Basha and Poojary, 2014).

Wang et al. (2009) examined the effects of CPF exposure on blood (serum) metabolites at low doses for male rats and found decreased levels of lactate, alanine, increased levels of NAc and no influence on glucose and different amino acids in exposed females. However, they found decreased levels of LDL/VLDL and increased glutamine levels in animals exposed to CPF. Both the present results and those of Wang's study suggest that low doses of CPF exposure have the potential risk of inducing neurotoxicity by disturbing cellular energy production and fatty acid metabolism, and in our case, this could be particularly true for females.

5. Conclusions and future guidelines

Taking all together, sub-chronic exposure to low doses of CPF during the late postnatal, preweaning developmental stage does not alter social behavior during adolescence and only modestly modulates

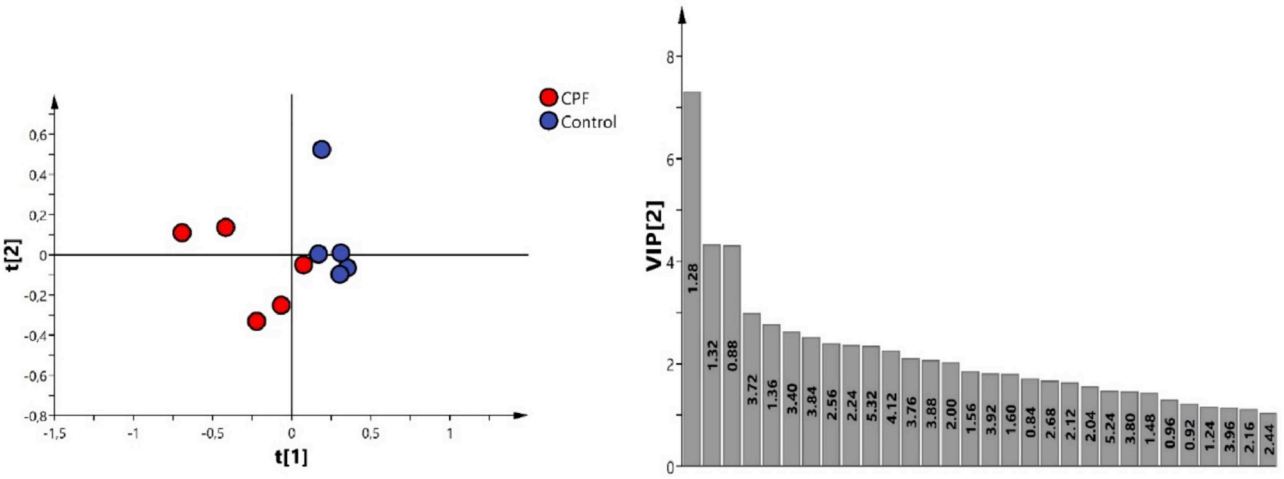

Fig. 6. Influences of CPF exposure on metabolic profile. a, left) PLS-DA scores plot generated from 1 H CPMG NMR data of plasma samples obtained from female rats belonging to the CPF-exposed group (red), and the control group (blue), at sampling point t4 (PNM7) and b, right) Variable importance in projection (VIP) scores plot obtained from the PLS-DA analysis (Var ID buckets), displaying the variables that most contribute to the discrimination observed between CPF-exposed and control plasma samples in female rats at sample point t4. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 5
CPF exposure influences on plasma metabolites. Metabolites that were shown to increase (+) or decrease (−) on plasma samples of female rats after CPF treatment at sampling point t4 (PNM7).

Bucket	Metabolite	Outcome
1.36, 1.28, 1.24, 0.92–0.84	LDL/VLDL	+
4.12; 1.32	Lactate	–
5.24, 3.92–3.72, 3.40	Glucose	–
2.68, 2.56	Citrate	–
2.24, 2.04, 2.00, 1.60, 1.56	FA	+
2.04	NAc	+
5.32	UFA	+
2.44, 2.16, 2.12	Glutamine	–
1.48	Alanine	–
0.96	Leucine	–
3.96	Serine	–

adult reactions to social novelty, with no effects on dominance status. Thus, the results regarding social behavior are limited and inconclusive, making it difficult to associate this exposure time and dosage with ASD. However, this exposure protocol altered gut microbiome composition at both genus and species taxonomic levels (two weeks after exposure) and induced a long-term (six and a half months after exposure) hyperlipidemic and hypoglycemic/hypogluconeogenesis profile with an apparent general decrease in cell energy production. In addition to CPF exposure, the present research also reveals a novel role for dominance in the reaction to social novelty. Although most of these results are novel and congruent with those in the existing literature, further research is needed in order to clarify the specific mechanisms underlying the alterations observed here, particularly for metabolite-related outcomes.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2020.109341.

Authors' declaration

All the authors have read the manuscript, agree that this work is ready for submission, and accept responsibility for its contents.

Authors' contribution

All the authors have contributed to this study. Mr. Cristian Perez-Fernandez completed the animal care, exposure protocol, behavioral tasks, gDNA extraction from stool samples, sacrifice protocol, statistical analyses and wrote the first version of the manuscript. Mr. Miguel Morales-Navas helped in all the behavioral tasks and statistical analyses as well as revised and improved the quality of the manuscript. Dr. José Miguel Aguilera-Saez, Dra. Ana Cristina Abreu and the professor Ignacio Fernández carried out (experimental procedure, data analyses, and figures and tables' design) the plasma metabolomic experiments and also revised the manuscript until its current form. Dra. Laia Guardia-Escote and Dr. José Antonio Garrido-Cárdenas helped in gut microbiome conceptualization and analysis as well as improved the quality of the manuscript. The professor Maria Teresa Colomina, Dra. Estela Giménez and the professor Fernando Sánchez-Santed set the original experimental design/conceptualization and hypothesis, supervised the experimental protocols and improved and create the final version of the manuscript.

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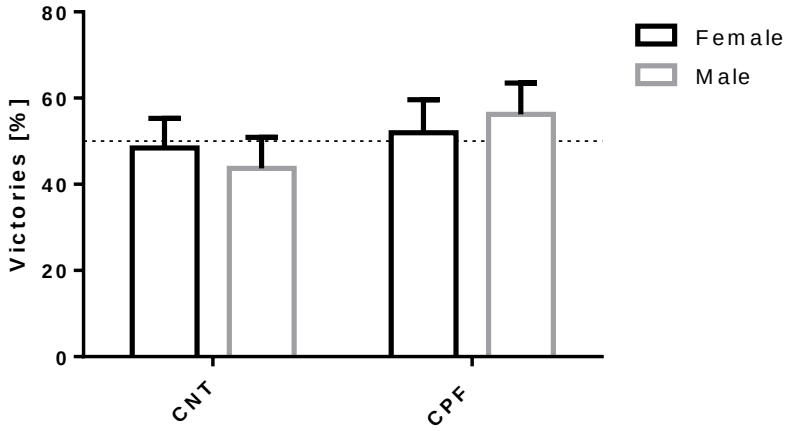
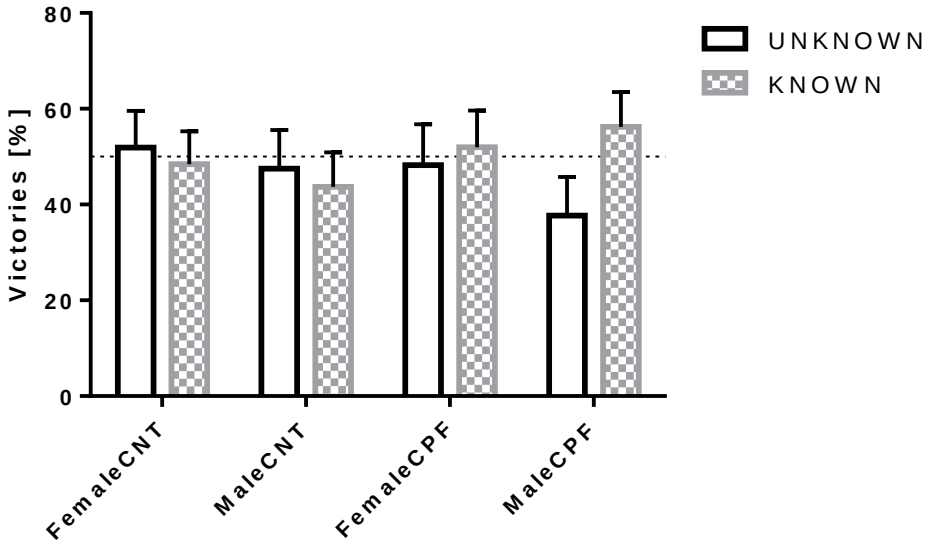
Supplementary Table 1

Number	Metabolite	Chemical shifts (ppm), multiplicities and coupling constants (Hz)
1	LDL/VLDL	0.82-0.91 (m), 1.21-1.38 (m)
2	Valine	0.98 (d, *J* = 7.1 Hz), 1.04 (d, *J* = 7.0 Hz)
3	Isoleucine	1.01 (d, *J* = 7.0 Hz), 0.95 (t, *J* = 7.2 Hz)
4	Leucine	0.96 (t, *J* = 6.3 Hz)
5	3-hydroxybutyrate	1.19 (d, *J* = 6.2 Hz), 2.30 (dd, *J* = 14.2 Hz, *J* = 6.2 Hz), 2.40 (dd, *J* = 14.2 Hz, *J* = 7.5 Hz)
6	Lactate	1.32 (d, *J* = 6.8 Hz), 4.11 (q, *J* = 6.8 Hz)
7	Alanine	1.47 (d, *J* = 7.2 Hz)
8	FA	1.54-1.62 (m), 1.97-2.04 (m), 2.21-2.26 (m)
9	Arginine	1.68-1.75 (m), 1.86-1.94 (m)
10	Acetate	1.91 (s)
11	Glutamate	2.04 (m), 2.13 (m), 2.35 (m)
12	NAc	2.04
13	Glutamine	2.13 (m), 2.44 (m)
14	Methionine	2.13 (s), 2.64 (t, *J* = 7.5 Hz)
15	Acetoacetate	2.27 (s)
16	Pyruvate	2.37 (s)
17	Succinate	2.40 (s)
18	Citrate	2.53 (d, *J* = 15.1 Hz), 2.65 (d, *J* = 15.1 Hz)
19	PUFA	2.78-2.83 (m)
20	Creatine	3.04 (s), 3.93 (s)
21	Choline	3.20 (s)
22	Serine	3.96 (m)
23	*β*-glucose	4.64 (d, *J* = 7.9 Hz)
24	*α*-glucose	5.23 (d, *J* = 3.7 Hz)
25	Glycine	3.55 (s)
26	UFA	5.25-5.35 (m)
27	2'-deoxyuridine	6.05 (d, *J* = 8.2 Hz), 6.27 (t, *J* = 6.7 Hz), 7.82 (d, *J* = 8.2 Hz)
28	Fumarate	6.52 (s)
29	Tyrosine	6.89 (d, *J* = 8.3 Hz), 7.19 (d, *J* = 8.3 Hz)
30	1-methylhistidine	7.05 (s), 7.75 (s)
31	Phenylalanine	7.32 (m), 7.37 (m), 7.42 (m)
32	Formate	8.45 (s)

Supplementary Figure 1

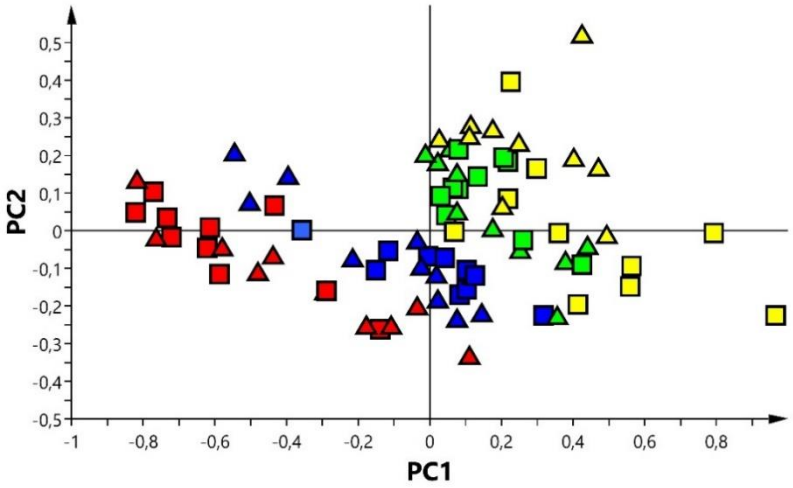


Supplementary Figure 2



Supplementary Figure 3

a.



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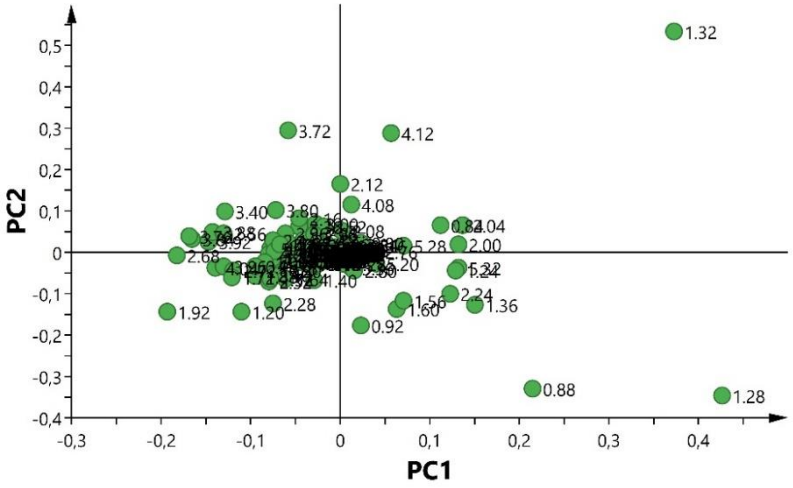

Supplementary Figure 4

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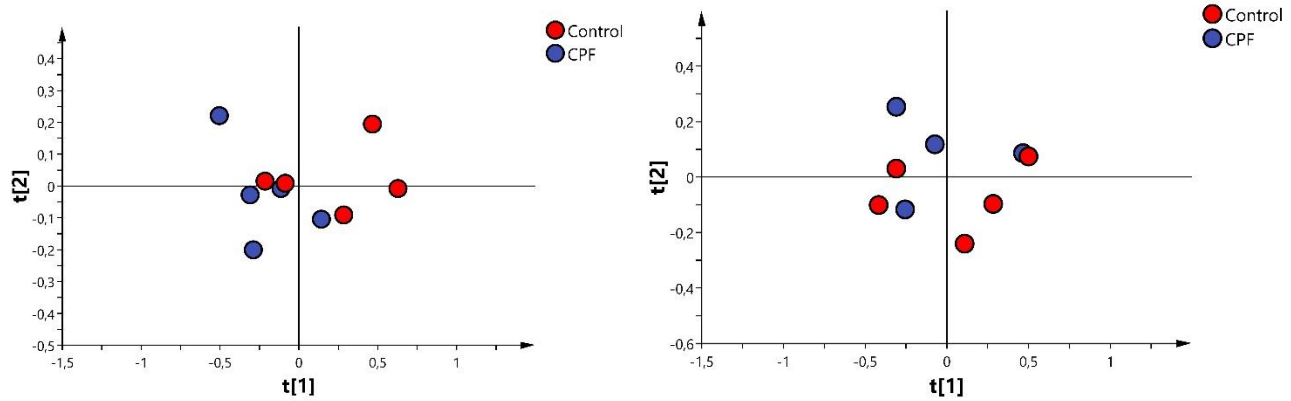

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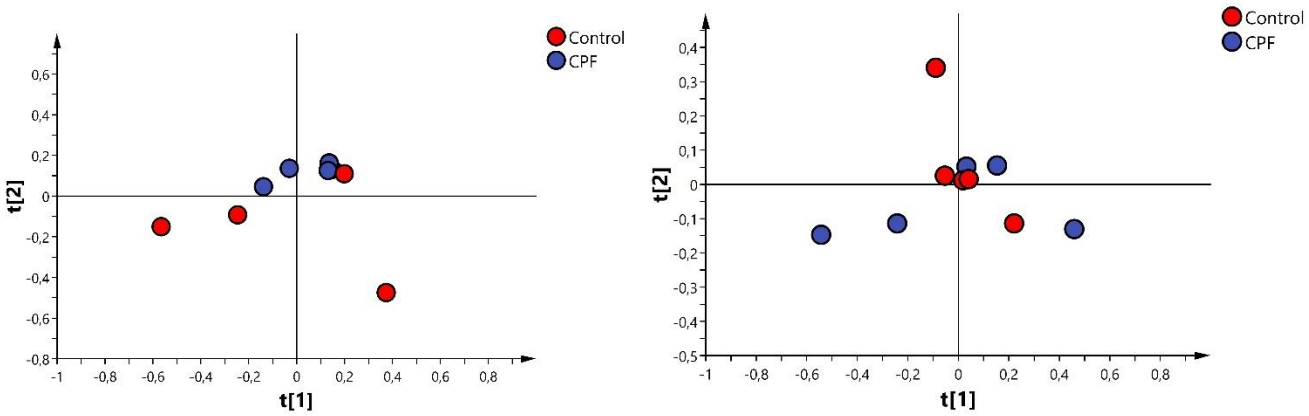

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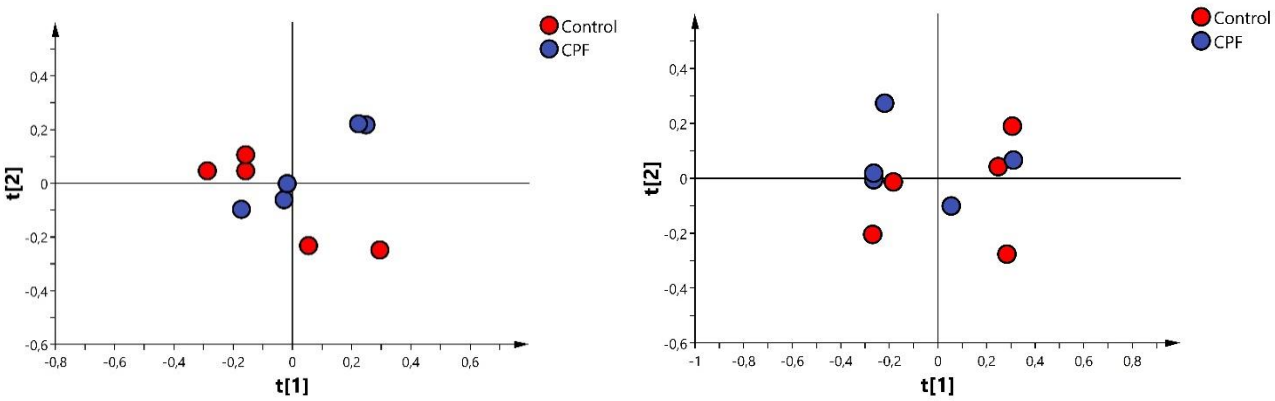

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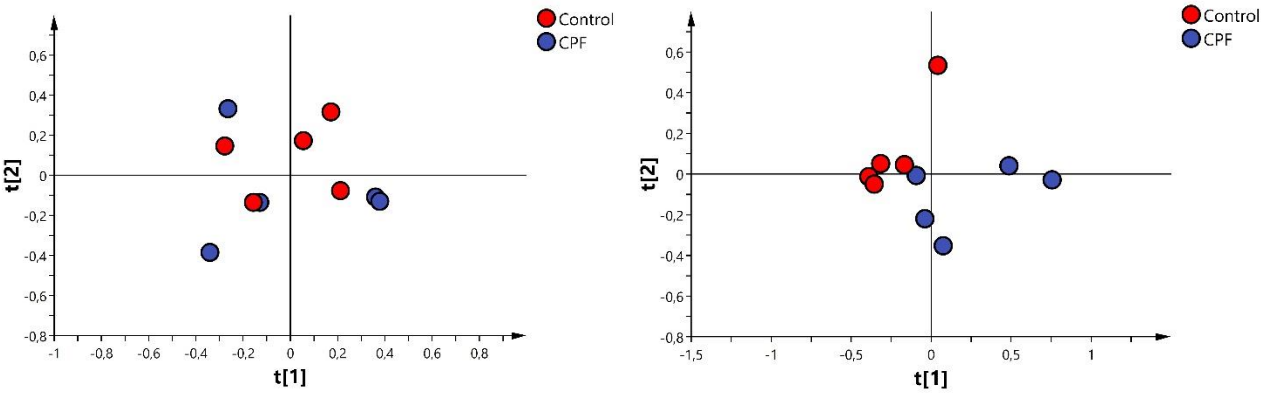
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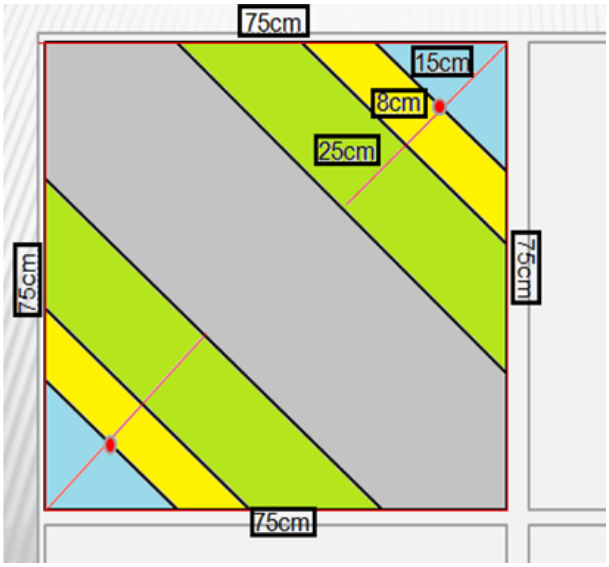
Supplementary Image 1.



Supplementary captions

Supplementary Table 1. ^{1}H NMR spectral information for metabolites identified on rat plasma samples. LDL = low-density lipoprotein; VLDL = very low-density lipoprotein; FA = fatty acid; NAc = *N*-acetylglycoprotein; PUFA = polyunsaturated fatty acid; UFA = unsaturated fatty acid.

Supplementary Figure 1. Percentage of victories after 9 matches versus known animals. Data are expressed by means and SEM.

Supplementary Figure 2. Percentage of victories after 9 matches versus both known and unknown rats. Data are expressed by means and SEM.

Supplementary Figure 3. PCA scores (a) and (b) loadings plots for 80 ^{1}H CPMG NMR spectra of rat plasma samples collected at different sampling time points - 16 days (red), 21 days (blue), 34 days (green) and 209 days -PNM7- (yellow) old - from rats belonging to the CPF-exposed group (□), and control group (Δ).

Supplementary Figure 4. PCA scores plot of 1H CPMG NMR data of plasma samples obtained from male (left) and female (right) rats belonging to the CFP-exposed group (white), and the control group (black), for sampling points t1 PND16 (a), t2 PND21 (b), t3 PND35 (c) and t4 PNM7 (d).

Supplementary Image 1. Drawn of one open field used to assess the social exploration of the rats. Blue areas represent the strangers' boxes, separated from the rest of the paradigm by a plexiglass-made, transparent wall with 6 small holes to facilitate odor transmission but not physical contact. Yellow and green areas were digitally created (no physical barriers). Yellow zones are defined as contact areas. For approximation zones, both the yellow and the green areas were summed. The distances indicated in the drawn were the same for both adolescent and adult rats except for the contact zone (8cm for adults and 5 cm for adolescents). 25cm, 8cm (or 5cm) and 15cm are referred to as the distance from the center of the plexiglass wall (red dot).

STUDY 3

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Research paper

Postnatal exposure to low doses of Chlorpyrifos induces long-term effects on 5C-SRTT learning and performance, cholinergic and GABAergic systems and BDNF expression

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ABSTRACT

Alterations in attention and inhibitory control are common features in several neurological disorders. Environmental factors such as exposure to pesticides have been linked to their appearance. Chlorpyrifos (CPF) is one of the most widely used organophosphate compounds in the world. CPF exposure during development seems to be critical for later behavioral and molecular disruptions during adult ages, although this depends on the specific period of development, where the preweaning period is one of the least studied. Despite the abundant empirical work made in the last decades on developmental CPF exposure, the systematic study of this on attention is sparse, and nonexistent concerning inhibitory control, without a single study on preweaning developmental stages. The present research explored the effects of the exposure to low doses of CPF that do not elicit a significant inhibition of the Cholinesterases during this developmental period on rats' behavior in the five-choice serial reaction time task. Behavioral manipulations (inter-trial interval and stimulus duration), pharmacological manipulations (cholinergic and GABAergic drugs) and brain gene expression analyses were also conducted. Exposure to CPF decreased the locomotor activity and enhanced the learning profile of the female rats, increased the impulsive rates, unmasked by a longer inter-trial interval, hypo-sensitized the cholinergic system and down-regulated the mRNA expression levels of the brain-derived neurotrophic factor in the dorsal striatum of the male rats. This happened without significant inhibition of the brain Acetylcholinesterase. All this new information corroborates that the exposure to a common pesticide at low doses during a key, but under-explored developmental period importantly affects different behaviors, neurotransmitter systems, and molecules that are altered in the main neurological disorders observed nowadays.

1. Introduction

Several concurrent psychiatric, neurological and neurodegenerative disorders have attention and/or inhibitory control alterations as a central or secondary feature of their clinical profile (Boxhoorn et al., 2018; Malhotra, 2019; Peckham et al., 2010; Schmitt et al., 2018; Christodoulou et al., 2012; Picazio et al., 2018). Although the genetic bases of these complex executive functions are relatively well studied, environmental factors such as early stress, socioeconomic status, and exposure to external contaminants have gained interest in recent decades (Banerjee et al., 2007; Liu et al., 2018; Gagne and Saudino, 2016; Perez-Fernandez et al., 2019a), with Organophosphate compounds (OP) being one of the most widely analyzed xenobiotic families with regard to this issue (Marks et al., 2010; Suarez-López et al., 2017; Perez-Fernandez et al., 2019a).

Chlorpyrifos (CPF) has been one of the most widely used OP for decades in both western countries and developing nations. It has been used for a variety of purposes ranging from agricultural pest control to industrial and residential functions, with the latter being partially banned in both the United States and the European Union (2001 and

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2008, respectively). CPF and, essentially, the main mechanism of toxicity in its oxon form is the irreversible inhibition of the Cholinesterases, primarily Acetylcholinesterase (AChE) at the central nervous system (CNS), which lead to a general increase of the cholinergic tone (Eaton et al., 2008). However, several in vivo and in vitro studies have found alternative molecular targets in the absence of AChE inhibition following low to very low CPF doses [non-cholinesterase inhibition dose (NChEI)], essentially during developmental stages (Burke et al., 2017).

In addition to the well-known influence of CPF on motor outcomes (Rauh et al., 2006; Malekirad et al., 2013; Ricceri et al., 2003; Perez-Fernandez et al., 2019b), various studies have found an interesting link between CPF exposure and alterations in learning, attention, and impulsivity/compulsivity traits in both humans (Rohlman et al., 2016, 2019; Rosenstock et al., 1991; Steenland et al., 1994; Stephens et al., 1995; Malekirad et al., 2013; Perez-Fernandez et al., 2019a) and rodents (Cardona et al., 2006, 2011; López-Granero et al., 2013, 2014; Montes de Oca et al., 2013; Sánchez-Santed et al., 2004; Peris-Sampedro et al., 2016; Basaure et al., 2017; Cañadas et al., 2005; Cohn and MacPhail, 1997; Terry, 2012; Terry et al., 2003; Samsam et al., 2005; Middlemore-Risher et al., 2010; Bushnell et al., 2001; Perez-Fernandez et al., 2019a).

These cognitive functions are commonly altered in different neurological and neurodegenerative disorders such as Alzheimer's (AD), Parkinson's (PD) and Amyotrophic Lateral Sclerosis diseases, as well as in neurodevelopmental pathologies such as autism spectrum (ASD) and attention/hyperactivity deficit disorders (Boxhoorn et al., 2018; Malhotra, 2019; Peckham et al., 2010; Schmitt et al., 2018; Christodoulou et al., 2012; Picazio et al., 2018). Furthermore, all of these have also previously been linked to exposure to CPF or other OPs (Sánchez-Santed et al., 2016; Shelton et al., 2014; Sagiv et al., 2018; Chang et al., 2018).

Interestingly, developmental exposure to low doses well below those needed to induce systematic toxicity, or with a NChEI profile in mammals, have also been linked to such neurological alterations, in both human (Dalsager et al., 2019; Sánchez Lizardi et al., 2008; Ruckart et al., 2004; Rauh et al., 2006; Dassanayake et al., 2009; Butler-Dawson et al., 2016; Suarez-López et al., 2017; van Wendel de Joode et al., 2016; Perez-Fernandez et al., 2019a) and preclinical research (Aldridge et al., 2005b; Basaure et al., 2017, 2019; Jett et al., 2001; Johnson et al., 2009; Gómez-Giménez et al., 2017; Levin et al., 2001, 2002; Perez-Fernandez et al., 2019a).

However, the molecular and neurobehavioral alterations following developmental CPF exposure are dependent on the neurodevelopmental stage, with significant differences observed between gestational and postnatal exposure regimes and, within these, between early and late windows of exposure (Eaton et al., 2008). The late postnatal, preweaning stage [around postnatal day (PND) 10] is equivalent to the human day of birth in terms of CNS development and is an essential period for synaptogenesis, myelination, and the maturation of the oxytocin and vasopressin systems (Tait et al., 2009; Semple et al., 2013). Consequently, its translational meaning in terms of perinatal brain damage is unquestionable. However, in terms of the vast published literature on CPF exposure during development, it is one of the least studied period. For our interest, there are only a few studies that have analyzed the influences of low doses of CPF on learning during this period (Basaure et al., 2019; Guardia-Escote et al., 2018; Jett et al., 2001; Levin et al., 2001). Moreover, we have not found a single study that analyzes the influences of this exposure protocol on attentional and/or inhibitory control.

Given the above considerations, we analyzed, in rats, the long-term effects of sub-chronic exposure to a well-known NChEI dosage during the preweaning developmental window on various neurobehavioral outcomes (attention, impulsivity, compulsivity and motricity) thought to underlie the previously mentioned neurological disorders. We suggest that there is a need for a more in-depth study of the influences of low doses of CPF on attentional and inhibitory control behaviors during this essential developmental stage. Given the fact that several preclinical studies have previously found that CPF exposure during development has an important sex dimorphic profile, and that this dimorphism is also present in many neurological disorders, we also included both sexes in the present experiment. On the basis of the data obtained from both human and rodents' studies we expect that this exposure protocol could induce different alterations in the mentioned behaviors. This behavioral information is completed with both pharmacological and gene expression experiments to provide some insights into the molecular mechanisms that could underlie these alterations. The drugs and genes focused on both the cholinergic and GABAergic systems as we have previously found that this exposure protocol alters these neurotransmitter systems and some of their components (Perez-Fernandez et al., 2019b). Furthermore, we added different serotonergic genes as they play an important role on impulsivity (Sholler et al., 2019; Winstanley et al., 2004), and the brain derived neurotrophic factor (BDNF) as it plays an essential role in the adequate functioning of the CNS in terms of brain plasticity and cell differentiation (Lykissas et al., 2007).

2. Materials and methods

2.1. Experimental animals

Nineteen timed pregnant female Wistar rats arrived at our laboratory and were individually housed on gestation day 16. A total of 190 pups (95 females) were born on gestation day 21 (PND0). At PND1, the animals were randomly distributed between mothers, with 5 animals of each sex allocated per dam, in order to ensure a higher degree of genetic variability per dam and similar amongst dams and to maintain the same litter size ($n = 10$) and sex ratio into each dam (50% male pups). At PND21, animals of the same sex were randomly distributed, with 4 per home cage. From these, a total of 85 rats were included in the present study, of which 20 (10 females and 10 males, half of which were exposed to CPF) were used to determine AChE activity 24 h after the last CPF exposure. The remaining 65 rats were included in the behavioral experiments (31 females and 34 males, 14 and 17 of which were exposed to CPF, respectively). All the rats used in the present experiment were naïve to the operating boxes. Various social (Crawley's test) and dominance (tube test) traits of these animals were previously analyzed (Perez-Fernandez et al., 2020). Rats were fed a standard diet (A04 Standard Free, Panlab) until PND74, after which their bodyweights were controlled with a maintenance diet to avoid obesity (during the first two weeks the males received 20 g daily and the females 18 g, and thereafter the males received 18 g males and the females 16 g). Three weeks before the beginning of the 5-CSRTT procedure, the animals were fed a restrictive diet for the entire experiment (described in the 5C-SRTT procedure section). Water was always made available ad libitum. The temperature and humidity of both the home and the testing room were set at 22 ± 2 °C and 50 ± 10%, respectively. The whole experimental timeline is displayed in Image 1. The present study forms part of the project ES040130002260 and was conducted in accordance with the Spanish Royal Decree 53/2013, the European Community Directive (2010/63/EU) for animal research and comply with the ARRIVE guidelines for animal research. All the experiments described in the present manuscript have been approved by the University of Almeria Animal Research Committee.

2.2. Neurotoxic agent

Female and male rats were randomly assigned to either Chlorpyrifos (CPF) exposure [O, O-Diethyl O-3,5,6-trichloropyridin-2-il phosphorotrioate (Pestanal, Sigma Aldrich, purity of 99.9%)] or to the vehicle group. The animals received the substances orally from PND10 to PND15 inclusively. For the experimental group, one mg/kg/ml/day of

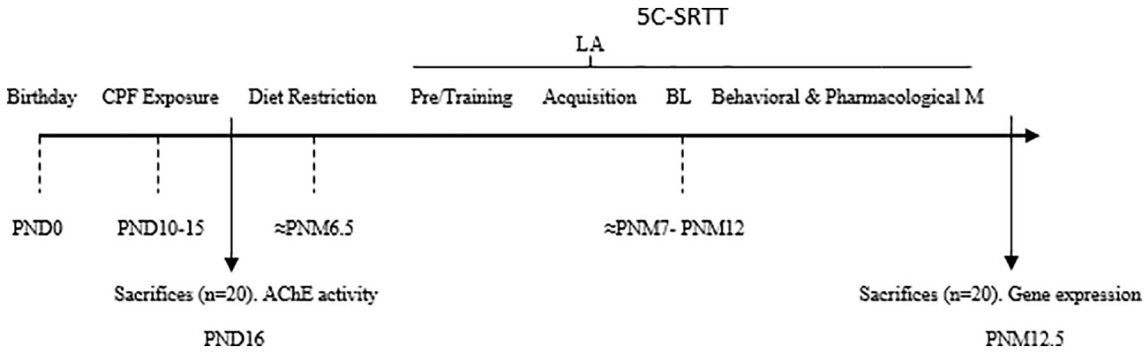

Image 1. Experimental timeline. A total of 190 rats were randomly assigned to CPF or corn oil oral exposure from PND10 to 15. 85 rats were included in this experiment. 20 were randomly selected and sacrificed 24 h after the last exposure (PND16) for AChE activity analysis. The remaining 65 rats were included in the 5-choice serial reaction time task (5C-SRTT). 59 learnt the task and were included in the statistical analyses. These 59 rats were included in the locomotor activity (LA) procedure, that was analyzed during the Acquisition phase for 30 min. Pre training was done around PND209. Acquisition started at PND215 and continued until the last animal reach the criteria (PND284). Behavioral manipulations (M) started the next day following baseline (BL) achievement and finished around PND311. Pharmacological challenges started at PND319 and finished around PND362. 2 weeks after 5C-SRTT was finished (≈ PNM12.5), 20 rats were randomly selected and sacrificed for brain gene expression analyses.

CPF was diluted in corn oil for ease of absorption (Timchalk et al., 2002). Control animals were exposed to the same vehicle at the same volume. This dose has previously been found to induce little-to-no ChEs inhibition (Savy et al., 2015), particularly during our developmental stage of interest (Carr et al., 2011, 2013), and it is close to that of the No-Observed-Adverse-Effect-Level revealed by experiments on sub-chronic exposure in rats (World Health Organization, 2009).

2.3. Drugs

Two different compounds were used in the 5C-SRTT procedure: (−)-Scopolamine Hydrobromide trihydrate (Sigma, lot number 106H0796, purity of > 98%), an antagonist of the muscarinic receptors in the cholinergic system, was administered at doses of 0.5, 0.250, 0.125 and 0.06 mg/kg, and Alprazolam, 8-chloro-1-methyl-6-phenyl-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine (Pfizer, lot number J3873, purity of ≥99%), an allosteric agonist of the GABA-A receptor, was administered at doses of 0.3, 0.2, 0.1 and 0.05 mg/kg. Physiological saline (NaCl 0.9%) was used as a solvent for Scopolamine, whereas Ethanol (15%), Propanediol (72%) and Physiological serum 0.9% NaCl (13%) were used for Alprazolam. Animals in the control condition received an injection of the respective solvent. All drugs were administered at a volume of 1 mL/kg.

2.4. Behavioral protocols

2.4.1. Locomotor activity

2.4.1.1. Paradigm description. Eight Plexiglas photocell beam-based activity cages (39x39x15cm) were used to evaluate locomotor activity. Behavior was automatically recorded with VersaMax® and data were collected with VersaData® Software (PLC Control System SL). Total distance (cm), vertical activity (arbitrary units), time in movement (*sec*), margin time (time spent in the periphery -sec-) and velocity (cm/s in movement) outcomes were analyzed for 30 min.

2.4.1.2. Experimental procedure. Rats were driven to the experimental room for habituation one hour before the test. The locomotor activity of males was evaluated in the odd numbered series and females during the even numbered series. Animals were randomly distributed throughout the experimental cages by treatment condition in order to avoid possible cage bias. The cages were cleaned with 70% ethanol before starting the first series and between series. The locomotor activity of the rats was assessed from 9 h to 15 h. The experimental room was dimly lit, and the temperature and humidity conditions were set according to those of the standard homeroom, as previously described.

2.4.2. Five choice serial reaction time task (5C-SRTT)

2.4.2.1. Paradigm description. 5C-SRTT was conducted in six standard sound-attenuated operant conditioning chambers with continuous background noise, equipped with facilities for the specific mobile arrangements needed for 5C-SRTT (for a more detailed description of the cages, please see Montes de Oca et al., 2013). Briefly, the animals were trained to respond rapidly (by introducing the nose) to a light stimulus inserted into a small hole in a wall of the Skinner box, surrounded by another four holes. When the animals responded appropriately (correct response), a dustless sweet pellet (TSE systems) was delivered into the magazine located on the wall opposite to the holes. If the rat did not respond (omission) or responded incorrectly (an incorrect response was recorded if the rat introduced its nose into any of the remaining four holes), the reward was not triggered and a 5-s time out period (environmental light off) was set. For 5C-SRTT training, behavioral manipulations and pharmacological challenges, animals finished the daily session once they had completed 100 trials or 30 min had elapsed. The dependent variables for study were: accuracy $\left[100 \times \left(\frac{\text{Correct Responses}}{(\text{Correct Responses} + \text{Incorrect Responses})}\right)\right]$, percentage of omissions $\left[100 \times \left(\frac{\text{Omissions}}{\text{Total trials}}\right)\right]$, premature responses, perseverative responses and latency to reward (averaged by trial). These outcomes evaluate attention (accuracy and omissions), impulsivity (premature responses), compulsivity (perseverative responses) and motivation (latency to reward).

2.4.2.2. Experimental procedure. The present protocol is an adaptation from Moreno et al. (2010). The rats received a progressively restrictive diet, which began 3 weeks before the experiment until they were maintained on 13-15 g/day (for males) or 10–12.5 g/day (for females) in order to maintain a body weight between 85 and 90% for the whole experiment and to ensure high rates of motivation for the reward. Following this, a total of 10 pellets/animal/day for 3 days were deposited into each of the home-cages in order to avoid neophobia. The rats were then habituated to the operating boxes and the whole experimental context for 2 days (10 min per day). On the second day, 2 sugar pellets were placed into each key zone of the apparatus (pellet magazine and each hole) so that the rats could learn to regard these places as the “action zones”. Once the animals were habituated to the apparatus, the pre-training phase began. This was defined by 2 days for Pre-training condition 1 (animals were automatically reinforced when they placed the nose into a hole) and another 2 days for Pre-training condition 2 (the same conditions as those of training but without time outs) from PND209 to PND214.

Once the pre-training phase was completed, the 5C-SRTT training

Table 1
5C-SRTT phases.

Phase	SD	ITI	LH	Criteria
1	8	10	5	CR ≥ 50
2	6	5	5	Acc ≥ 80%
3	2.5	5	5	Acc ≥ 80% - %Om ≤ 20%
4	1.25	5	5	Acc ≥ 80% - %Om ≤ 20%
5	1	5	5	Acc ≥ 70% - %Om ≤ 25%
Baseline performance	Average of three consecutive days accomplishing the Phase 5 criteria			

Main characteristics of the different phases of difficulty in the 5C-SRTT procedure. From left to right, Phase number, stimulus duration (SD), inter-trial interval (ITI), limited hold (LH) and the criteria in order to pass to the next phase in terms of correct responses (CR), accuracy (Acc) and percentage of omissions (%Om).

began at PND215. Briefly, rats had to achieve a baseline (BL) performance at different stages of training, which differed in terms of levels of difficulty. The target baseline criteria and the previous training stages are described in Table 1. Once animals achieved baseline criteria rates on three consecutive sessions (last animal at PND284), these data were averaged for every single outcome studied, representing the baseline performance of the rat. The behavioral manipulation phase then began in which we varied the stimulus duration (SD) (0.25, 0.5, 1 and 1.5 s) and the inter-trial interval (ITI) (2, 5 and 7 s) from the day immediately following BL achievement until PND311. The experiment finished with a series of pharmacological challenges using both Scopolamine hydrobromide and Alprazolam (see drugs section), starting around PND319 and finishing at PND362. Both behavioral and pharmacological manipulations were carried out on Tuesdays and Fridays, allowing for at least 72 h of wash-out between each manipulation. A complete randomized Latin square design was chosen for the different challenges. Both Scopolamine and Alprazolam were administered (i.p.) 30 min before recording the animals' behavior. The whole behavioral protocol lasted from PNM 7 to 12.

On each day, the rats were transported to the experimental room 40 min before the beginning the experimental sessions. Each Skinner box was cleaned using 70% ethanol before the first series and between each subsequent series. The experimental series were alternated according to sex (the first two series with males, followed by two for females, and so on). Animals were randomly allocated to the experimental boxes based on the treatment condition for controlling for box bias. The experiment was conducted between 9 h and 14 h once the animals had learned the procedure. Temperature and humidity were set as previously described.

2.5. Sacrifice protocol

20 Animals (10 females and 10 males, 5 each sex exposed to CPF and the remaining to corn oil) were sacrificed by rapid decapitation 24 h after the postnatal exposure (AChE analysis subsample -PND16-) and 2 weeks after behavioral analyses had been completed (animals that had completed the locomotor and 5C-SRTT procedures - ≈ PNM12.5-). The brains of the rats were quickly removed from the skull and dissected. Dorsal striatum samples were collected when relevant to the 5C-SRTT derived outcomes (Robbins, 2002), as its functioning has been linked to attention (Agnoli and Carli, 2011), impulsivity and compulsivity (Lipton et al., 2019; Agnoli and Carli, 2012), as well as its role in motricity is well-known (Kravitz and Kreitzer, 2012). All areas were directly placed into RNAse-free tubes (1.5 mL) and flash-frozen immediately after extraction. All samples were stored at −80 °C until use. All utilities, spaces, and materials were autoclaved and treated with RNAse ZAP ® (Sigma Aldrich) to avoid RNA degradation.

2.6. Biochemical procedures

2.6.1. Acetylcholinesterase activity assessment

Frontal cortex AChE activity was assessed 24 h after the last day of exposure (10 females and 10 males, 5 animals of each sex exposed to CPF). Briefly, the samples were homogenized in 0.1 M PBS (pH 8) with 1% Triton X-100, 1/10 (*w/v*). After centrifugation (15,000 ×*g*, 15 min), the supernatant was taken and placed into a fresh tube. AChE activity was measured following Ellman's method (Ellman et al., 1961) with slight modifications, using a 96-well microplate reader (DTX 880, Multimode Detector, Beckman Coulter). Following centrifugation, the supernatant was diluted with 0.1 M PBS (pH 8.0), 1/10 (*v*/v), and 10 mL of the diluted sample was mixed with 5.5-dithiobis-2-nitrobenzoic acid [60 uL, in 0.1 M PBS (pH 8), with a final concentration of 0.33 mM] and 206 uL of 0.1 M PBS (pH 8.0). After shaking, the mix was incubated for 300 s at 37 °C, and 15 uL of the butyrilcholinesterase blocker tetra isopropyl pyrophosphoramide (final concentration of 50 uM) was added. Finally, the enzymatic reaction started by the addition of 9 uL of acetylthiocholine iodide in 0.1 M PBS (pH 8) (final concentration of 0.5 mM). Blank wells were composed of all the reagents and the samples except for the acetylthiocoline iodide. The reaction rate was monitored at 37 °C for 22 min. Absorbance was measured (405 nm) with 30-s intervals with a 3-s shake before each reading (45 cycles). Once the slopes had been analyzed, two optimal cycles (60 s) were chosen for statistical analysis. Enzyme activity was calculated as an increase in absorbance over time according to the formula given by Ellman et al. (1961) using the molar absorption coefficient of the yellow reaction product at 412 nm. Protein concentration was measured following the Bradford method with absorption rates at 592 nm (Bradford, 1976). The activity was normalized to total protein concentration.

2.6.2. Gene expression analysis: Reverse transcription quantitative Polymerase chain reaction (RT-qPCR)

Briefly, RNA samples were extracted from the brain tissue (dorsal striatum) of a total of 20 animals randomly selected (10 females and 10 males, with half of each group exposed to CPF) using the TRIZOL® method (Sigma Aldrich), following the manufacturer's instructions. RNA was quantified using a Qubit ® (Fisher Scientific). RNA quality was analyzed with agarose gel electrophoresis. RNA samples (1.4 μg) were then retro-transcribed to cDNA following the supplier's protocol (Maxima first strand, Fisher Scientific). cDNA samples were then diluted 1/4 and RT-qPCR was conducted in a Thermocycler (Applied Biosystems). All reactions contained the pair of primers, the cDNA, the nuclease-free water (Ambion), and the Sybr Green Master Mix (Applied Biosystems). Specific primers from an intron section of the gene gapdh were used to check the lack of gDNA contamination in each individual sample. Gapdh gene (exon sections) was used as housekeeping since it has previously shown good performance in our laboratory in Wistar rats. The efficacy of each pair of primers was analyzed with a standard curve (1:10 serial dilution). Melting curves were also analyzed to discard unspecific amplifications. Samples that expressed > Ct30 and/or showed unspecific secondary melting signals were discarded. All data were obtained and analyzed using StepOne software real-time PCR Systems (v2.2.2, Applied Biosystems). Data transformation and analysis are described in the Statistical analyses section. All the primers are described in more detail in Table 2.

2.7. Statistical analyses

The data from the frontal AChE activity (nmol/min/mg) were analyzed with a two-way analysis of variance (ANOVA), with T*reatment* (two levels, control and CPF) and S*ex* (two levels, female and male) as factors. The AChE data of the CPF exposed animals were then normalized to their respective controls in order to display the percentage of inhibition. As we considered that the multivariate analyses adjusted per

Table 2
Primers selected for the RT-qPCR study.

Gene	Gene name (Rattus)	Forward primer	Reverse primer	Source
GAPDH (Intron)	Gapdh	ctgggtggctcaaggaata	cacacgcatcacaaaaaggt	Own design
GAPDH (Exon)	Gapdh	cttcaccaccatggagaag	catggactgtggtcatgag	Own design
Nicotinic α7	Chrna7	tatcaccaccatgaccctga	cagaaaccatgcacaccagt	Chamoun et al., 2016
M1 Receptor[a]	Chrm1	catggagtccctcacatcct	gggcatcttgatcaccactt	Own design
M2 Receptor	Chrm2	caagacccagtatctccaagtctg	cgacgacccaactagttctacagt	Chamoun et al., 2016
ChAT	Chat	atggccattgacaaccatcttctg	aacaaggctcgctcccacagcttc	Lips et al., 2007
VAChT	Slc18a3	gccacatcgttcactctcttg	cggttcatcaagcaacacatc	Lips et al., 2007
AChE-S	Ache	gtgagcctgaacctgaagcc	tcctgcttgctatagtggtc	Jameson et al., 2007
GABA-A α1	Gabra1	gcccaataaactcctgcgtatc	attcggctctcacagtcaacct	Fujimura et al., 2005
GABA-A α2	Gabra2	ccaggatgacggaacattgc	ggaaagtcctccaagtgcattg	Fujimura et al., 2005
GAD1	Gad1	gtgagtgccttcagggagag	cgtcttgcggacatagttga	Own design
GAD2	Gad2	ctgagaagccagcagagagc	agagtgggcctttctccttc	Own design
KCC1	Slc12a4	catgatttcccgctctttg	ccgtacacccgcatgttatt	Own design
KCC2	Slc12a5	aggtggaagtcgtggagatg	cgagtgttggctggattctt	Jaenisch et al., 2010
NKCC1[a]	Slc12a2	catggtgtcaggatttgcac	gatattgtccttacatagag	Cho et al., 2013
5HT2a	Htr2a	aacggtccatccacagag	aacaggaagaacacgatgc	Kindlundh-Högberg et al., 2006
5HT2c	Htr2c	ttggactgagggacgaaagc	ggatgaagaatgccacgaagg	Kindlundh-Högberg et al., 2006
BDNF	bdnf	ggtcacagcggcagataa	ccgaacatacgattgggtag	Own design

From left to right, the name of the Gene, ID, forward primer, reverse primer and source. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase. M1 & 2: Muscarinic 1 & 2. ChAT: Choline acetyltransferase. VAChT: Vesicular acetylcholine transporter. AChE-S: Acetylcholinesterase Isoform S. GABA-A-α1 & 2: Gamma-Aminobutyric acid receptor A, alpha1 & 2 subunits. GAD 1 & 2: Glutamate decarboxylase 1 & 2. KCC1 & 2: Potassium-chloride cotransporter 1 & 2. NKCC1 Sodium-potassium-chloride cotransporter 1. 5HT2a and c: Serotonergic receptor 2 a & b. BDNF: Brain-derived neurotrophic factor.

[a] Indicates that the data resulting from the referred genes were discarded because generalized secondary peaks in the melting curve (M1r) or lack of reliable expression (> Ct 30, NKCC1).

domain [e.g. locomotor activity (all the motor outcomes), attention (accuracy and percentage of omissions) and inhibitory control (premature and perseverative responses)] are not suitable in the present study for different reasons [a) e.g. we do not think that CPF will alter all the behaviors associated for a specific domain, as previously demonstrated (please see the different studies cited in Perez-Fernandez et al., 2019b, as well as the main results of this study), b) some of these behaviors could be categorized in a different domain (e.g. center and margin time could be anxiety-like instead of motor-like behaviors -Seibenhener and Wooten, 2015-, omissions could be a motivation-like instead of attention-like behavior -Asinof and Paine, 2014-, amongst others), and c) it is well-known that CPF can alter impulsivity without affecting compulsivity and vice-versa (e.g. Montes de Oca et al., 2013) as these behaviors are inter-related but they are not strictly inter-dependent, with specific and distinct physiological substates at their basis (Dalley et al., 2011)], we analyzed them individually.

The different locomotor variables were individually analyzed with a further two-way ANOVA using the same factors. Acquisition of the 5C-SRTT was firstly analyzed with a repeated measures ANOVA (rmANOVA) to check the general learning profile, using the different phases as the within-subject variable and the above-mentioned factors as between-subject variables. Individual analysis of every phase was also conducted with a two-way ANOVA, with the above-mentioned factors. The baseline performance of each outcome was analyzed with a further two-way ANOVA, again with sex and treatment as the factors. Behavioral and pharmacological challenges were analyzed separately with an individual rmANOVA for each outcome, using *manipulation* (*SD or ITI*) or D*ose* (the different doses of each drug) as within-subject variables and the above-mentioned factors as between-subject variables. For the gene expression procedure, the average of the Ct from each target gene was normalized to the average of its housekeeping Ct (ΔCt) and then normalized to the average of each control group (ΔΔCt). These data were then transformed to obtain the fold change from the control average (2^ΔΔCt). Each gene was individually analyzed using a one-way ANOVA, using T*reatment* as the factor. The statistical error was set at $p < .05$. All data are represented by means and SEMs in both figures and tables. Individual plots in the molecular analyses are also included to display the individual distribution. A statistical description of all the significant outcomes is provided in the text, including the F values, degrees of freedom, p values, means ($\bar{x}$), adjusted (adj) $\bar{x}$, adj $\bar{x}$ difference, confidence intervals (CI), adj CI and size effects defined by the partial eta squared (η_p^2) and the Cohen's d (d) values for the ANOVA and post-hoc multiple comparisons results, respectively. The analyses were carried out with the SPSS v25 software (IBM), and the figures were designed with Prism v6 (GraphPad). The tables and image were designed with Excel Office 365 (Microsoft).

3. Results

A total of 10 animals per exposure condition (5 females and 5 males from each condition) were finally included for AChE activity determinations. From the 65 animals that started the 5C-SRTT protocol, 6 out of them did not reach the baseline criteria and were discarded (1 female control, 2 males control, 2 exposed females and 1 exposed male), thus the performance of the remaining 59 rats was included in the analyses. 28 rats were female and 31 male. From these, 12 female and 16 male rats were exposed to CPF (16 and 15 were control, respectively). These animals were included for all the analyses except in the SD manipulations [n = 57, 27 females (11 exposed and 16 control) and 30 males (16 exposed and 14 control)] and the Alprazolam challenge [n = 55, 27 females (12 exposed and 15 control) and 28 males (13 exposed and 15 control)], as the performance of 2 and 4 animals was lost, respectively. All these 59 rats were included for the locomotor activity assessment. Finally, a total of 10 animals per exposure condition (5 females and 5 males from each condition) were included for the brain gene expression experiment, but some samples were discarded for the final analyses as they showed unspecific signals in the melting curve or expressed beyond the Ct30 (indicated in the associate Figure's caption for each gene).

3.1. AChE activity. Lack of brain AChE inhibition following CPF exposure

CPF exposure did not significantly inhibit frontal AChE activity 24 h after the last exposure (Fig. 1).

3.2. Locomotor activity. Decreased velocity in exposed females

Different locomotor activity outcomes in an open field were

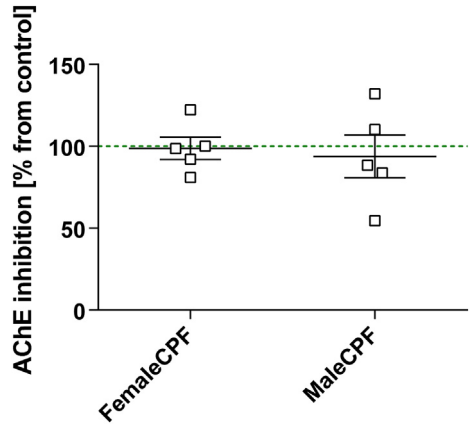

Fig. 1. AChE inhibition. Percentage of inhibition (from control) of AChE activity 24 h after the last exposure to CPF in Frontal cortex. Data are expressed as means and SEMs, and individual plots are depicted.

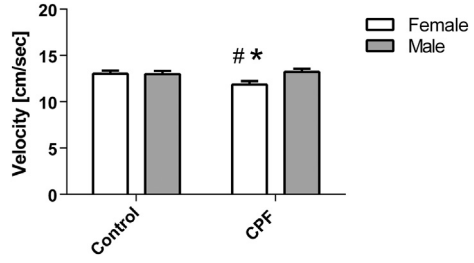

Fig. 2. Locomotor activity. Long-term influences of CPF on velocity. Significant threshold was set at $p < .05$. # indicates significant differences between different exposure conditions in the same sex. * indicates significant differences between sexes within the same exposure condition. Data are expressed as means and SEMs.

analyzed during the acquisition phase of the 5C-SRTT. CPF exposure did not alter total distance covered, time in movement, vertical activity, or time in the margin/center (Supplementary Table 1). However, a significant *Sex x Treatment* interaction [F(1,55) = 4.292, $p = .043$; $\bar{x}$= 12.84, 95% CI (12.5, 13.2), $\eta_p^2 = 0.07$] was found for velocity (Fig. 2). Post hoc analyses revealed that the exposed females [adj $\bar{x}$= 11.85, 95% CI (11.01, 12.61)] were generally slower than both non-exposed females [adj $\bar{x}$= 13.03, 95% CI (12.37, 13.69)] [$p = .023$; adj $\bar{x}$ difference = 1.18, adj 95% CI (0.17, 2.19), d = 0.90] and the exposed male rats [adj $\bar{x}$= 13.24, 95% CI (12.58, 13.90)] [$p = .008$; adj $\bar{x}$ difference = 1.39, adj 95% CI (0.38, 2.40), d = 1.05].

3.3. 5 Choice serial reaction time task

3.3.1. Acquisition phase. CPF exposure improved females' learning rates

CPF exposure did not influence the acquisition curve of the 5C-SRTT in general terms (Fig. 3a). However, when analyzing each of the SD criteria, CPF improved females' learning rates during the initial stages (Fig. 3b and c). The subsequent ANOVA revealed a significant *Sex x Treatment* interaction for both the first stage (number of sessions needed to progress from SD8 to SD6) and the second stage (sessions needed to progress from SD6 to SD2.5) [F(1,55) = 7.815, $p = .007$; $\bar{x}$= 3.54, 95% CI (2.61, 4.47), $\eta_p^2 = 0.12$ and F(1,55) = 6.159, $p = .016$; $\bar{x}$= 5.12, 95% CI (4.01, 6.23), $\eta_p^2 = 1.01$, respectively]. Post hoc analyses revealed that the usual differences observed in control animals, where males [for the first stage, adj $\bar{x}$= 2.47, 95% CI (0.68, 4.26); for the second stage, adj $\bar{x}$= 4.07, 95% CI (1.90, 6.24)] learned faster than females [for the first stage, $\bar{x}$= 5.38, 95% CI (3.63, 7.11) and for the second stage, adj $\bar{x}$= 7.00, 95% CI (4.90, 9.10)] [$p = .023$; adj $\bar{x}$ difference = 2.91, adj 95% CI (0.42, 5.40), d = 0.84 and $p = .057$; adj $\bar{x}$ difference = 2.93, adj 95% CI (−0.85, 5.95), d = 0.70, for the first and second stage, respectively], were abolished by exposure to CPF by improving the learning rates of exposed females [for the first stage, adj $\bar{x}$= 1.83, 95% CI (−0.17, 3.84) and for the second stage, adj $\bar{x}$= 3.17, 95% CI (0.742, 5.59)] in comparison with control females [$p = .010$; adj $\bar{x}$ difference = 3.54, adj 95% CI (0.89, 6.19), d = 1.02; and $p = .020$ and adj $\bar{x}$ difference = 3.83, adj 95% CI (0.63, 7.04), d = 0.91 for the first and the second stages, respectively]. However, these effects of CPF disappeared throughout the learning process.

3.3.2. Baseline performance. CPF exposure did not alter basal performance

CPF exposure did not alter the basal performance of the rats for either attentional outcomes (accuracy and percentage of omissions) or inhibitory control (premature and perseverative responses). However, sex differences were observed in the behavior of the animals [F(1,55) = 13.349, $p = .001$; $\bar{x}$= 11.61, 95% CI (10.2, 13.1), $\eta_p^2 = 0.20$], where females showed a higher percentage of omissions compared with males [adj $\bar{x}$= 14.22, 95% CI (12.22, 16.22) vs. adj $\bar{x}$= 9.21, 95% CI (7.33, 11.09), respectively] [$p = .001$; adj $\bar{x}$ difference = 5.01, adj 95% CI (2.26, 7.76), d = 0.95] (Fig. 4 a-d).

3.3.3. Behavioral manipulations. Increase in the inter-trial interval revealed the highest rates of impulsive action in the exposed animals

The influences of the manipulation of SD and ITI are displayed in both the supplementary data shown in Table 2 (SD) and Table 3 (ITI). In terms of SD manipulation, SD shortening should lead to decreased accuracy and an increased number of omissions, whilst a longer SD should produce improvements in performance (Bari et al., 2008; Asinof and Paine, 2014). A decrease in the basal SD (from 1 to 0.25 and 0.5 s) considerably reduced the accuracy shown by all animals and increased the percentage of omissions (only for the shorter condition). In contrast, an increase in the SD (from 1 to 1.5 s) improved the accuracy rates for females [adj $\bar{x}$= 78.27 95% CI (74.34, 82.20) vs. adj $\bar{x}$= 87.51, 95% CI (84.92, 90.10), respectively] but not for males [adj $\bar{x}$= 82.93, 95% CI (79.25, 86.60) vs. adj $\bar{x}$= 85.43, 95% CI (83.01, 87.85), respectively] [for female rats, $p < .001$; adj $\bar{x}$ difference = 9.24, adj 95% CI (4.14, 14.33), d = 1.07 and for male rats, $p = .639$; adj $\bar{x}$ difference = 2.51, adj 95% CI (−2.26, 7.270), d = 0.30], as revealed by a *SD x Sex* interaction [F(3,159) = 3.055, $p = .030$; $\bar{x}$= 73.48, 95% CI (71.2, 75.8), $\eta_p^2 = 0.05$].

Although the increase of SD had little effect on omissions, the significant *SD x Sex* interaction [F(2.3, 122.1) = 4.578, $p = .009$; $\bar{x}$= 6.08, 95% CI (4.54, 7.62), $\eta_p^2 = 0.08$] revealed that male rats did not significantly increase their omission rates in the shorter manipulation compared with the basal SD1 condition [adj $\bar{x}$= 5.49, 95% CI (3.06, 7.92) vs. adj $\bar{x}$= 3.23, 95% CI (1.14, 5.33), respectively], an effect that was observed in the females [adj $\bar{x}$= 14.16, 95% CI (11.56, 16.75) vs. adj $\bar{x}$= 7.58, 95% CI (5.34, 9.82), respectively] [for males, $p = .401$; $\bar{x}$ difference = 2.26, 95% CI (−1.22, 5.74), d = 0.37 and, for females, $p < .001$; $\bar{x}$ difference = 6.57, 95% CI (2.85, 10.30), d = 1.05, respectively from SD1].

Similarly, the decrease in the SD induced an increase in premature responses, whilst there was little effect of an increase in SD on this factor. A significant *Sex x SD* interaction was found [F(3,159) = 3.897, $p = .010$; $\bar{x}$= 25.72, 95% CI (21.9, 29.5), $\eta_p^2 = 0.07$]. Post hoc analyses revealed that this manipulation did not affect the female rats at both SD0.25 and 0.5 compared with SD1 [adj $\bar{x}$=27.34, 95% CI (21.58, 33.09) and adj $\bar{x}$ =25.77, 95% CI (20.26, 31.28), respectively vs. adj $\bar{x}$= 24.99, 95% CI (20.07, 29.91)] but produced important increases in impulsive action in male rats [adj $\bar{x}$=34.84, 95% CI (29.46, 40.22) and adj $\bar{x}$ =28.22, 95% CI (23.07, 33.37), respectively vs. adj $\bar{x}$=19.57, 95% CI (14.97, 24.16)] [for males, for SD0.25 vs. SD1, $p < .001$; adj $\bar{x}$ difference = 15.28, adj 95% CI (8.59, 21.96), d = 1.12 and for SD0.5 vs. SD1, $p = .010$; adj $\bar{x}$ difference = 8.65, adj 95% CI (1.52, 15.79), d = 0.65]. Finally, a decrease in SD produced a significant decrease in compulsivity in terms of perseveration, without further influences on

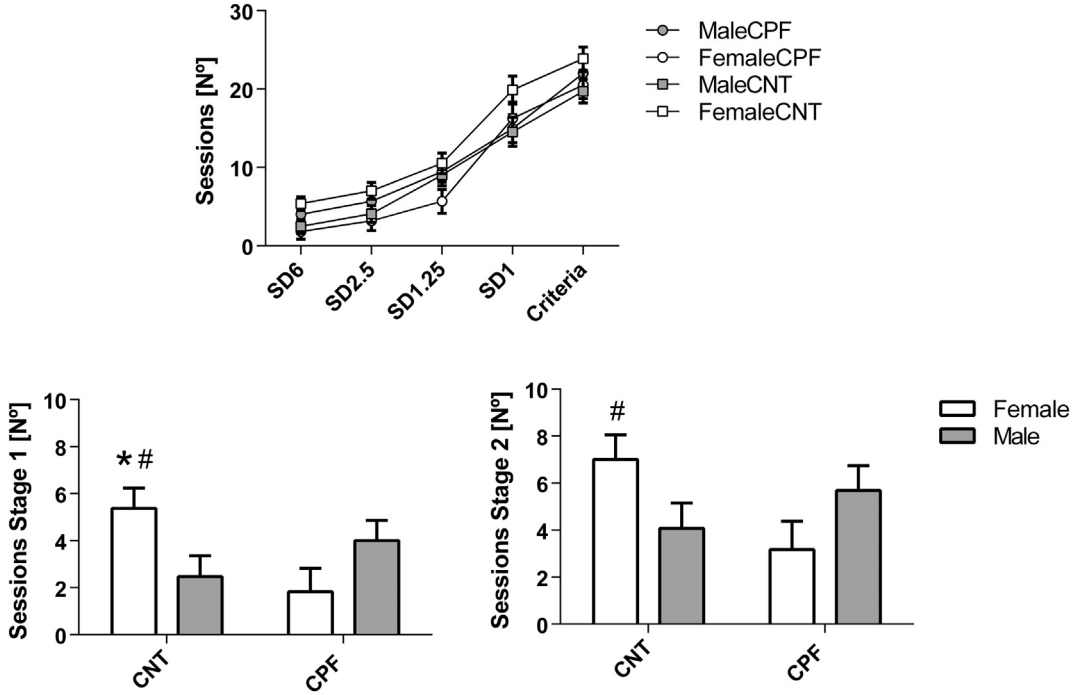

Fig. 3. Acquisition curve & learning of the 5C-SRTT. Number of sessions needed to reach one stage of difficulty across all the learning stages until basal criteria was achieved (a, up). Number of sessions needed to reach the stage 1 (SD8) (b, down-left). Number of sessions needed to reach the stage 2 (SD6) (c, down-right). * indicates significant differences between sexes at the same exposure regime. # indicates significant differences between exposed and control rats of the same sex. Significance was set at $p < .05$. Data are expressed as means and SEMs.

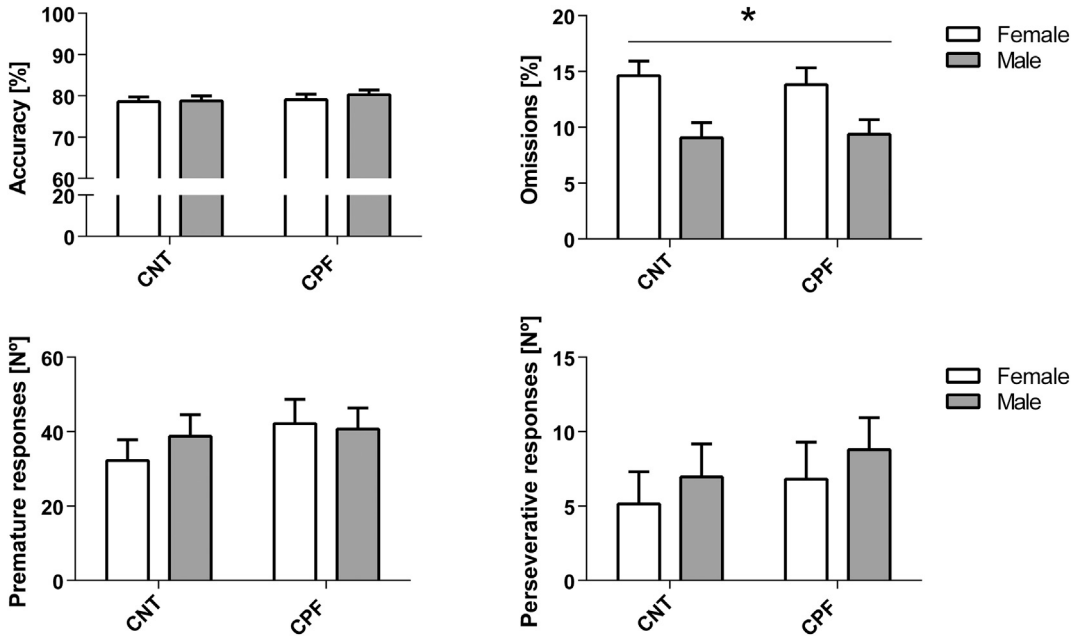

Fig. 4. Baseline performance in the 5C-SRTT. Average of three consecutive days in SD1 criteria for accuracy (a, up-left), percentage of omissions (b, up-right), premature (c, down-left) and perseverative responses (d, down-right). * indicates significant differences between sexes. Significance was set at $p < .05$. Data are expressed as means and SEMs.

any other related factor.

The expected outcomes of ITI manipulation are an increase in premature responding rate (longer ITI) and decreased accuracy and increased omissions (shorter ITI) (Bari et al., 2008; Asinof and Paine, 2014). ITI manipulation -both increase and decrease- generally reduced accuracy and increased the percentage of omissions. However, the shorter ITI condition produced a significant decrease in the number of premature responses while the longer (ITI7) had the opposite effect when compared with the standard ITI5. Interestingly, the rmANOVA revealed a significant *Treatment x ITI* interaction [$F(1.5, 84.1) = 4.640$, $p = .020$; $\bar{x} = 24.97$, 95% CI (20.9, 29.1), $\eta_p^2 = 0.08$]. Post hoc analyses revealed that whilst no differences were found in ITI2 and 5 between exposure conditions, an increase of the ITI to 7 s triggered higher rates of impulsivity in CPF exposed rats [adj $\bar{x} = 64.91$, 95% CI (54.34, 75.47)] when compared with their control counterparts [adj $\bar{x} = 48.77$, 95% CI (38.82, 58.71)] [$p = .030$, adj $\bar{x}$ difference = 16.14, adj 95% CI

Table 3
ITI manipulation.

	Female CNT	Male CNT	Female CPF	Male CPF	Outcome
			Accuracy (%)		
ITI2	79.18 ± 3.32	72.41 ± 3.71	83.13 ± 2.62	77.34 ± 2.34	
ITI5	83.38 ± 2.47	83.45 ± 3.04	87.41 ± 1.94	82.67 ± 2.34	
ITI7	82.34 ± 2.62	75.67 ± 2.59	84.23 ± 2.09	77.32 ± 2.12	
			Omissions (%)		
ITI2	25.81 ± 3.89	18.33 ± 3.50	13.08 ± 2.41	14.25 ± 3.84	
ITI5	5.56 ± 0.95	2.76 ± 0.69	3.75 ± 0.84	2.00 ± 0.47	
ITI7	9.87 ± 3.87	9.34 ± 4.41	6.16 ± 2.06	7.39 ± 4.00	
			Premature responses (N°)		
ITI2	0.56 ± 0.33	2.40 ± 1.03	1.00 ± 0.56	1.19 ± 0.40	
ITI5	12.44 ± 1.89	22.00 ± 6.21	14.75 ± 2.44	19.56 ± 5.01	
ITI7	39.06 ± 4.52	58.47 ± 9.15	63.50 ± 7.74#	66.31 ± 6.94#	CPF > CNT
			Perseverative responses (N°)		
ITI2	10.88 ± 3.10	32.36 ± 8.63	13.08 ± 3.18	38.31 ± 21.33	
ITI5	21.25 ± 5.90	20.53 ± 6.76&	12.58 ± 3.01	35.81 ± 19.99&	
ITI7	12.50 ± 3.77	10.60 ± 4.12&	6.75 ± 3.56	27.88 ± 18.62&	M- ITI7 < 5

Influences of inter-trial interval (ITI) manipulation on the different outcomes. $p < .05$. # means significant differences between Chlorpyrifos (CPF) and CNT rats. & means significant differences between males (M) and females (F). Data are expressed as means and SEM.

(1.63, 30.63), d = 0.58]. Finally, perseveration was only affected by an increase in the ITI with a strong reduction of this compulsive trait. The main analysis also revealed a significant *Sex x ITI* interaction [F(2,110) = 4.854, p = .010; $\bar{x}$ = 20.83, 95% CI (9.43, 32.20), η_p^2 = 0.08]. Post hoc analyses showed that whilst the ITI manipulation did not significantly alter the number of perseverative responses in females in comparison with the basal ITI5, the ITI7 produced a significant decrease in these responses in males compared with ITI5 [adj $\bar{x}$ = 19.24, 95% CI (4.52, 33.96) vs. adj $\bar{x}$ = 28.17, CI (11.71, 44.64), respectively] [p = .007; adj $\bar{x}$ difference = 8.94, adj 95% CI (1.99, 15.88), d = 0.21)].

3.3.4. Pharmacological manipulations. CPF exposure induced a long-term hyposensitivity of the cholinergic system

There was not an effect concerning *Sex*, thus the Fig. 5 represents both male and female rats all together. Scopolamine exposure had little effect on accuracy in general terms, with only a slightly improved performance being observed following the lowest dose and significantly poorer performance following the largest dose (Fig. 5a). For CPF exposure, a significant *Treatment x Dose* interaction was found in the main analysis [F(3.2, 173.6) = 2.930, p = .033; $\bar{x}$ = 83.46, 95% CI (80.7, 86.2), η_p^2 = 0.05]. Post hoc analyses revealed that CPF exposed rats were less affected (better performance) following both 0.125 mg/kg [adj $\bar{x}$ = 86.62, 95% CI (82.14, 91.10)] and 0.250 mg/kg doses (adj $\bar{x}$ = 86.10, 95% CI (80.81, 91.40)] compared with their control counterparts (adj $\bar{x}$ = 80.25, 95% CI (76.04, 84.47) and adj $\bar{x}$ = 79.65, 95% CI (74.67, 84.64), respectively] [for 0.125 mg/kg, p = .043; adj $\bar{x}$ difference = 6.37, adj 95% CI (0.21, 12.52), d = 0.54, and for 0.250 mg/kg, approaching significance, p = .081; adj $\bar{x}$ difference = 6.45, adj 95% CI (−0.82, 13.72), d = 0.46].

This apparent hyposensitivity of the CPF exposed rats is confirmed by the analyses of the relations between doses, where in CNT rats there were significant differences between those given saline [adj $\bar{x}$ = 84.73, 95% CI (82.14, 87.32)] and both the lowest dose [adj $\bar{x}$ = 88.40, 95% CI (85.77, 91.03)] [improved performance, p = .05; adj $\bar{x}$ difference = 3.67, adj 95% CI (0.00, 7.35), d = 0.51] and the highest [adj $\bar{x}$ = 76.56, 95% CI (71.78, 81.34)] (worse performance, p = .015; adj $\bar{x}$ difference = 8.17, adj 95% CI (1.05, 15.28), d = 0.77] as well a generally poorer performance following all doses in relation to 0.065 mg/kg for saline, 0.125 mg/kg [p = .02; adj $\bar{x}$ difference = 8.15, adj 95% CI (2.09, 14.20), d = 0.83], 0.250 mg/kg [p = .03; adj $\bar{x}$ difference = 8.75, adj 95% CI (2.13, 15.37), d = 0.79] and 0.5 mg/kg [p < .001; adj $\bar{x}$ difference = 11.84, adj 95% CI (5.43, 18.25), d = 1.11). However, in the CPF animals there were no significant effects of Scopolamine exposure at any dose.

Scopolamine increased the percentage of omissions made by the rats in a dose-dependent manner, except for the lowest dose where rats even showed a reduced omission rate (Fig. 5b). Interestingly, a significant *Sex x Dose* interaction was found [F(2.6140.4) = 3.931, p = .014; $\bar{x}$ = 83.46, 95% CI (11.2, 16.3), η_p^2 = 0.07]. Post hoc analyses revealed that the usual higher rate of omissions observed in females compared with males following saline administration [adj $\bar{x}$ = 4.37, 95% CI (2.94, 5.79) and adj $\bar{x}$ = 2.03, 95% CI (0.69, 3.37), respectively] [p = .02; adj $\bar{x}$ difference = 2.34, adj 95% CI (0.38, 4.29), d = 0.83] on baseline performance is abolished following both 0.125 [adj $\bar{x}$ = 11.07, 95% CI (6.93, 15.22) and adj $\bar{x}$ = 12.68, 95% CI (8.77, 16.58), respectively] [p = .575; adj $\bar{x}$ difference = 1.60, adj 95% CI (−4.09, 7.30), d = 0.15] and 0.250 mg/kg doses of Scopolamine [adj $\bar{x}$ = 24.53, 95% CI (18.00, 30.07) and adj $\bar{x}$ = 17.21, 95% CI (12.00, 22.42), respectively] [p = .059; adj $\bar{x}$ difference = 7.32, adj 95% CI (−0.28, 14.92), d = 0.5]. On the other hand, both the lowest [adj $\bar{x}$ = 3.98, 95% CI (2.90, 5.06) and adj $\bar{x}$ = 2.00, 95% CI (0.98, 3.02), for females and males, respectively] and the highest [adj $\bar{x}$ = 36.86, 95% CI (30.61, 43.12) and adj $\bar{x}$ = 23.65, 95% CI (17.76, 29.54), for females and males, respectively] doses significantly maintained this pattern of results [p = .01; adj $\bar{x}$ difference = 1.98, adj 95% CI (0.50, 3.47), d = 0.70 and p = .003; adj $\bar{x}$ difference = 13.22, adj 95% CI (4.62, 21.81), d = 0.80, respectively]. However, no significant effects were found concerning *Treatment* condition.

However, Scopolamine exposure generally increased impulsivity except at the lowest dose (Fig. 5c). A significant *Treatment x Dose* interaction was found in the main analysis [F(3.5192.1) = 2.585, p = .046; $\bar{x}$ = 17.73, 95% (13.3, 22.2), η_p^2 = 0.05]. Post hoc analyses revealed that the control rats were more impulsive than their exposed counterparts, but only following the largest dose of Scopolamine [adj $\bar{x}$ = 12.28, 95% CI (5.02, 19.54) and adj $\bar{x}$ = 25.97, 95% CI (19.13, 32.80), respectively] [p = .008; adj $\bar{x}$ difference = 13.69, adj 95% CI (3.71, 23.66), d = 0.72]. As observed for accuracy, CPF rats showed hyposensitivity to the Scopolamine challenge. This also reached significance following the largest dose, since the control rats showed a significant increase in their rates of impulsivity when compared with the rates observed at the lowest dose [adj $\bar{x}$ = 11.92, 95% CI (8.36, 15.47)] [p = .003; adj $\bar{x}$ difference = 14.05, adj 95% CI (3.39, 24.71), d = 0.93], a finding that was not observed in the CPF treated animals. Finally, Scopolamine reduced perseveration in a dose-dependent manner, which was not affected by either the exposure condition or the sex of the animals (Fig. 5d).

There was no influence of *Sex* following Alprazolam administration

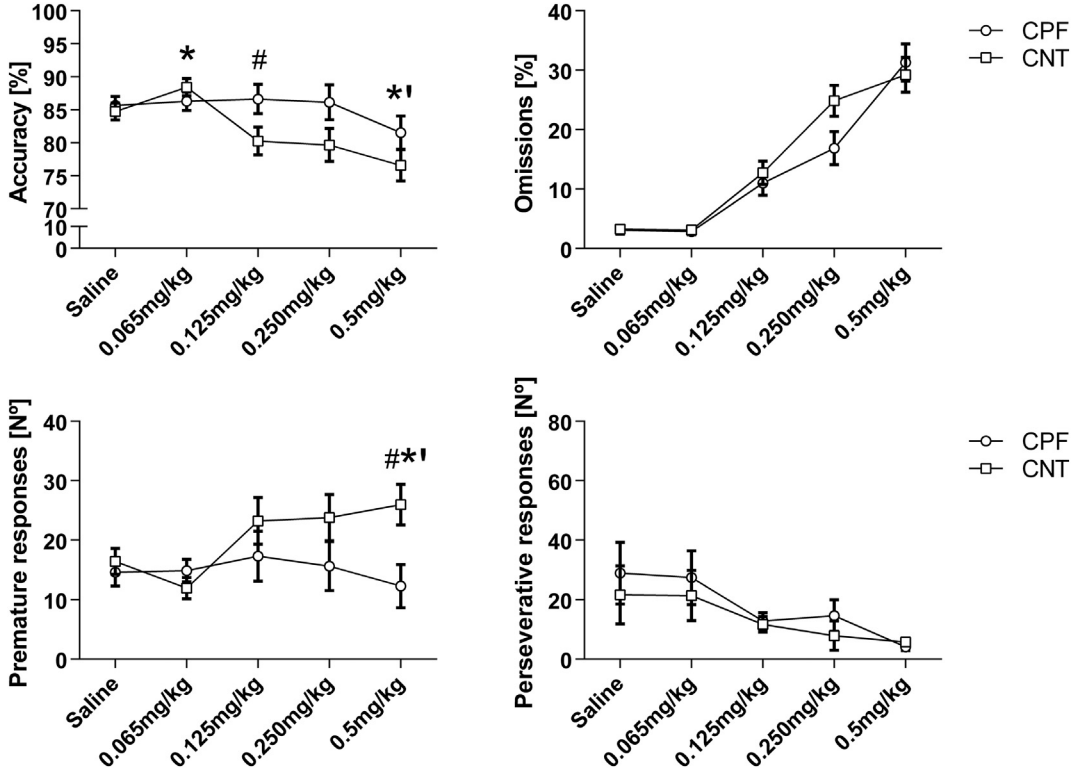

Fig. 5. Scopolamine. Effects of Scopolamine hydrobromide exposure on rats' (both males and females together) accuracy (a, up-left), percentage of omissions (b, up-right), premature (c, down-left) and perseverative responses (d, down-right). # indicates significant differences between CPF and vehicle treated rats. * indicates that the control rats significatively performed better when exposed to the lowest dose in relation to the rest dosages and saline. *' indicates that the control rats performed significatively worse following the largest dose in relation to saline. Significance was set at $p < .05$. Data are expressed as means and SEMs.

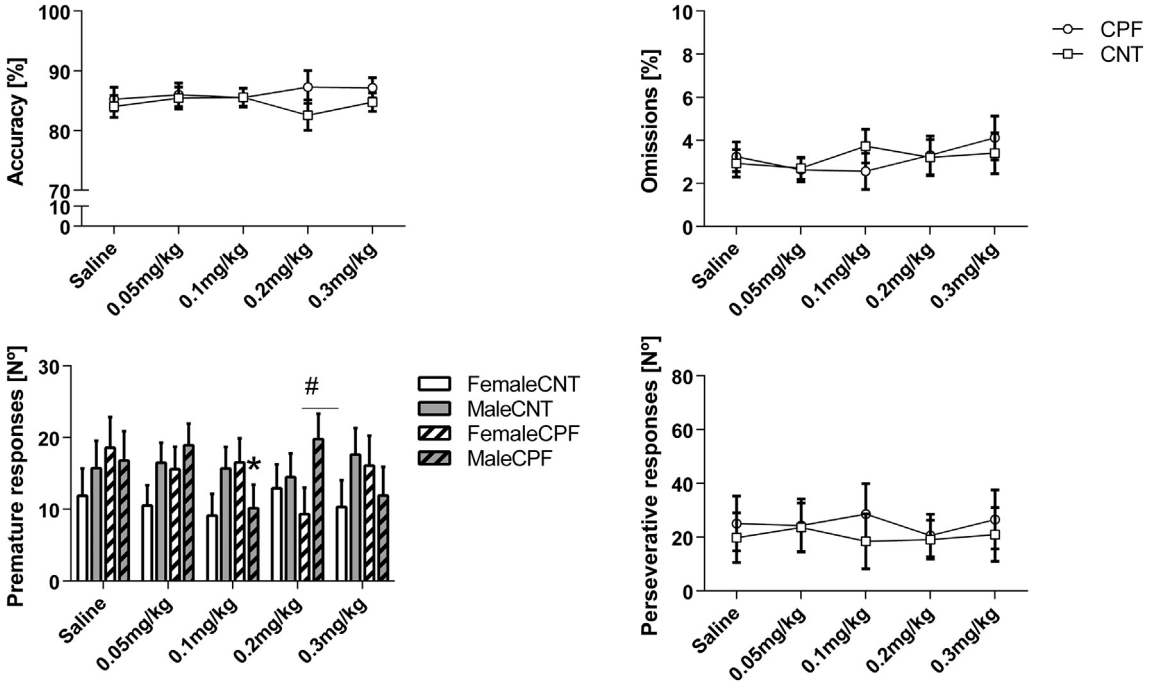

Fig. 6. Alprazolam. Effects of Alprazolam exposure on rats' (both males and females together except in Figure c) accuracy (a, up-left), percentage of omissions (b, up-right), premature (c, down-right) and perseverative responses (d, down-left). # indicates significant differences between CPF treated rats following 0.2 mg/kg of Alprazolam. * indicates significant decreased premature responses of the CPF treated males compared with their own rates at the lowest dose of the benzodiazepine. Significance was set at $p < .05$. Data are expressed as means and SEMs.

in the different behaviors analyzed except for the premature responses, thus the Fig. 6 (a,b and d) displays both male and female rats' performance together, while the Fig. 6c shows both sexes regarding the exposure condition. Alprazolam administration had little effect on accuracy rates, percentage of omissions and perseverative responding in relation to *Dose*, whilst no *Sex* and/or *Treatment* interaction was found

(Fig. 6a, b, and d). However, exposure to this drug altered premature responding (Fig. 6c), where a significant *Sex x Treatment x Dose* interaction was found in the main analysis [F(3.2163.4) = 2.775, p = .040; $\bar{x}$ = 14.34, 95% (10.90, 17.80), η_p^2 = 0.05]. Post hoc analyses revealed that CPF exposed males were more impulsive than their exposed female counterparts following 0.2 mg/kg dose of benzodiazepine [adj $\bar{x}$ = 19.77, 95% CI (12.65, 26.89) and adj $\bar{x}$ = 9.33, 95% CI (1.92, 16.75), respectively] [p = .047; adj $\bar{x}$ difference = 10.44, adj 95% CI (0.15, 20.72), d = 0.82], an effect that was not observed in the control rats at that dose [adj $\bar{x}$ = 14.47, 95% CI (7.83, 21.10) and adj $\bar{x}$ = 12.93, 95% CI (6.30, 19.57) for males and females, respectively] [p = .744; adj $\bar{x}$ difference = 1.53, adj 95% CI (−7.85, 10.91), d = 0.12]. In addition, exposed males also showed a decrease in premature responding rates following a 0.1 mg/kg dose [adj $\bar{x}$ = 10.15, 95% CI (3.63, 16.67)] when compared with the lowest dose [adj $\bar{x}$ = 18.92, 95% CI (12.86, 24.99)] [p = .048; adj $\bar{x}$ difference = 8.77, adj 95% CI (0.05, 17.49), d = 0.78], an effect that was not observed in any of the other groups.

3.4. Gene expression analyses. Long-term brain derived neurotrophic factor mRNA down-regulation following postnatal CPF exposure in males

In the male rats, CPF exposure induced a strong long-term down-regulation of the BDNF mRNA levels in the dorsal striatum [*Treatment*, F(1,7) = 14.721, p = .006; $\bar{x}$ = 0.84, 95% CI (0.69, 0.98), η_p^2 = 0.69; for control, adj $\bar{x}$ = 1.00, 95% CI (0.87, 1.14), and for CPF, adj $\bar{x}$ = 0.71, 95% CI (0.58, 0.83)] [adj $\bar{x}$ difference = 0.30, adj 95% CI (0.11, 0.48), d = 2.56], an effect that was not observed in females (Fig. 7). However, CPF exposure had little influence on the remaining genes studied here (Supplementary Figs. 1–14).

4. Discussion

Late postnatal, preweaning (PND10 to 15) exposure to NChEI doses of CPF induced long-term neurological and neurocognitive alterations concerning locomotor activity and the learning profile of female rats. In particular, altered attentional and impulsive behavior was revealed by both behavioral and pharmacological manipulations, along with down-regulation of the mRNA levels of BDNF at the dorsal striatum in males. This is, to the best of our knowledge, the first time that these long-term neurological effects have been associated with developmental CPF exposure, particularly following this dose range and postnatal stage, during which basic neurodevelopmental processes take place.

CPF decreased the speed of female rats, which were slower than control females and exposed males. Studies conducted during adolescence and/or adulthood have reported similar effects in rats following exposure to low-medium doses of CPF, for both speed and total distance covered (Adedara et al., 2018). Interestingly, the authors also found that the administration of Diphenyl diselenide (an antioxidant and neuroprotective compound) blocked these motoric alterations induced by CPF, pointing towards an AChE and lipid peroxidation-mediated effect. Whilst an association between decreased motor speed and OP exposure has received stronger empirical support from studies in adult humans (Mackenzie Ross et al., 2010; Starks et al., 2012; Malekirad et al., 2013; Steenland et al., 1994; Rosenstock et al., 1991), developmental research is sparse (Harari et al., 2010). In short, the present research extends previous findings in adults by demonstrating altered motor speed following CPF exposure at lower dosages and in the immature brain. However, the different variables associated with speed in the 5C-SRTT (latencies to correct, incorrect responses and/or reward) were not affected throughout the experiment according to the treatment condition (data not shown). This could be an initial sign of altered motricity in older ages, something that we have confirmed in our laboratory with the use of this exposure protocol (unpublished data). The abundant body of evidence showing motor alterations in multiple neurodegenerative pathologies observed in the clinical neurology field is unquestionable. Therefore, the present findings could be taken as further evidence of the relationship between exposure to external agents and the neurotoxicological profile during essential stages of development of the CNS along with the neurological sequelae that increase the likelihood of developing these types of disorders.

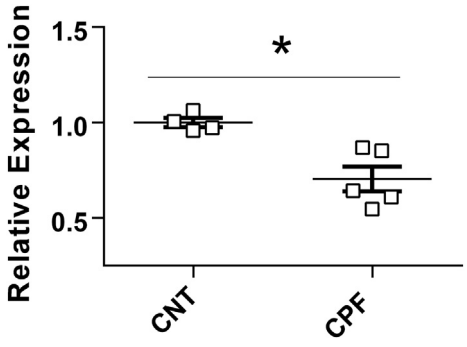

Fig. 7. BDNF mRNA relative expression. Effects of postnatal CPF exposure on dorsal striatum BDNF mRNA relative expression in 1-year old male rats. Expression data from one control male was discarded as the original Ct was non-reliable (> Ct30), thus the males' n = 9, (4 control and 5 exposed). * indicates significant differences between CNT and CPF rats. Significance was at set p < .05. All data are expressed as means and SEMs and individual plots are depicted.

The acquisition of the 5C-SRTT showed that learning was facilitated in the exposed females by improving their performance in the early/easier stages, generally reaching the levels of performance shown by control males, which could possibly be taken to indicate a certain degree of masculinization. Interestingly, Aldridge et al., 2005b described similar effects when assessing working and reference memory with rats following the same dosage, but at an earlier stage of development (PND1–4). Similarly, Levin et al. (2001) found that CPF exposure during this period altered learning in males and enhanced the performance of females, but no effects were observed as a consequence of late postnatal, preweaning exposure. This could indicate that the learning enhancing effects of low levels of CPF exposure can be triggered in a relatively extensive postnatal developmental window. Furthermore, their data showed the animals' behavior from PND 64 to 110 and from PND32 to 62 (approximately). In our case, the rats were aged 7-months at the beginning of the task, and 8-months during the acquisition phase. This completes previous Aldridge's conclusions on both critical stages and long-lasting effects and applies to other cognitive functions beyond memory to attention. All this information points towards conclusions that are compatible with the findings observed here; postnatal exposure to NChEI doses of CPF can alter learning abilities, a cognitive function generally disturbed in several neurological disorders (Disterhoft et al., 2004; Olson et al., 2019; Rahn et al., 2012).

Exposure to these NChEI doses of CPF during the postnatal, preweaning stage did not alter the basal performance of the animals in the present experiment, contrary to what has been observed when using other exposure protocols with larger doses during adulthood (Middlemore-Risher et al., 2010; Montes de Oca et al., 2013; Peris-Sampedro et al., 2016). However, hidden alterations were found following both behavioral and pharmacological manipulations. The increase of the ITI from 5 to 7 s increased the number of premature responses in the exposed rats compared with the control group. Although we were unable to find any other developmental study, Terry et al., (2014) found that exposure to low doses of diisopropylfluorophosphate (another OP agent) for 30 consecutive days in adulthood also increased the premature rates once exposure had finished (washout period) and only following an increased ITI. Thus, the results of both studies point to the possibility that exposure to both OPs can alter the animals' ability to control an increased demand for inhibition, a sign commonly observed in a multitude of neurological disorders (Bidzan et al., 2012; Ruitenberg et al., 2018; Irwin et al., 2007).

Concerning the pharmacological challenges, the administration of different doses of Scopolamine hydrobromide produced substantial alterations in both attentional and impulsive behaviors in control rats. However, rats exposed to CPF were insensitive to this challenge. We have previously found this hyposensitivity to a cholinergic challenge with Scopolamine in rats exposed to CPF following this same regime but at younger ages and measuring locomotor outcomes (Perez-Fernandez et al., 2019b). Interestingly, Levin et al. (2001) found that low doses of Scopolamine triggered completely different effects depending on sex and exposure conditions in a memory task in animals exposed during this preweaning stage, although they used 5 times the dosage that we administered in the present study. Increasing drug dose generated little changes in male rats, regardless of whether or not they were exposed to CPF. However, control female rats made more errors as the dose increased, whilst the opposite pattern of results was observed for the exposed females. The results of both studies point towards the possibility that exposure to NChEI doses of CPF during this developmental period hypersensitizes the cholinergic system in the long-term, possibly by the modulation of other components of the cholinergic system [i.e. M2 receptors as previously proposed (Perez-Fernandez et al., 2019b)] and/or the assumption of cholinergic functions by other neurotransmitter systems (Levin et al., 2001; Aldridge et al., 2005a, 2005b). Given the fact that certain neurodegenerative disorders such as AD are commonly characterized by a mismatched and progressively deteriorating cholinergic system (Hampel et al., 2018), the hypoactivation of this system observed in exposed animals could make theoretical sense and have clinical consequences.

The GABAergic system plays an important role in many neurological diseases (i.e. epilepsy), and psychiatric disorders (i.e. anxiety) (Ting Wong et al., 2003). Furthermore, it has been linked to different neurodegenerative disorders (Calvo-Flores Guzmán et al., 2018; Błaszczyk, 2016). Interestingly, this system has also been linked to specific types of impulsivity (Ucha et al., 2019; Schulte et al., 2017), but its role in CPF neurotoxicity is less clear (Montes de Oca et al., 2013; Sánchez-Amate et al., 2002; Cardona et al., 2006; Gómez-Giménez et al., 2018; Perez-Fernandez et al., 2019b). In terms of the GABAergic challenge described in the present manuscript, Alprazolam exposure increased premature responding in exposed (CPF) males compared with exposed (CPF) females following a dose of 0.2 mg/kg, whilst the control groups behaved similarly at that dose of Alprazolam. Interestingly, 0.1 mg/kg decreased the impulsivity rates of the exposed males from the lowest dose, an effect that was not observed at any other dose and in any other group. This indicates the selective profile of Alprazolam with regard to the dosage used, making males more susceptible to showing failures of inhibition than females when the GABAergic system is challenged.

This exposure protocol has previously been linked to a hypersensitized GABAergic system by using Alprazolam in an open field test (Perez-Fernandez et al., 2019b), and still represents the only developmental CPF study, along with that of Gómez-Giménez et al. (2018), which analyzed the state of the GABAergic system following administration of NChEI. However, in the case of our previous report, the hypersensitivity was clearer and primarily observed in exposed female rats. Given the different nature of the behaviors analyzed in this study and that of Perez-Fernandez et al. (2019b), along with the different doses of Alprazolam used, these differences are not surprising.

From all the genes analyzed in the dorsal striatum of 12 months-old rats, we found that CPF exposure significantly decreased the expression of one of these in males, that is, the brain-derived neurotrophic factor (BDNF). BDNF is the most important neurotrophic molecule in the mammalian CNS and plays an essential role in its adequate functioning in terms of brain plasticity and cell differentiation (Lykissas et al., 2007). BDNF levels have been also linked to specific alterations in motricity, attention and impulsivity rates, both human (MacHughen et al., 2010; Corominas-Roso et al., 2013; Martinotti et al., 2015) and experimental rodents (Boger et al., 2011; Snigdha et al., 2011; Weston et al., 2014). Alterations in this family of neurotrophins have been linked to several neurological pathologies -also linked to CPF exposure- where altered attention and or increased impulsivity are the core symptoms, as in the case of AD (Fumagalli et al., 2006a), PD (Fumagalli et al., 2006b), ASD (Skogstrand et al., 2019), amongst others (Bathina and Das, 2015).

Only a few studies have analyzed the effects of CPF exposure on BDNF expression and/or activity in adulthood (Lee et al., 2016; Mahmoud et al., 2019), whilst three studies have examined these effects following developmental exposure (Betancourt and Carr, 2003; Slotkin et al., 2008), including preweaning stages (Betancourt et al., 2007). With regard to the developmental studies, Betancourt and Carr (2003) exposed the rats to 1.5 or 3 mg/kg/day of CPF from PND1 to 6 and found no effects on BDNF activity in the forebrain of pups after either 4 consecutive days of exposure or 1 or 6 days after exposure. Similar early exposure (1 mg/kg/day from PND1 to 4) was given by Slotkin et al. (2008). These authors found that CPF exposure both up (differentiated cells) and down-regulated (undifferentiated cells) gene expression in cell culturing by in vitro procedures, but not in in vivo procedures. Finally, and of particular interest for our current work, the preweaning (4 or 6 mg/kg/day from PND10 to 20) study conducted by Betancourt et al. (2007) found that CPF triggered a generalized up-regulation of both mRNA and protein (unbounded) expression in the hippocampus and cortex. Interestingly, total BDNF protein levels increased in the hippocampus and decreased in the cortex. The disparity between the results of these studies, some of them related to the present work, could be due to differences in methodology, particularly the dosage of CPF used, the time of exposure, and the time lapse between exposure and sample collection. The present study is the first to demonstrate long-term (12 months after exposure) alterations in BDNF gene expression in the brain, more specifically in the dorsal striatum.

Finally, we did not find differences in the mRNA expression levels at the dorsal striatum for most of the analyzed genes concerning CPF exposure. These genes are part of the cholinergic (M2 receptor, Nicotinic α7 receptor, ChAT, and VAChT), GABAergic (GABA-A-α1/2 receptor isoforms, GAD1/2, and KCC1/2) and serotonergic systems (5HT2a and c receptors). Alterations of these cholinergic genes and/or their respective proteins have been previously linked to the developmental exposure to CPF (e.g. Perez-Fernandez et al., 2019b; Basaure et al., 2019; Guardia-Escote et al., 2018; Slotkin and Seidler, 2007). In the case of the GABAergic genes, there is, to the best of our knowledge, only one developmental study that found that CPF exposure up-regulated the α2 isoform of the GABA-A receptor (Perez-Fernandez et al., 2019b), remaining the least studied neurotransmitter system concerning CPF exposure by far. Finally, other empirical works have found selective alterations in the 5-HT2 receptor and isoforms (e.g. Slotkin and Seidler, 2007; Aldridge et al., 2004; Aldridge et al., 2005a). The lack of effects in the present research may be due to important methodological differences such as the developmental period of exposure, the dose of CPF used and the animals' age when the samples were collected.

5. Conclusions and future guidelines

Exposure to low NChEI doses of CPF during the late postnatal, preweaning neurodevelopmental window induced long-term alterations in various behavioral and molecular outcomes that are essential features of certain neurological and neurodegenerative pathologies. These alterations were characterized by an enhanced learning profile and decreased speed in the exposed females, increased impulsivity when some features of the behavioral task were manipulated (ITI), general hyposensitivity to a cholinergic challenge (Scopolamine) in both attentional and impulsivity-related outcomes, a pattern of increased premature responding following specific doses of Alprazolam in exposed males and, finally, a significant down-regulation of the BDNF mRNA expression levels in the dorsal striatum of the exposed males in comparison with their control counterparts. All of this information is novel in terms of the exposure protocol chosen, and primarily extends

and confirms previous observations following the use of other doses and/or other developmental windows. Future research should provide a more in-depth analysis of the molecular bases of these changes and extend the gene analyses to other brain regions.

Authors' contribution

C Perez-Fernandez completed the experimental procedures and animal care, the statistical analyses and wrote the first version of the manuscript. M Morales-Navas helped in the experimental procedures and animal care and improved the manuscript quality with his revision. L Guardia-Escote and E Giménez helped with the molecular procedures and improved the final version of the manuscript. MT Colomina and F Sánchez-Santed conceptualized the experimental design, acquired the funding, provided all the resources needed for the resolution of the present research, and improved the final version of the present manuscript with their revisions.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.expneurol.2020.113356.

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Supplementary material

Table 1.

	Control (mean + SEM)	CPF (mean + SEM)	ANOVA	Adj mean differences	Adj 95% CI for mean differences
Time in movement	476.9 ± 18,5	460.28 ± 19.6	F(1,55)= 0.380, p= 0.540	16.60	-37.34, 70.54
Distance traveled	6260.81 ± 299.6	5963.76 ± 318.3	F(1,55)= 0.462, p= 0.500	297.05	-578.91, 1173
Margin time	1287.5 ± 30.7	1231.06 ± 32.6	F(1,55)= 1.592, p= 0.212	56.47	-33.24, 146.18
Rearing frequency	795.76 ± 41.4	792.63 ± 44	F(1,55)= 0.003, p= 0.959	3.14	-117.98, 124.26

Supplementary Table 1. Influence of CPF exposure on total distance (cm), time in movement (sec), time in margin (sec), and vertical activity (frequency). Statistical output of the ANOVA is displayed for *Treatment* factor. Statistical significance was set p< 0.05. Data are expressed as means, SEMs, adjusted mean differences and adj 95% confidence interval (CI) for the mean differences.

Table 2.

	Female CNT	Male CNT	Female CPF	Male CPF	Outcome
Accuracy (%)					
SD0.25	53.12 ± 2.47	54.26 ± 2.33	58.05 ± 1.94	57.59 ± 2.51	
SD0.5	71.88 ± 2.42	69.74 ± 2.82	73.25 ± 1.90	71.56 ± 2.84	
SD1	76.76 ± 2.87	84.08 ± 1.64	79.79 ± 4.00	81.77 ± 2.12	
SD1.5	84.89 ± 1.09	83.98 ± 2.40	90.13 ± 1.60	86.89 ± 1.69	F- SD1.5>1
Omissions (%)					
SD0.25	15.65 ± 2.16	5.72 ± 1.36	12.66 ± 2.08	5.26 ± 1.27	F- SD0.25>1
SD0.5	7.60 ± 1.15	2.75 ± 0.84	5.59 ± 1.11	4.13 ± 0.84	
SD1	8.80 ± 2.17	3.71 ± 1.02	6.36 ± 1.59	2.75 ± 0.74	
SD1.5	7.28 ± 2.18	2.65 ± 0.99	5.18 ± 0.83	1.75 ± 0.41	
Premature responses (Nº)					
SD0.25	23.13 ± 2.99	37.50 ± 4.21	31.55 ± 5.04	32.19 ± 3.67	M- SD0.25>1
SD0.5	23.63 ± 3.36	30.50 ± 3.97	27.91 ± 3.11	25.94 ± 3.97	M- SD0.5>1
SD1	20.06 ± 2.98	16.07 ± 2.45	29.91 ± 3.87	23.06 ± 3.79	
SD1.5	18.63 ± 2.64	30.14 ± 6.60	24.82 ± 4.24	21.44 ± 3.39	
Perseverative responses (Nº)					
SD0.25	5.63 ± 1.74	7.64 ± 2.60	5.18 ± 1.93	11.69 ± 5.14	
SD0.5	15.81 ± 5.11	13.93 ± 5.21	7.73 ± 3.07	22.75 ± 9.52	
SD1	15.38 ± 4.00	18.21 ± 4.93	10.18 ± 2.95	24.38 ± 10.34	
SD1.5	15.88 ± 4.84	16.79 ± 4.86	10.36 ± 3.32	21.38 ± 9.11	

Supplementary Table 2. Influence of stimulus duration (SD) manipulation on 5C-SRTT performance. Statistical significance was set p< 0.05. Data are expressed as means and SEMs.

Figure 1.

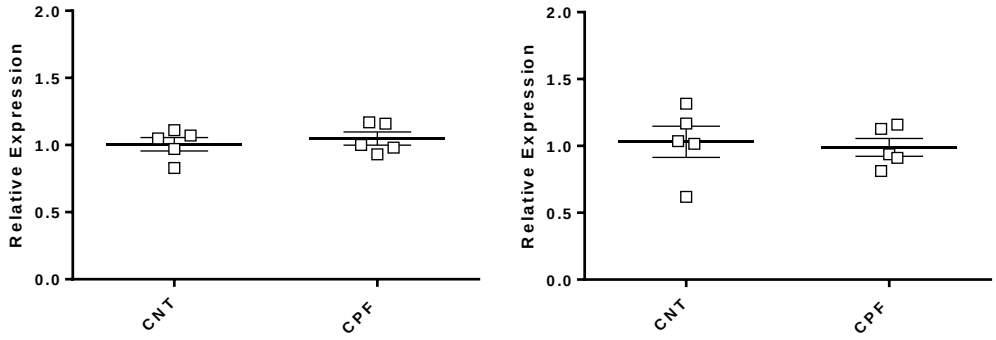


Figure 2.

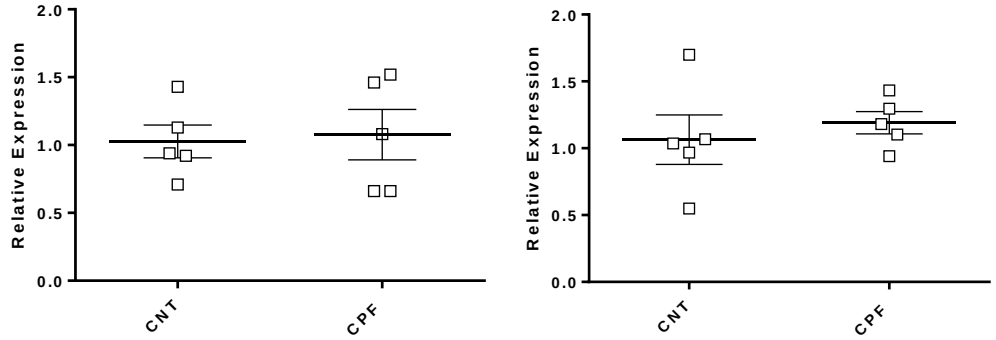


Figure 3.

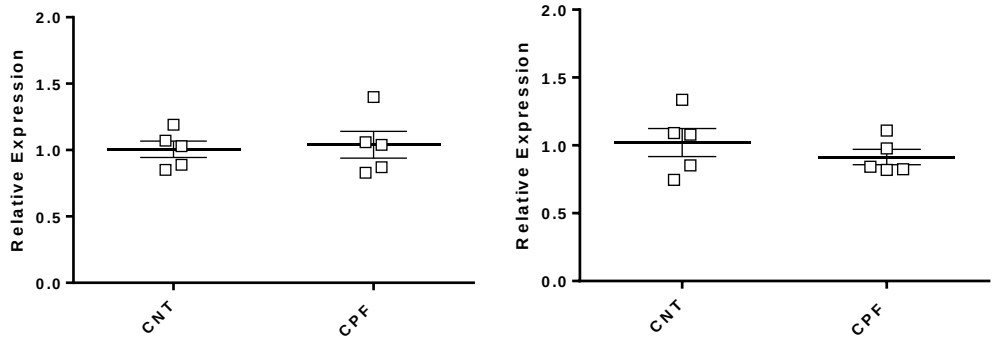


Figure 4.

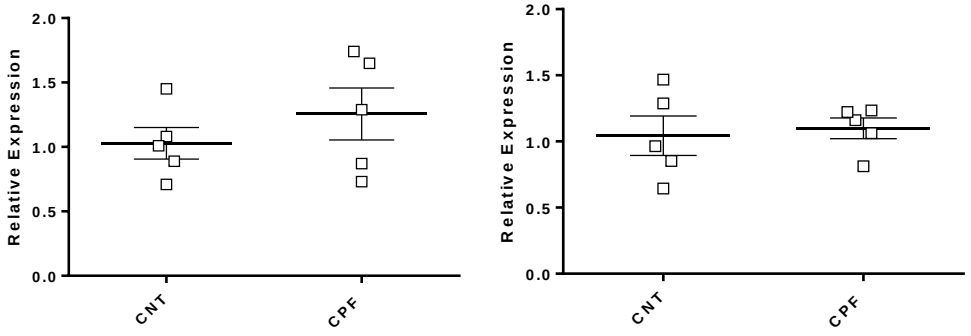
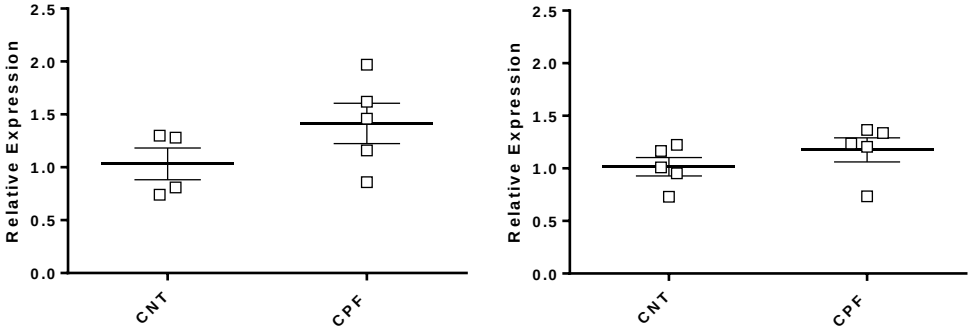


Figure 5.



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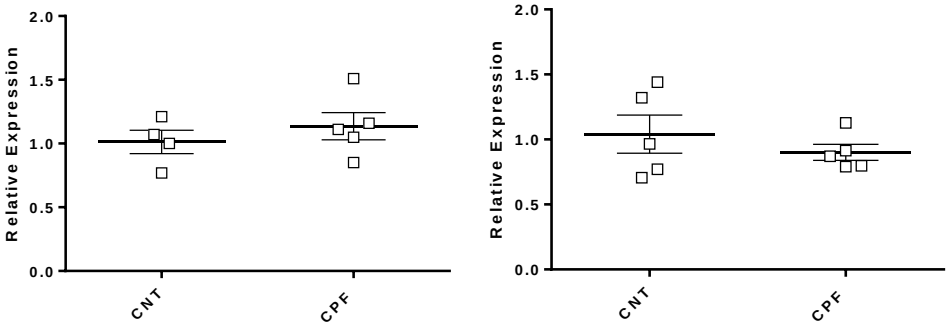

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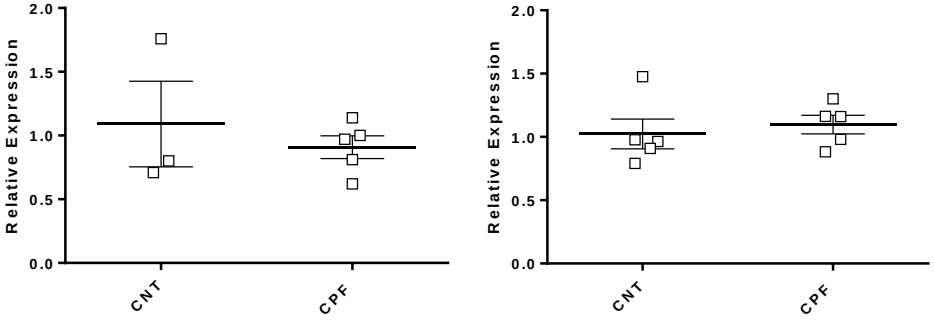

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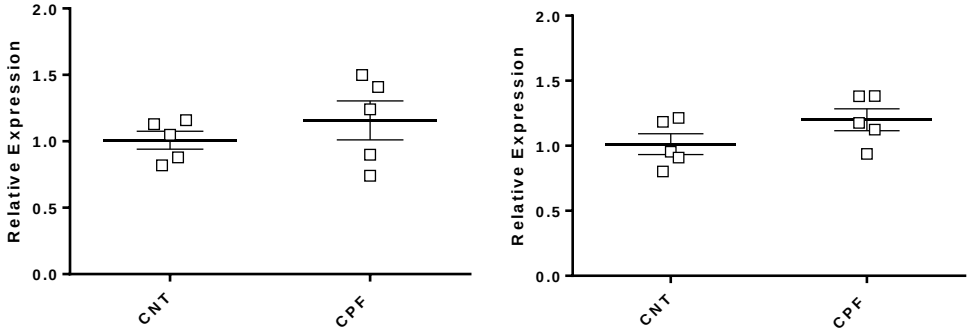

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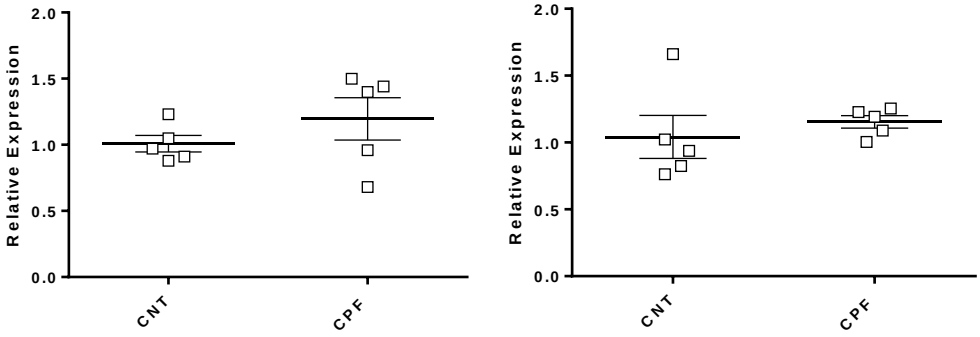

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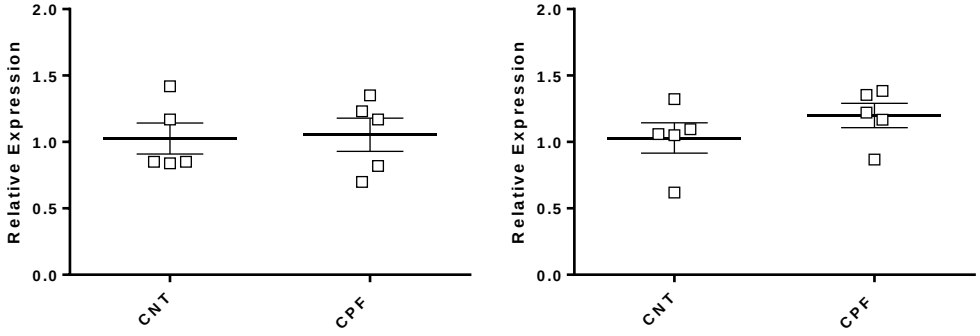

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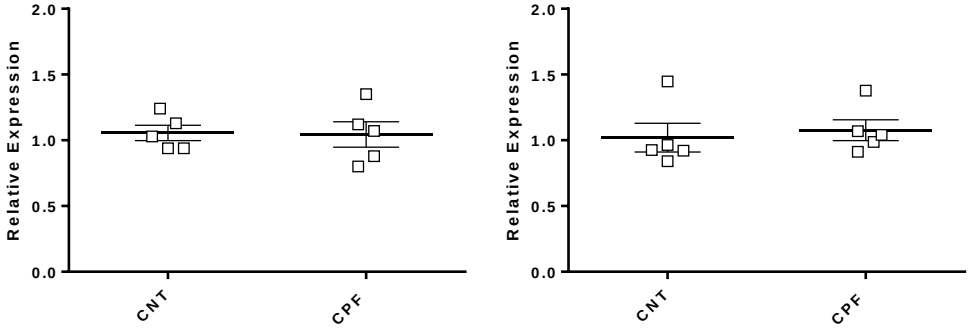

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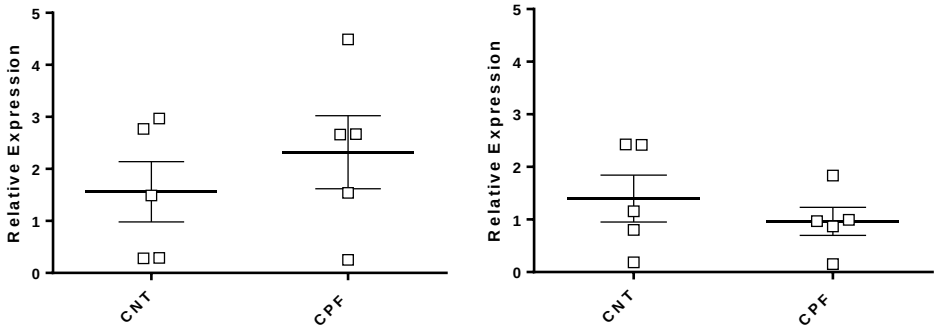

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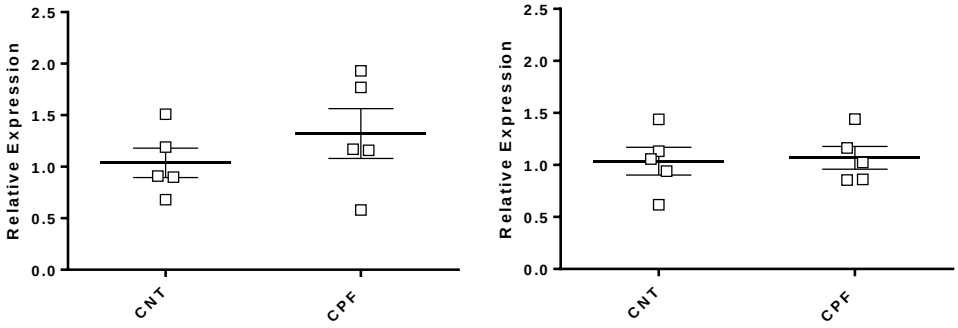

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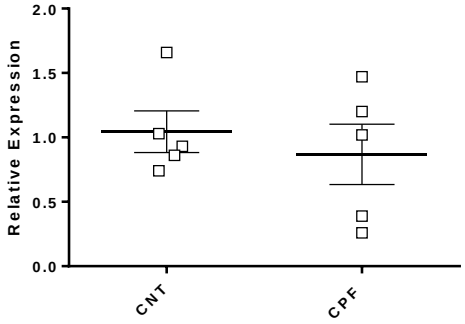

Figures 1-14. RT-qPCR analyses. Relative expression (mRNA) in rats' dorsal striatum of M2 receptor (1), ChAT (2), Nicotinic 7 receptor (3), VAChT (4), AChE-S (5), GABA-A-α1 subunit (6), GABA-A- α2 subunit(7), GAD1 (8), GAD2 (9), KCC1 (10), KCC2 (11), HT-2a receptor (12), HT-2c receptor (13), and BDNF (14) genes of both female (left) and male (right) 12-months old rats. Only the BDNF data referred to the female animals are displayed as the males' figure is displayed in the manuscript. Data are expressed as means and SEMs. Individual plots are depicted.

STUDY 4

Pesticides and aging: preweaning exposure to Chlorpyrifos induces a general hypomotricity state in late-adult rats.

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Please, **do not use color** for any figures and tables in print

1. Introduction

The period from the late-adulthood to the old age is a stage in the human life characterized by the progressive decline of different physical and cognitive domains (Goodpaster et al., 2006; Harada et al., 2013). This common decline depends on the lifestyle assumed by the people, where the continuous stimulation of the physical (e.g. regular aerobic exercise) and cognitive (e.g. studying, reading and other intellectual occupations) functions generally lead to a more attenuated and less severer altered profile (Barnes JN, 2015; Kramer et al., 2006). Added to this, some of the most disabling disorders concerning the malfunctioning of the central nervous system (CNS) begins to develop their early clinical symptoms in these ages, as it is the case of neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson disease (PD), amongst others (Hou et al., 2019).

Both aging and, especially, the mentioned neurodegenerative disorders have different altered behaviors as their main clinical features. From these, alterations in memory function are generally considered as the most disable and earlier altered (Jahn H, 2013; Chiaravalloti et al., 2014), along with motricity (Scarmeas et al., 2004; Armstrong and Okun, 2020). Furthermore, depression and anxiety-related symptomatology are also commonly observed within these ages and disorders (Cortés et al., 2018; Aminian and Strafella, 2013). Interestingly, many of these alterations in the normal aging, as well as the most important clinical features of the indicated disorders, can be generally early detected at late adulthood, namely early-onset development (Filley et al., 1986), at even younger ages in the case of Parkinson's disease (Giovannini et al., 1991).

The developmental profile of the "normal aging" as well as the different neurodegenerative disorders has an important genetic root which controls the final physiological and behavioral output and their evolution (Rodríguez-Rodero et al., 2011). However, the context plays an essential role in the regulation of these genetic vulnerabilities, as previously mentioned. The continuous exposure to different xenobiotic agents is gaining especial interest in the last decade (e.g. Kim et al., 2015; Paul et al., 2018), amongst all the different environmental factors presumable involved. From these, the organophosphate compounds (OP) are a group of neurotoxicological agents generally used as pesticides for multiple targets (Gupta RC, 2006). Chlorpyrifos (CPF) is one of the OP pesticides most widely used worldwide from decades ago (Eaton et al., 2008).

CPF, like the rest of OP compounds, exerts its neurotoxicological profile by irreversibly inhibiting the Acetylcholinesterases (AChE), eminently in the CNS, which triggers a general increase in the cholinergic tone in both the central and the peripherical nervous systems (Eaton et al., 2008). However, alternative molecular targets have been proposed (e.g. Burke et al., 2017). Some of these alternative mechanisms comprise several proteins from the most important neurotransmitter systems, and are unrelated to the inhibition of the Cholinesterases (ChE) as the doses used were well below those needed to induce systemic toxicity (NChEI profile), especially when exposed during sensitive period to CPF toxicity like development (some of the most important studies are summarized in Perez-Fernandez et al., 2019).

Regarding the behaviors described here, developmental CPF exposure has been linked to long-term changes in learning and memory (Levin et al., 2001, 2002; Jett et al., 2001; Aldridge et al., 2005; Icenogle et al., 2004; Johnson et al., 2009; Cole et al., 2012; Gómez-Gimenez et al., 2017; Guardia-Escote et al., 2018, 2019; Wang et al., 2018 Basaure et al., 2019a; Alipour et al., 2019), anxiety (Peris-Sampedro et al., 2020; Carr et al., 2017, 2020; Silva et al., 2017; Venerosi et al., 2008, 2010; Braquenier et al., 2010; Ricceri et al., 2006) and motricity (Please see Perez-Fernandez et al., 2019) in rodents. However, the molecular and behavioral effects induced by the exposure to NChEI doses of CPF during development depends on the specific developmental stage that it occurs (Eaton et al., 2008). This makes essential the study of the effects of CPF exposure at different NChEI dosages into the different developmental windows.

The postnatal, preweaning exposure protocol [around postnatal day (PND) 10], is especially relevant based on its translational meaning and the specific neurodevelopmental benchmarks that takes place on it (defined in the methods' section). However, the study of the long-term effects of NChEI doses (<1mg/kg/day) of CPF during this preweaning stage is sparse (for good examples, please see Carr et al., 2011, 2013, 2014, 2015, 2020) and it counts with less empirical evidence compared to the early postnatal and, especially, the gestational stages. Finally, the exposure to this dose on different behavioral and molecular outcomes during this developmental period in the rats' memory, anxiety and motricity functions at late-adulthood (< 15 months-old) has not been explored yet, focusing this assessment to as old as 9 months-old (Guardia-Escote et al., 2018).

Based on the information provided, the specific analysis of the influences of a ubiquitous toxicological compounds such as CPF is justified. For this reason, we wanted to know if the exposure to NChEI doses of CPF during this preweaning stage (that is, exposure to low doses during a short period of time) can be linked to specific deeper alterations in memory, anxiety and motricity in late-adult rats, ages at which the early clinical features of the different pathologies and the initial evidences of dysfunctional aging take place in. We consider that even low doses during short periods like this will be enough to induce long-term subtle alterations on the above-mentioned behaviors at ages where these deficits are commonly unmasked.

2. Materials and methods

2.1. Experimental animals

The present study used late adults aged wistar rats (> 15 months-old), both males and females (n=16 and 15, respectively). Briefly, a total of 19-timed pregnant female rats arrived at our laboratory 5 days before the expected delivery date, housed individually. The day of delivery was considered as the postnatal day 0 (PND0). At PND1, all pups were separated from their biological mothers, randomly mixed and pseudo-randomly distributed between dams (n=10, 5 males and 5 females per mum). At PND10, the pups were randomly assigned to the control condition or CPF, with around the half of the pups of each sex from each dam assigned to one condition or the other (n= 2-3 per sex/per dam exposed to CPF). At PND21, all the pre-adolescent rats were weaned 4 per cage of the same sex, with a proper representation of both treatment conditions in each home cage (2 control and 2 CPF exposed animals per cage). A total of 7 females (8 control rats) and 8 males (8 control rats) were exposed to CPF. Animals received a maintaining diet of 17g and 20g (females and males, respectively) from the age of 7 months old, consuming water ad libitum. Concerning the home room living parameters, the temperature was set 22ºC±2 and humidity 50%±10 for the whole live of the rat. Animals had a 12 light our cycle (lights on at 8:00h). The **Image 1** summarizes the whole experimental protocol described in the present manuscript. This work is part of the project ES040130002260. It was conducted in accordance with the Spanish Royal Decree 53/2013 and the European Community Directive (2010/63/EU) for animal research. The present study was also approved by the Animal Research Committee from the University of Almería.

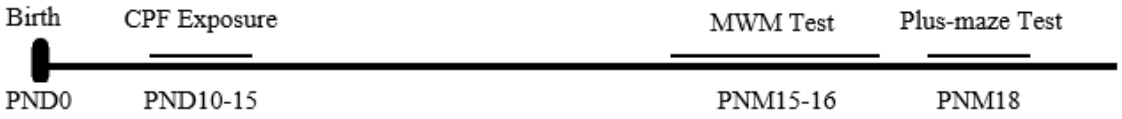

Image 1. Experimental procedure. Rats' day of birth was considered as the postnatal day (PND) 0. Both male and female pups were randomly assigned to CPF or vehicle exposure from PND10 to 15. At the age of 15 months-old (PNM15), rats begun the Morris Water maze testing (MWM) and completed the last phase with around 16 months of age (PNM16). At the age of 18 months-old (PNM18), the rats' anxiety-like and motor performance was assessed in the Plus-maze test.

2.2. Neurotoxic agent and exposure protocol

Chlorpyrifos (CPF) [O, O-dietil O-3,5,6-trichloropyridin-2-il fosforotioato (Pestanal, Sigma Aldrich)] was orally administered from PND10 to PND15, both inclusive. Animals from both sexes were randomly assigned to vehicle (corn oil) or CPF, using a dose of 1mg/kg/mL per day. The importance of this stage in neurodevelopmental terms has been previously defined (Perez-Fernandez 2019, 2020a and b). Briefly, during this period different neurological processes reach their developmental peak (synaptogenesis, myelination, glial cells growth, oxytocin and vasopressin systems maturation, amongst others) and PND7-10 in rats is, at some degree, equivalent to around birthday in humans in neurodevelopmental terms (Venerosi et al., 2006; Semple et al., 2013; Tait et al., 2009; Tau and Peterson, 2010). Added to this, this preweaning period is the least studied compared other gestational and early neonatal periods. CPF was diluted in corn oil as this vehicle facilitates the absorption rates of the OP (Timchalk et al., 2002), using it for the control condition in the same volume (1mL/Kg). There is empirical evidence that this dose does not cause a significant inhibition of the ChEs (Savy et al., 2015), also when exposed during this developmental period (e.g. Carr et al., 2011, 2015) and empirically corroborated in animals from this same cohort in previous publications both for frontal ChEs (Perez-Fernandez et al., 2019) and AChEs (Perez-Fernandez et al., 2020b). Finally, this dosage is defined as the No-Observed-Adverse-Effect-Level concerning sub-chronic oral exposure regimens in the rat (World Health Organization, 2009).

2.3. Behavioral procedures

2.3.1. The Morris Water Maze task

2.3.1.1. Description of the apparatus

A black pool (50cm height, 150cm diameter) was used in the present study. Briefly, the pool was filled around 1.5cm above a black platform. The pool was digitally divided in 4 quadrants. The black platform was always placed in the same position for every single animal, although 2 different (opposite) positions were used in the present study (half of the animals of each sex and exposure condition were trained in one of them and the remaining in the other) to control position bias. Different external stimuli were distributed around the pool in order to facilitate contextual cues. Water temperature was always maintained at 22ºC. All animals were driven to the testing room 1 hour before the beginning of the test. The testing was always done between 9h and 13h. The temperature and humidity of the testing room was the same as described for the home room living conditions. Illumination of the testing room was set as dim-light.

2.3.1.2. Experimental procedure

Animals were around 15 months-old at the beginning of the test, and they finished it with around 16 months-old. The MWM protocol described here follows the guidelines defined in de Bruin et al., (1994), with some minor changes. Animals were driven to the testing room 1 hour before the beginning of the test. The experiment was divided in 5 stages: *Acquisition, Probe, Reinstating, Reversal learning* and *Visual control stages*. Feces were removed after every single trial. The order of the animals was counterbalanced for exposure condition in order to avoid hour of the day bias. The *Acquisition* (original spatial learning) was completed after 8 consecutive sessions of 4 trial per session, the *Probe* (reference memory) was a single trial of 30 seconds, the *Reinstating* was a session of 4 trials and the *Reversal* (perseveration, inflexibility and new learning) and *Visual* (visual function integrity) *stages* were characterized by 3 sessions of 4 trials each session. Every trial consisted in leaving the rat at some of the 4 quadrants (pseudo-randomly selected and different depending of the session, but the same within each session) and letting it to freely explore the pool. As the water is an aversive context for rats, it will try to escape, being the hidden platform the only opportunity to this. The time between the animal's dropping into the pool and it reaches the platform is the escape latency. Each trial had a time-limit of 90 seconds. If the animal did not reach the platform within this period, it was placed on the platform for 30 seconds. Once the animal escaped (or is placed in the platform) and the 30 seconds period was finished, the animal was briefly dried and let resting for another 30 seconds and once again placed into the pool for a new trial, completing 4 trials per day.

Once the animals learnt the task after 8 consecutive days of *Acquisition*, the platform was removed and the time of exploring behavior on each quadrant was assessed for 30 seconds, named the *Probe stage*. Following this, another session composed of 4 trials was completed using the same platform localization as the *Acquisition stage*, namely the *Reinstating stage*. Once animals mostly recovered their learning rates, the position of the platform was changed to the opposite quadrant regarding the original position in the *Acquisition stage*, forcing to a new learning process (*Reversal stage*) for 3 consecutive sessions, 4 trials per session. Finally, the visual performance of the animals was analyzed in order to discard CPF-induced alterations in this level, namely *Visual control stage*. The level of the water was reduced, letting visible around 1.5 cm of the platform. The external border of the platform was marked with a clear grey adhesive to facilitate the object visualization. The animals completed this phase after 3 consecutive sessions, 4 trials per session. Contrary to the other stages, the position of the platform was changing across from trial to trial.

The main dependent variables in the MWM were the escape latencies (sec) for learning and spatial memory performance and total distance covered (cm), velocity (cm/sec) and immobility (frequencies) for locomotor activity state control. The escape latency was also used as a control variable in the *Visual stage* but concerning the visual system function integrity. All this data was automatically recorded and analyzed with the software Ethovision® version 3.1. (Noldus).

2.3.2. The Plus-Maze test

2.3.2.1. Description of the apparatus

For the present experiment, a standard plus-maze apparatus was used. Briefly, the apparatus consisted in 4-armed structure with a center square. 2 of these arms had walls around them (closed arms), while the remaining 2 were only composed by the floor (open arms). Within this test, the stress situation is defined by the conflict triggered by the pro-exploratory instinct of the rat and the aversive situation generated by the lack of physical protection in the open arms vs. the closed arms. That is, the anxiety state of the rat is linked to the time that it is in a lesser-protected, open space (open arms) vs. the time in the protected space (closed arms). The longer the animal spends is in the open arms, the lesser anxious is. All animals were driven to the experimental room 1 hour before the beginning of the test. Temperature and humidity were set following the home room conditions. Illumination was set as Dim-light.

2.3.2.2. Experimental procedure

The rats were 18 months-old at the time of the Plus-maze assessment. The animals were driven to the testing room and habituated to the room for 1 hour. Following this, they were introduced to the center of the paradigm and their behavior was recorder only once for 5 minutes. The order of the animals was counterbalanced for exposure condition in order to avoid hour of the day bias. The anxiety-related dependent variables were the total time in the open arms and the number of entries in the open arms. The motor control variables were the number of entries in the closed arms (frequencies), the total distance covered (cm), the immobility (frequencies), velocity (cm/s) and, eminently and as a general motor factor, the general mobility rate (sec). All these variables were automatically recorded and analyzed with Ethovision® version 3.1. (Noldus).

2.4. Statistical analyses

For the WMW experiment, both the escape latency and the motor variables (total distance covered, velocity and immobility) were equally analyzed depending on the specific stage. A repeated measures Analysis of the Variance (rmANOVA) was conducted for the *Acquisition stage*, with *Session* and *Trial* as the within-subject variables (with 8 and 4 levels, respectively) and *Sex* and *Treatment* as the between-subject variables (with 2 levels each one). These between-subject variables were subsequently used in all the analyses. a two-way ANOVA was conducted for the *Probe stage*, with the above-mentioned factors. Another rmANOVA was conducted for the *Reinstating stage*, as this session was compared to the latest session of the *Acquisition stage*, with a within-subject variable *Session* (with 2 levels, session 8 and reinstating) and *Trial* (4 levels) and the mentioned between-subject factors. Both the *Reversal learning stage* and the Visual stage were separately analyzed with a rmANOVA, with *Session* and *Trial* as the within-subject variables (3 and 4 levels, respectively). For the Plus-Maze experiment, both anxiety and motor-related behaviors were equally analyzed with individual two-way ANOVAs for every single variable (time in open arms, entries to open arms, entries to closed arms, total distance covered, velocity and general mobility), with the two above-mentioned variables as the between-subject factors. All analyses were carried out with the SPSS® software version 24 (IBM). Significance threshold was set <0.05. Figures were designed with GraphPad Prism® software version 6.1. Statistical description of the significant data is described within the text. Mean and SEMs are displayed in the Figures.

3. Results

3.1. CPF and Spatial memory in pre-old age rats.

3.1.1. MWM. Cognitive variables

All animals learnt the position of the platform by the sessions, needing around 76 seconds in the first session to 18 in the latest (**Figure 1**). The ANOVA showed a significant effect concerning the variable *Session* [F(7,189)= 49.788, p<0.001]. Post hoc analyses revealed a significant improve (reduced escape latencies) of all the sessions from the session 1 (always p<0.001). However, there was not influence by neither *Treatment x Session* nor *Treatment x Sex x Session interactions.*

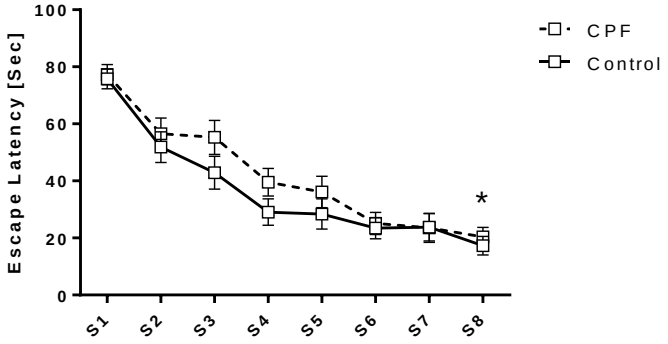

Figure 1. Acquisition of the MWM. S1-8 defines the sessions 1-8. * indicates that all rats needed less time to reach the platform at the S8 compared the first session. Data are expressed with means and SEM.

Added to this lack of effects on learning performance, spatial memory consolidation was not affected based on the treatment condition assessed in the Probe test stage (**Figure 2**). There were not influences of exposure condition, *Treatment x Session* nor *Treatment x Sex x Session* interactions.

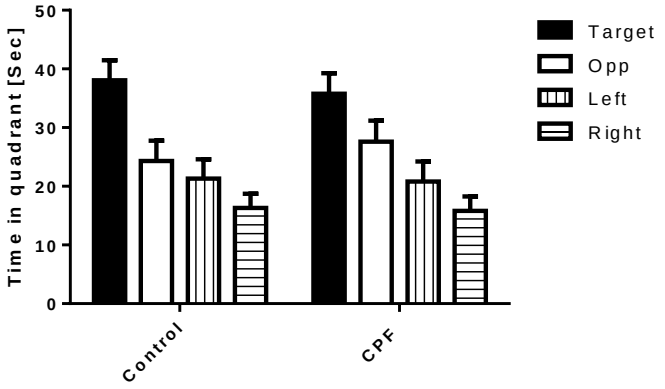

Figure 2. Probe stage. Time exploring each quadrants once the platform is removed for a 30-sec period. Target quadrant represent the section where the platform has been placed during the acquisition. The Opp quadrant is the section placed right at the opposite direction of the target area. Data are expressed with means and SEM.

Otherwise, all the rats mostly recovered the learning rates achieved at the last session of acquisition stage during reinstating (**Figure 3**). However, a significant effect in the main analyses was found for *Treatment x Session* interaction [F(1,27)= 4.342, p= 0.047] when the reinstating performance was related to the session 8 of the acquisition. Post hoc analyses revealed that the control rats' performance during reinstating was significatively affected by the Probe stage (p=0.019), something non-observed in the exposed animals (p=0.638).

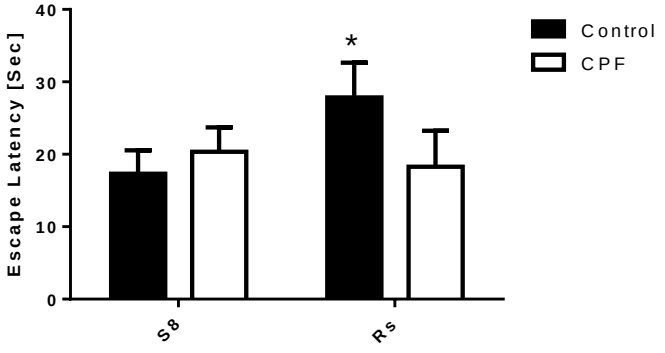

Figure 3. Reinstating stage. Effects of the platform removal (Probe) in the learning profile during the reinstating stage (Rs) from the last session (S8) of the acquisition. * indicates significant differences concerning the performance of the control animals in both sessions. Data are expressed with means and SEM.

Concerning the reversal stage, animals performed as expected by increasing their escape latency rates in the first session by rapidly improving them in the second and third session (**Figure 4**). The ANOVA showed this effect in relation to *Session* [F(1.6,44.3)=

7.059, p= 0.004]. Post hoc analyses revealed that all rats improved their performance during the 3 compared to the first and second days of reversal (p=0.006 and 0.024, respectively). Once again, there was not significant effects concerning neither Treatment *x Session* nor *Treatment x Sex x Session*.

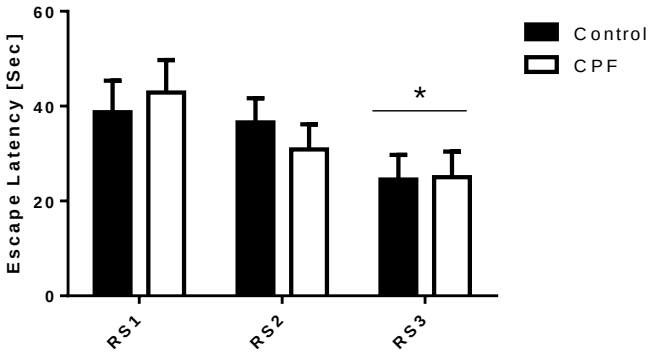

Figure 4. Reversal stage. Effects of the change of the platform position in the animals' performance for three sessions. RS refers to reversal session. * indicates significant differences compared to RS1. Data are expressed with means and SEM.

A visual task was conducted in order to control possible influences of CPF on vision (**Figure 5**). The ANOVA revealed a significant effect of *Session* [F(2,54)= 5.134, p= 0.009], where animals improved their performance by repetition of the task in the last session compared the first one (p= 0.005). Once again, there were no influences of exposure condition or any other complex interaction at main analyses, discarding that the results previously discussed could be partially due to visual alterations.

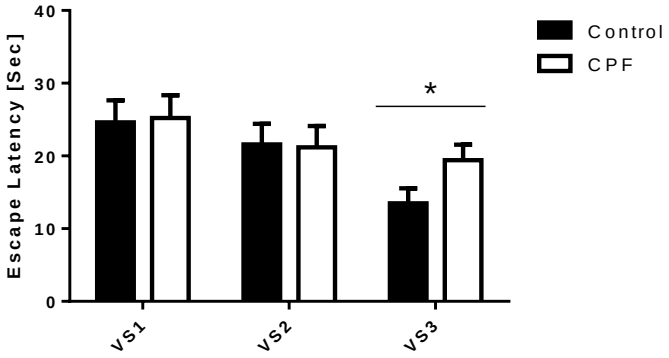

Figure 5. Visual stage. Checking of the rats' visual system integrity by unhiding and highlighting the platform for three consecutive sessions. VS refers to visual session. * indicates significant differences compared to VS1. Data are expressed with means and SEM.

3.1.2. MWM. Motor variables.

Total distance covered, velocity and immobility frequencies were analyzed in order to ensure that the spatial memory modulations induced by CPF are due to specific cognitive alterations and not a general behavioral mismatching. During acquisition, CPF exposed rats showed a hyper-activated motor state based on the total distance covered compared to the control animals in the two first sessions that was progressively corrected throughout the learning stage **(Figure 6a)**. The ANOVA revealed a significant effect in *Session* [$F(7,189)= 23.980$, $p<0.001$] and the interaction *Treatment x Session* [$F(7,189)= 3.144$, $p= 0.004$]. Post hoc analyses revealed that the rats significatively decreased their total distance covering from the session 4 to onwards compared the first day of training ($p=0.035$ -session 4-, 0.003 -session 5- and <0.001 -sessions 6, 7 and 8-). CPF exposed animals did covered more distance than their control counterparts in the two first sessions ($p<0.001$ and = 0.015, respectively), as well as they quickly reached a decline in their activity (from session 4 to onwards, $p=0.001$ -session 4- and <0.001 -remaining sessions-) compared the first day of training, meanwhile this was only observed in the last two sessions in the control rats ($p=0.033$ and $p=0.002$, respectively). Similarly, CPF exposed rats were faster in the earliest sessions compared control animals, with a significant *Treatment x Session* interaction [$F(7,189)= 3.330$, $p= 0.002$] (**Figure 6b**). Post hoc analyses revealed that the exposed rats were significatively faster than the controls only during the first session ($p=0.028$), with a significant velocity increase throughout the sessions (from the first session, $p<0.001$, $p=0.011$ in the session 5), something also observed in their control counterparts (form the first session, $p<0.001$ in all cases). Finally, there were no significant main effects in immobility concerning *Treatment, Treatment x Session or Treatment x Sex x Session* interactions (Supplementary Figure 1).

During the Probe and reinstating test stages, there were no differences between groups based on the different locomotor variables analyzed neither for *Treatment* nor *Treatment x Sex* or *Trial* interactions, with the exception of *Treatment x Trial* interaction for immobility frequencies during the reinstating [$F(2.2,59.5)= 3.318$, $p=0.039$]. However, there were not significant effects at the post hoc analysis (Supplementary Figures 2 and 3).

Otherwise, the animals covered less distance by the sessions in the reversal stage, without significant influences of *Treatment*. Velocity was also not affected

(Supplementary Figure 4). However, immobility frequencies were progressively reduced by *Sessions* [F(2,54)= 11.520, p<0.001] (**Figure 6c**). This was affected by the treatment condition, showing a significant main effect in the interaction *Treatment x Session* [F(2,54)= 4.048, p= 0.023]. Post hoc analysis revealed that the high rate of immobility was focused on the session 1 in the exposed animals, which significantly reduced their immobility by sessions compared the first day of reversal (p=0.003 and 0.001, respectively), something non-observed in the control rats (p= 0.985 and 0.132, respectively). Interestingly, a significant interaction *Treatment x Trial* was also found [F(2.1,57.9)= 4.468, p=0.014] (**Figure 6d**). Post hoc analyses revealed that the exposed rats did significantly more episodes of immobility at every single first trial throughout sessions in the reversal stage than their control counterparts (p=0.018). This higher rate of immobility state was progressively normalized to the control animals' performance in the subsequent trials, showing a significant reduction in the exposed rats in the third and fourth trial compared the first one (p=0.006 and 0.007, respectively), something non-observed in the control rats (p=0.557 and 1.000, respectively).

Finally, the motor behavior of the rats during the visual test stage also followed the expected pattern of higher activity rates at the first session compared the last one in total distance and immobility rates, but without any influence concerning the treatment condition. Interestingly, exposed animals showed a general decreased velocity compared their control counterparts taking all the three "visual" sessions together [F(1,27)= 5.083, p= 0.032] (**Figure 6e**). The remaining non-significant outcomes are shown in Supplementary Figure 5.

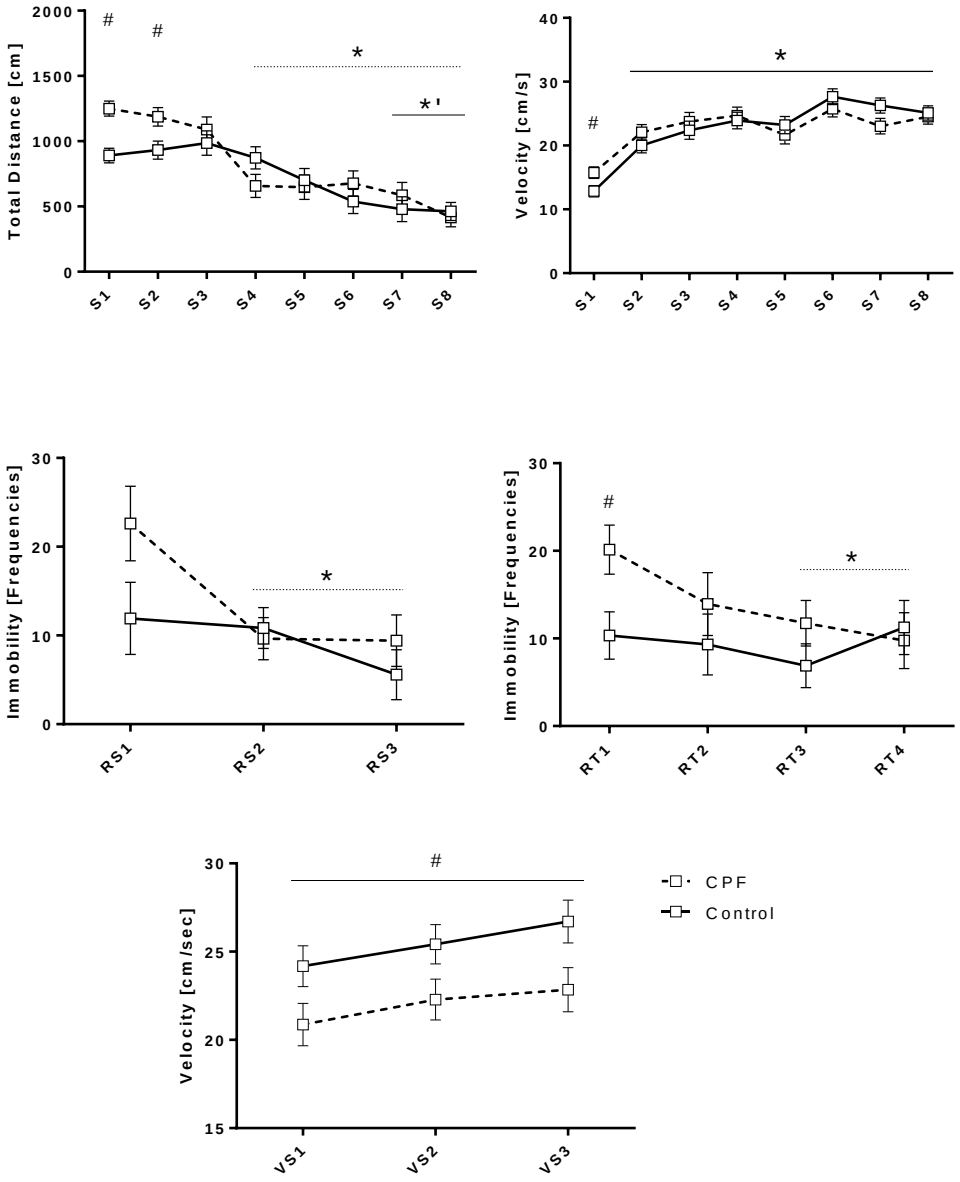

Figure 6. Motricity in MWM. Locomotor variables significatively affected by the CPF exposure during the acquisition (a and b, up left and right), reversal (c and d, middle left and right) and visual (e, down) stages of the MWM. RT refers to reversal trial. --*-- indicates significant differences compared to the first session (or trial) of the stage only for the exposed rats.-*- indicates significant differences compared to the first session of the stage for both groups. -*'- indicates significant differences compared to the first session of the stage only for the control rats. # indicates significant differences between control and CPF rats. Data are expressed with means and SEM.

3.2. CPF and anxiety in pre-old age rats

3.2.1. Plus-Maze test. Anxiety and motor variables.

All rats expended more time exploring the closed arms than the open arms, as expected. **Figure 7** shows both the total time in and the number of entries (**7a**) to the open arms (**7b**). CPF exposure did not affect the anxiety-related behaviors of the rats at any of these variables. CPF exposure lead to a general decrease of motor activity. All the

locomotor variables analyzed (total distance, velocity, entries to closed arms and general mobility) showed this pattern, although only general mobility was found as significant concerning *Treatment* [F(1,37)= 5.044 p=0.031] (**Figure 7c**). The remaining motor-related outcomes are described in the Supplementary Figure 6.

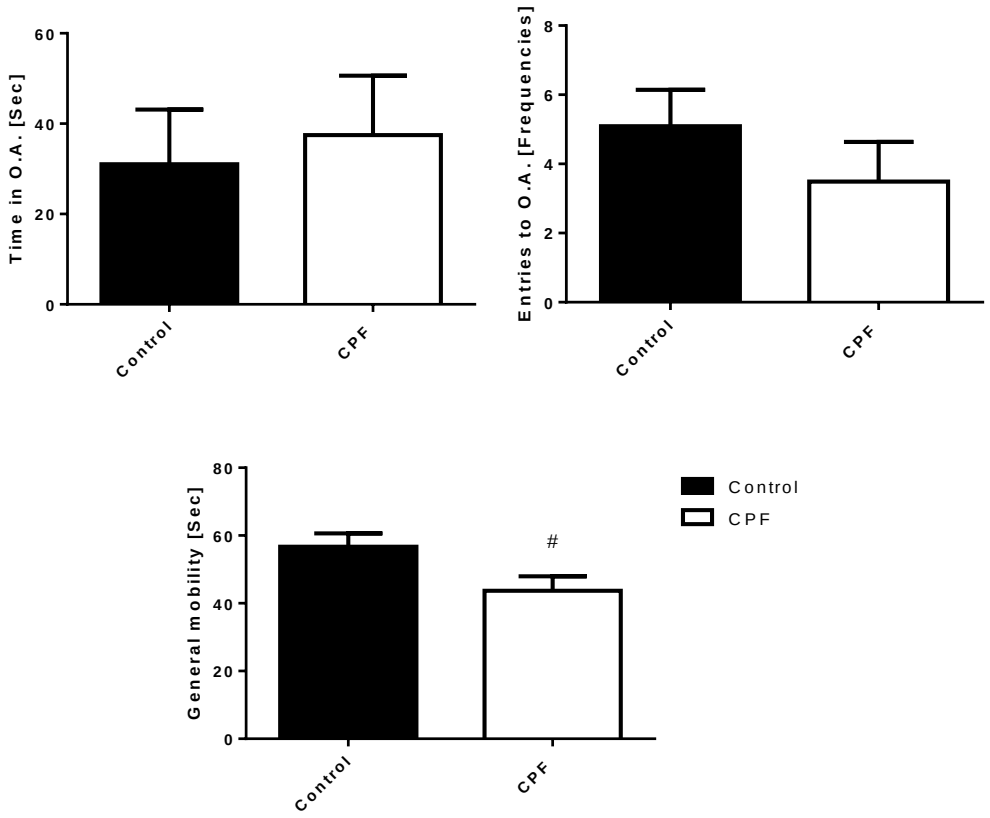

Figure 7. Plus-maze. Anxiety (a and b, up left and right, respectively) and motor-related (c, down) variables. O.A. refers to open arms. # indicates significant differences between control and CPF exposed rats. Data are expressed with means and SEM.

4. Discussion

The preweaning exposure to NChEI doses of CPF did not alter the spatial and reference memory functioning in late-adult rats. It only induced a hyposensitive reaction to temporal disruptions on learning (Probe test). Added to this, anxiety-like behaviors were not different between the control and exposed animals at these ages. However, the exposed rats were generally hypoactive in terms of motricity. This is the first time that, to the best of our knowledge, the long-term effects of developmental CPF exposure have been analyzed in such late ages for these behaviors, core in several neurodegenerative disorders.

Previous empirical studies found that gestational exposure induced alterations in both working and reference memory performance (Icenogle et al., 2004), specifically in females when exposure occurs at later gestational stages (Levin et al., 2002). Neonatal studies also found poorer working and reference memory in exposed males (Levin et al., 2001; Alipour et al., 2019) and females (Aldridge et al., 2005). However, spatial learning (*Acquisition stage*), reference memory (*Probe stage*), compulsive traits and flexibility for learning new contingencies (*Reversal stage*) and visual functions were not altered by the exposure to developmental CPF in the present preweaning study.

Focusing on the preweaning exposure, different studies have found no influences of CPF exposure on learning, attention and/or memory (Levin et al., 2001; Cole et al., 2012), as we do, but most of them found the expected depauperated performance (Jett et al., 2001; Guardia-Escote et al., 2018, 2019; Wang et al., 2018; Basaure et al., 2019). Concerning rats' models, these deficits seem to be task-specific as they have been found following a MWM (Jett et al., 2001; Wang et al., 2018) but not the Radial-arm maze protocol (Levin et al., 2001). However, the differences between the Jett et al., (2001) and Wang et al., (2018) and the present study make impossible to extract proper conclusions as their doses were higher and assessed the rats' behavior at very young ages compared to our late age model.

Following the *Probe stage,* animals completed a *Reinstating* session in order to recover the presumable depauperated performance to baseline levels. The control rats were importantly affected by the platform removing during the *Probe stage*, as observed in their performance during the *Reinstating,* with longer escape latencies compared the last session of the *Acquisition stage*. However, this was not the case of the CPF exposed animals. These rats were not affected by this interference in learning. This hyposensitivity to learning disruptions is surprising and we have found nothing concerning this in the existent literature.

In fact, this disruption can be conceptualized as a brief extinction (Vorhees and Williams, 2006). And it has been proposed that the cholinergic system plays an essential role in the extinction learning, at least with the fear component (Wilson and Fadel, 2017). We previously demonstrated that other CPF exposed rats following this same exposure protocol had a hyposensitized cholinergic system at both young adulthood (Perez-Fernandez et al., 2019) and adulthood (Perez-Fernandez et al., 2020b) following pharmacological challenges in different motor and attentional paradigms. It is possible

that the malfunctioning of the cholinergic system in those animals postnatally exposed to the OP agent could be at the basis of this abnormal reaction to the *Probe test*. However, the lack of empirical references and theoretical models make impossible to reliably discuss this information.

The exposure to CPF had no effect on anxiety-like behaviors assessed by the time in and number of entries to the open arms in a Plus-maze test. We were able to find 8 preclinical studies that analyzed the influences of developmental CPF on anxiety-like behaviors (Peris-Sampedro et al., 2020; Carr et al., 2015, 2020; Silva et al., 2017; Venerosi et al., 2008, 2010; Braquenier et al., 2010; Ricceri et al., 2006). From these, 5 studies used a preweaning exposure protocol to NChEI doses of CPF, generally leading to a decreased anxiety in the exposed rodents. However, the ages of anxiety-like behaviors assessment in these studies raged from pre-adolescence to 5 months-old adults. This essential difference could be at the basis of the lack of concordance between the results of the present study and the consensus observed in the empirical literature.

Exposed rats showed a general hypomotricity in both MWM and Plus-Maze tasks, except for the early sessions of the acquisition phase, although these stages could be reflecting an increased spontaneous motricity due to the exposure to a new context. This hypoactivity is the first time that this effect following developmental CPF exposure has been found in pre-old age rats. The general reduction of the locomotory activity is a sign commonly observed in normal aging and different neurodegenerative disorders such as AD and PD, amongst others. Several studies have linked the developmental exposure to low doses of CPF with alterations in motricity and motor reflexes following both gestational (Lan et al., 2017; De Felice et al., 2015; Ricceri et al., 2006; Levin et al., 2002; Icenogle et al., 2004; Silva et al., 2017) and early postnatal (Dam et al., 2000; Levin et al., 2001) exposure protocols, although this has not been always observed (Venerosi et al., 2006, 2009; Icenogle et al., 2004 in the Fig.8 test; Ricceri et al., 2003).

To our particular interest, preweaning exposure has also been linked to this sort of motor alterations, both increasing (Ricceri et al., 2003; Dam et al., 2000; Levin et al., 2001; Guardia-Escote et al., 2019) or decreasing (Lee et al., 2015; Venerosi et al., 2008) the locomotor rates of the rodents. From these, only two studies (Dam et al., 2000 and Levin et al., 2001) used rats as experimental animals, both observing an hyperactivated profile during adolescence and adulthood in the exposed rats but using 5 times the dose

of CPF used in the present research. However, and supporting this CPF-induced hyperactivity in young ages following a preweaning NChEI protocol, we have previously found this increased spontaneous activity increase in adolescent rats, "siblings" of those used in this study and that followed the same exposure protocol (Perez-Fernandez et al., 2019). More recently, other authors have also given support to using the same dose chosen in the present work (Carr et al., 2020).

The main limiting factor concerning the comparisons of our data with all these studies is, once again, the age of assessment. From all these works, the age range was from few days after birth to as far as 4 months old, far away from the ages used in the present research. That is, attending to the data obtained in our laboratory following this exposure protocol, that CPF exposure during preweaning stages seems to induce a motor hyperactivation that gets progressively into decline by age, observed at pre-old age. Another support of this hypothesis is that we also found a decrease in velocity in the exposed females at around 9 months old (Perez-Fernandez et al., 2020b), presumably showing an initial proof of the later general decline found in the present study.

5. Conclusions and future guidelines

Postnatal, preweaning exposure to NChEI doses of CPF induced a long-term general hypomotricity in pre-old age rats, without affecting anxiety and spatial memory functions. This exposure long-term unsensitized the rats to brief learning disruptions (*Probe test*), something non-observed before. Based on the previous empirical data obtained in previous studies in our laboratory and other laboratories demonstrating that this exposure regime induces hypermotricity during the adolescence and early adulthood in the rat, we propose that the effects of low doses of developmental CPF on motricity are age-dependent, characterized by an initial motor excitation that progressively turns into a general degradation of the motricity, primarily observed in pre-old age and, presumably, in old age. As the motor function alteration is a core sing in most of the neurodegenerative disorders known currently, and the aging process is also characterized by a variable decline of this function, the present study supports the notion that the exposure to this sort of contaminants could be a environmental factor for the development of these pathologies and a worst aging profile, especially worrisome in the aged developed nations. Future research should be focus on validate this hypothesis of the biphasic effect of the CPF exposure concerning age by regularly checking the

motricity in different paradigms during the life-span of the rat. Finally, the lack of sensitivity to the learning disruption by the Probe test could be further explored following different learning procedures including extinction stages to know if this effect is consistent or a mere isolated performance.

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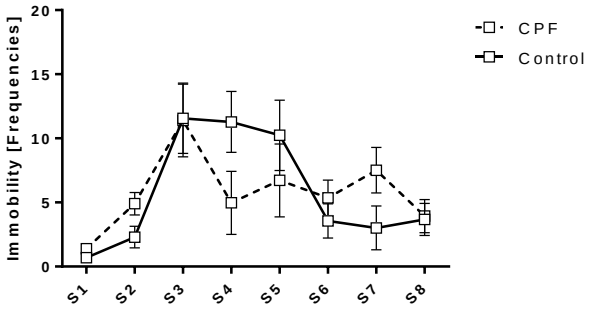
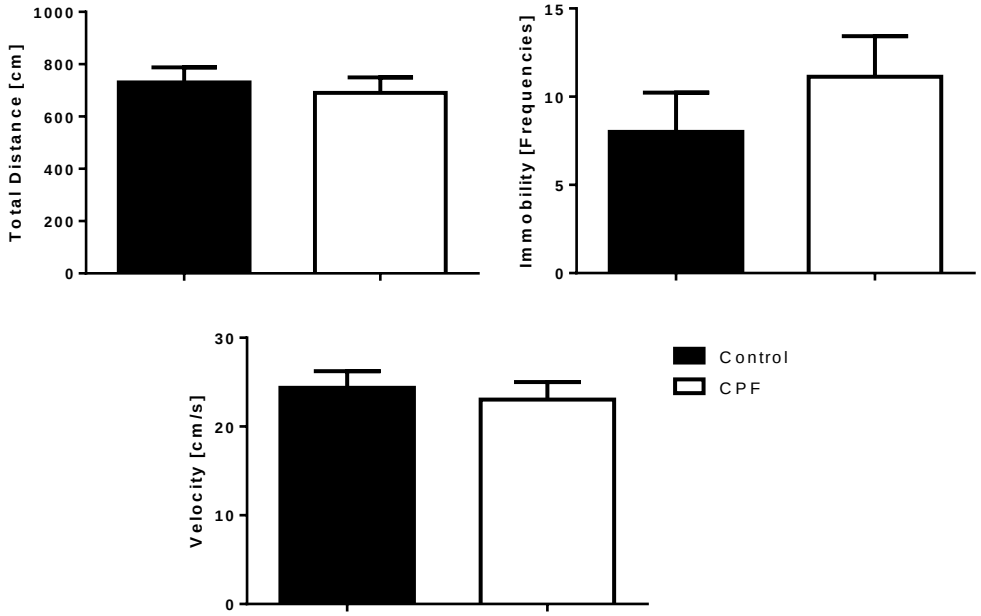
Supplementary Figure 1



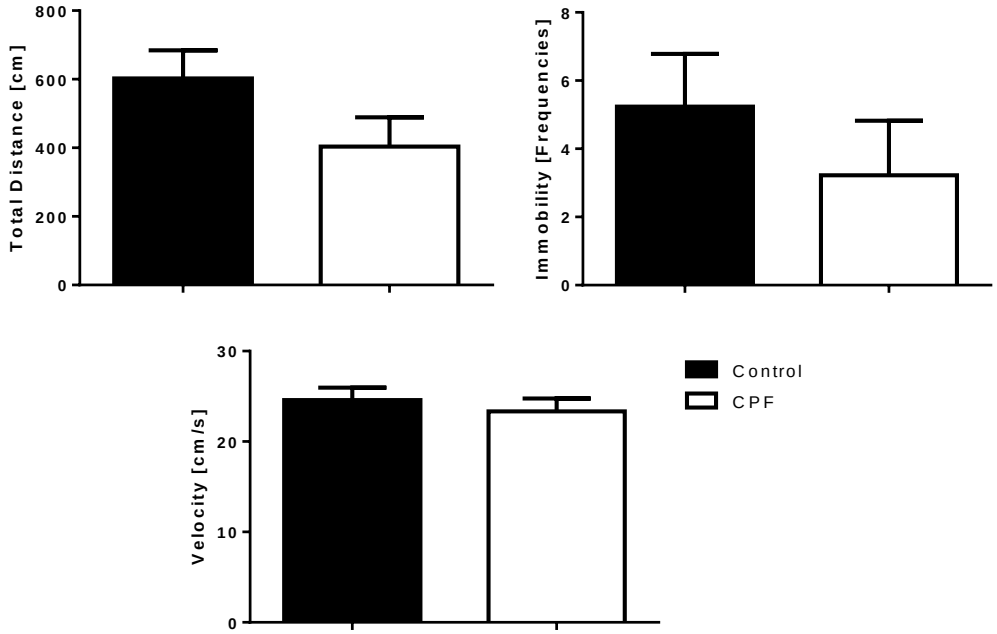
Supplementary Figure 1. Motricity rates during the Acquisition stage of the Morris water maze. Immobility throughout the sessions.

Supplementary Figure 2



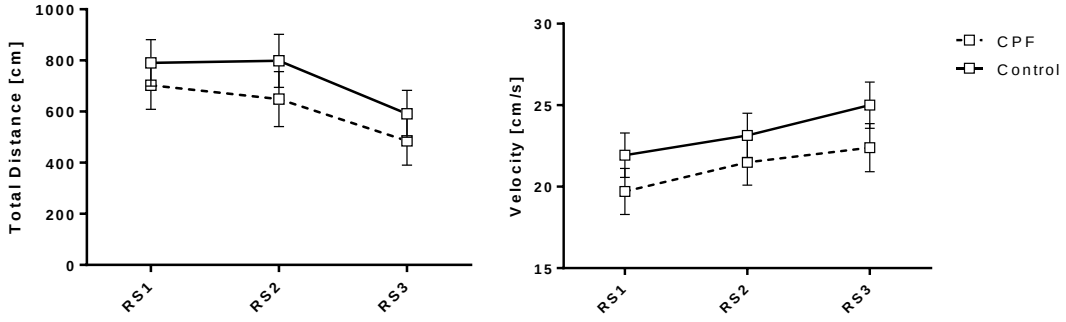
Supplementary Figure 2. Motricity rates during the Probe stage of the Morris water maze. Total distance covered, immobility and velocity.

Supplementary Figure 3



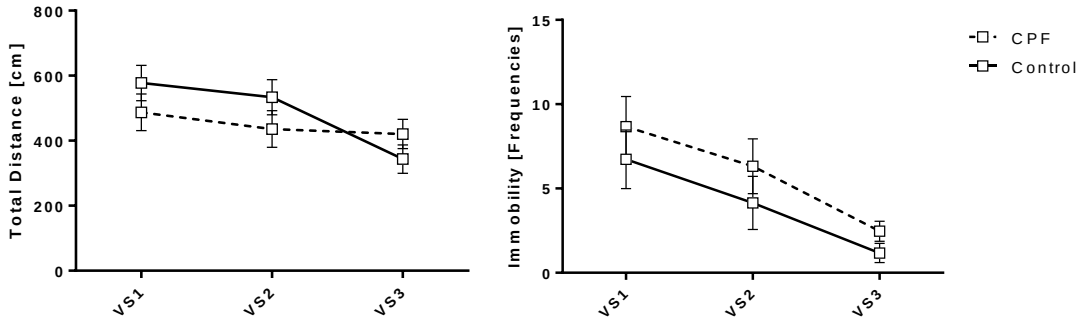
Supplementary Figure 3. Motricity rates during the Reinstating stage of the Morris water maze. Total distance covered, immobility and velocity.

Supplementary Figure 4



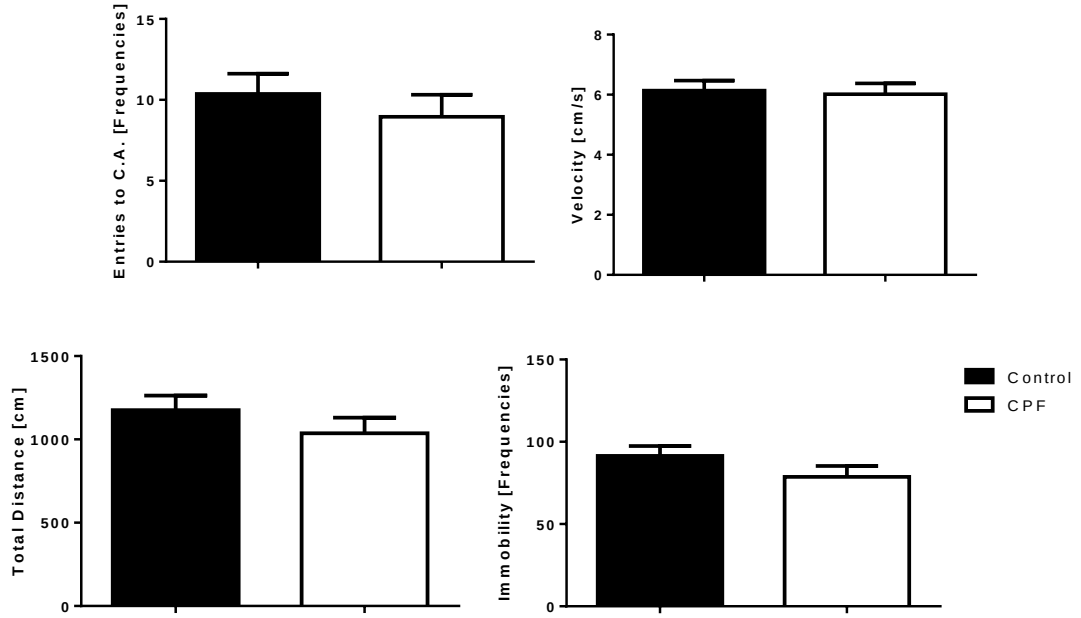
Supplementary Figure 4. Motricity rates during the Reversal stage of the Morris water maze. Total distance covered and velocity throughout the sessions. RS means reversal session.

Supplementary Figure 5



Supplementary Figure 5. Motricity rates during the Visual stage of the Morris water maze. Total distance covered and immobility throughout the sessions. VS means visual session.

Supplementary Figure 6



Supplementary Figure 6. Motricity rates during the Plus maze. Entries to closed arms (C.A.) (up-left), total distance covered (down-left), velocity (up-right) and immobility (down-right) during a 5-min period.

4. General Discussion

The general goal of the experiments described here was to analyze the effects of exposure to one of the most widely used pesticides in the world, CPF, during a specific postnatal developmental window in the rat, which is equivalent to the human perinatal period in terms of neurodevelopment, and that lacks from extensive empirical research in the current literature. The present set of experiments has demonstrated that exposure to NChEI doses of CPF during short periods of time at the postnatal, preweaning developmental stage induces medium and long-term alterations in multiple behavioral and molecular outcomes throughout the rat's life-span, and in some cases, revealing examples of sex-dimorphism. In short, the experimental articles extracted from this doctoral thesis support the notion that doses of CPF below those required to activate its main mechanism of toxicity (AChE inhibition) have the potential to alter different behavioral domains and molecular elements and processes by mismatching of other systems or other components of the cholinergic system. **Table 9** summarizes the extent to which the different hypotheses are supported (those described in the section Justification, hypothesis, and objectives).

Hypothesis	Hypothesis support
Study 1	
This exposure protocol will not significatively inhibit (<20% from control) brain ChE activity, based on previous literature	
CPF exposed animals will be more sensitive to stress derived from exposure to a novel space (spontaneous activity). The same could be expected in relation to stress induced by pain (i.p. injection), although there is not previous empirical data on this regard	
CPF will induce alterations in the endocannabinoid system. It is also possible that it will affect the serotonergic and dopaminergic, and cholinergic systems based on previous literature. The effects on the remaining systems is also possible but there is less empirical support based on previous literature	
CPF will induce alterations in expression levels of different genes based on previous literature	
CPF will induce dysbiosis, presumably with special sensitivity on Lactobacillus, Bacteroides, Bifidobacterium and Clostridium bacteria families, based on previous literature	
Study 2	
CPF exposure will alter (reduce) social interaction in both adolescents and adult rats (contrary results in previous literature)	
CPF exposure will alter (reduce) dominance status	
Dominance status will be an important factor for other social domains	
Alterations in motricity will not be an important factor that could explain the possible differences in sociability amongst groups	
CPF will induce dysbiosis, presumably with special sensitivity on Lactobacillus, Bacteroides, Bifidobacterium and Clostridium bacteria families, based on previous literature	
CPF will alter different systemic metabolic pathways and specific metabolites, with a special emphasis on the lipid systems, as previously observed in the literature	
Study 3	
CPF exposed animals will be more sensitive to stress derived from the exposure to a new space (spontaneous activity), as observed in previous literature	
CPF exposure will lead to altered (poorer) learning rates, opposite results could be found in females, based on previous literature with adult exposure	
CPF exposure will alter (impair) the different cognitive functions, as observed in previous literature with other exposure protocols.	
CPF exposed animals will be particularly sensitive to the change of important variables in the context of a learnt task, as observed in previous literature with adult exposure	
CPF exposed animals will have alterations in both neurotransmitter systems	
Various genes involved in the cholinergic system, GABAergic system, inhibitory control, and general CNS functioning are expected to be altered in the CPF animals	
Study 4	
CPF will alter learning rates as observed in previous literature concerning other exposure protocols and earlier in life	
CPF will alter learning spatial memory consolidation and will be sensitive to learning disturbances as observed in previous literature with adult exposure and earlier in life	
CPF will induce a compulsive-like pattern following changes in contingencies as observed in previous literature with adult exposure	
CPF will have no effect on visual performance	
CPF will alter anxiety state, as observed in other developmental studies and earlier in life	
CPF will induce alterations in locomotor outcomes	

Table 9. Degree of support obtained for the initially-proposed hypotheses. Green, yellow, and red squares indicate that the hypotheses were completely, partially, or not confirmed, respectively.

4.1. Alternative mechanisms of action of developmental Chlorpyrifos on the central nervous system

4.1.1. Alternative mechanisms within the cholinergic system: the role of the muscarinic autoreceptors beyond AChE

Beginning from this conception, we have found that most of the neurotransmitter systems were not altered in CPF-exposed animals when using different drug challenges. However, both the cholinergic and the GABAergic systems of the CPF exposed animals responded differently in comparison with their control counterparts when disrupted by the use of Scopolamine hydrobromide (muscarinic antagonist) and Alprazolam (GABA-A receptor allosteric agonist), respectively (Study 1 and 3) (Perez-Fernandez et al., 2019b; Perez-Fernandez et al., 2020b). Exposure to different doses of Scopolamine produced a dose-dependent increase in the total distance covered by all of the rats (Study 1) (Perez-Fernandez et al., 2019b). Control rats showed a substantial increase in their motor rates, even following the lowest dose (0.5mg/kg), achieving an early ceiling-effect as they slightly increased their motor rates at 1mg/kg. However, the exposed rats were clearly less affected following the lowest dose and, to a lesser extent, the highest dose, this being more evident in females, although sex-differences were not significant. In our view, this is clearly indicative of a hyposensitized cholinergic system in the exposed animals, as they did not reach the normative levels found in their control counterparts, requiring a higher dose of the drug to reach the rates shown by the controls.

These effects were observed when using motor outcomes and testing at early adulthood ages (around 2 months old), more than 45 days after the exposure to CPF (Study 1) (Perez-Fernandez et al., 2019b). However, this was not the only case where we empirically demonstrated that CPF alters this neurotransmitter system in the absence of AChE inhibition. A clear hyposensitized cholinergic system in the CPF-exposed animals was found using this same drug when measuring 5C-SRTT performance (concerning attentional and impulsivity-related outcomes) close to one year after the exposure to CPF was finished (Study 3) (Perez-Fernandez et al., 2020b). Given our earlier observation that the effects of CPF on the cholinergic systems are more marked when using lower doses of Scopolamine than those used in the first set of experiments, we used different doses within the range of 0.06-0.5 mg/kg. Scopolamine (with the exception of the lowest dose) decreased the accuracy and increased the premature responding rates of the control rats in a dose-dependent manner, while its effects on CPF-exposed animals was nonexistent

for the impulsivity action-related outcome, whilst effects on attentional-related measures were partially abolished.

Taking together, these results, when added to those previously found by other authors using higher doses during the preweaning stage (Levin et al., 2001) or gestation (Levin et al., 2002; Icenogle et al., 2004) that also found this hypo-response in the CPF animals following a cholinergic challenge by assessing different memory functions, support the notion that the exposure to CPF during development alters the long-term integrity of the cholinergic system assessed across several cognitive domains (motricity, memory, attention and inhibitory control). These effects possibly suggest the decreased control of the cholinergic system over different functions in favor to other such as the serotonergic system, as other authors have proposed (Levin et al., 2001; Aldridge et al., 2005b).

This hyposensitivity of the cholinergic system could be explained by general hypoactivation. A general hypoactivity of this system, as well as its progressive degradation, is one of the molecular hallmarks of Alzheimer's disease (Hampel et al., 2018). Furthermore, exposure to different pesticides has been associated with this disorder, including CPF and other OPs (Sánchez-Santed et al., 2016). A general hypoactivity of the cholinergic system could be explained, at least in part, by a decrease in the cholinergic tone, something that we have not studied in the present set of experiments. However, we found that CPF exposure induced a long-term up-regulation of the striatal muscarinic 2 receptors (M2r), as reported in Study 1 (Perez-Fernandez et al., 2019b). Aside from its novelty (the only developmental study that showed similar results is that of Slotkin & Seidler, 2007b), this result is relevant, as this receptor is a cholinergic protein that generally works on the presynaptic neuron as an auto/heteroreceptor (Mrzijak et al., 1996; Douglas et al., 2002). When working as an autoreceptor, M2r activation leads to a decrease in the release of acetylcholine (Douglas et al., 2002). Based on our experimental findings, we can argue that preweaning exposure to NChEI doses of CPF induces a hypofunctional cholinergic system, that is possibly mediated by the over-functioning of specific muscarinic receptors that commonly regulate the cholinergic activity in the mammalian brain. In order to confirm this suggestion, direct measures of the acetylcholine levels at the CNS should be performed.

4.1.2. Alternative mechanisms outside the cholinergic system: the under-explored GABAergic system as a relevant candidate

Aside from the effects of CPF on different components (non-cholinesterase related) of the cholinergic system, we also found that the exposed rats behaved differently to their control counterparts when challenged with Alprazolam, an indirect agonist of the GABA-A-receptor (Perez-Fernandez et al., 2019b). Briefly, in Study 1, the control rats showed a biphasic effect of the drug (inverted U-shaped dose-response pattern), with increased motor rates at the lower dose and expected sedation (decreased behavior) at the highest dose, which was evident in the female rats. However, the exposed females did not show the expected hyperactivity at the lowest dose, but instead showed a decrease in their motor rates, this decrease being more marked in both exposed males and females merged at the highest dose, in comparison with their control counterparts. Thus, the exposure to CPF induced a long-term hypersensitized GABAergic system since the exposed animals needed a lower dose of the drug to show the behavioral rates expected at higher doses, as found in their control counterparts. We also found that the GABAergic system is altered by using this drug in the exposed rats close to one year after the CPF exposure, as shown by impulsive-like behaviors (Study 3) (Perez-Fernandez et al., 2020b). However, the dynamics observed in this case did not allow us to establish a clear dose-response pattern, which limits our ability to draw any firm conclusions. These findings, in comparison with those found in the cholinergic system, are completely novel and relevant, given the scarcity of evidence in the current literature regarding the relationships between CPF and the GABAergic systems (Sánchez-Amate et al., 2002; Cardona et al., 2006; Montes de Oca et al. 2013; López-Granero et al., 2016), particularly during development (Gómez-Giménez et al., 2018).

Added to this, we found that the mRNA expression of a specific subunit (α2) of the GABA-A-receptor was also up-regulated in the frontal cortex in the exposed rats (Study 1) (Perez-Fernandez et al., 2019b). The implications of these higher expression levels for this subunit on the observed GABAergic hypersensitivity are unclear, although theoretically might be expected (more molecular targets for the interaction of the surrounding GABA neurotransmitter, whose physiological implications are increased by facilitation of its activation with an allosteric agonist, as in is the case of Alprazolam). This is, to the best of our knowledge, the first time that CPF has been linked to selective alterations of the GABA-A-α2 subunit, regardless of the dose (NChEI or AChE

inhibition-linked dose), exposure protocol (acute, sub-chronic or chronic) and age of exposure (during development or adulthood). Interestingly, this specific subunit has been extensively linked to the abuse of alcohol and other substances (Engin et al., 2012; Gonzalez-Nunez, 2015; Olsen and Liang, 2017). This is relevant, since previous studies from our laboratory found that acute exposure to high doses of CPF increased alcohol-consumption rates (Carvajal et al., 2014). Although the methodological differences between studies are evident, it is reasonable to suppose that these consumption rates could be partially due to specific alterations in this GABAergic subunit, although in the absence of sufficient empirical data, we are unable to draw any firm conclusions in this regards.

4.1.3. Alternative mechanisms outside the cholinergic system: The unexpected lack of effects on the endocannabinoid system

Throughout this doctoral thesis, we have repeatedly referred to the preweaning developmental stage as one of the least studied developmental ages regarding CPF exposure, at least in comparison with gestational models. Although it is true that it is mostly under explored, there is something that has been systematically associated with NChEI doses of CPF during this period: the specific modulation of various components of the endocannabinoid system (Carr et al., 2011, 2013, 2014, 2017). Briefly, these authors have systematically found that CPF exposure at doses in the range of 0.5-1mg/kg leads to a reduction in different enzymes that are important for the adequate functioning of the endocannabinoid system, such as the Monoacylglycerol lipase and the Fatty acid amide hydrolase, and that this triggers a net increase in different endogenous agonists such as Anandamide and 2-Arachidonoylglycerol. Although not demonstrated by the authors (as far as we know), this agonist increase could lead to a regulation of the cannabinoid 1 (CB1) receptors in different brain areas, thus affecting different physiological (e.g., glutamate and GABA release) and behavioral outcomes. This should be added to other studies that found a direct effect of CPF on the CB1 receptors (Quistad et al., 2002; Baireddy et al., 2011; Liu et al., 2015; Liu and Pope, 2015).

However, we were not able to find a differential impact of the agonism of CB1 by the administration of WIN 55, 212-2 on different behavioral outcomes (feeding and feeding efficiency rates) in the exposed rats (Study 1) (Perez-Fernandez et al., 2019b). It is true, however, that administration of this compound selectively decreased the locomotor rates of the control female rats during the time of exposure, something that was not observed in the exposed females. This could be taken to indicate a hyposensitized endocannabinoid

system, which could partially explain the (nonsignificant) results observed in the feeding efficiency rates, where exposed females decreased their food consumption concerning their body weight in a more muffled way compared with the clear decrease observed in their control counterparts. However, in the absence of specific statistical differences between groups, our conclusions can only be speculative at this stage. Given the consistent results reported by Carr et al. (2011, 2013, 2014, 2017), we consider that this lack of significant effects on the endocannabinoid system observed in our experiments is more likely to be due to methodological limitations in the behaviors analyzed, the drug used, the selected dose or a combination of these factors.

4.1.4. Beyond the neurotransmitter systems: BDNF as a molecule that is sensitive to the exposure to NChEI doses of developmental Chlorpyrifos

Although the present thesis focuses on looking for alternative molecular targets for CPF exposure within the different components of the main neurotransmitter systems, it is well-documented that CPF can alter several of these molecule, whose functional role is essential for the integrity of the CNS. Developmental exposure to CPF has been linked to selective alterations on different components of the oxidative stress, lipid peroxidation, neuroinflammation, and intra-cell pathways associated with the cyclic adenosine monophosphate, along with mitochondrial activity dysregulation, alteration of glial cell activity, amongst others (Schuh et al., 2002; Saulsbury et al., 2009; Xu et al., 2017; Hussein et al., 2018). Interestingly, it has also been linked to the modulation of different neurotrophic factors (Lee et al., 2016; Mahmoud et al., 2019; Betancourt & Carr, 2003; Betancourt et al., 2007; Slotkin et al., 2008), where BDNF plays an essential role in the mammalian nervous system by regulating plasticity along with cell proliferation and differentiation (Lykissas et al., 2007).

In Study 3, we found that this exposure protocol down-regulated the expression of the striatal *BDNF* gene in 12-months-old males (Perez-Fernandez et al., 2020b). This is the first time that CPF exposure has been linked to such a long-term misregulation of the *BDNF* gene expression. However, there are three developmental studies that also pointed to this effect following different exposure protocols (Betancourt & Carr, 2003; Betancourt et al., 2007; Slotkin et al., 2008), the latter using the preweaning exposure protocol, finding no effects (Betancourt and Carr, 2003), increases (Betancourt et al., 2007, hippocampus; Slotkin et al., 2008, differentiated cells) and decreases (Betancourt et al., 2007, cortex; Slotkin et al. 2008, undifferentiated cells) in BDNF levels following CPF

exposure. Based on the wide range of functions associated with BDNF in the mammalian CNS, the relevance of this finding is unquestionable. Taken together, the present study provides empirical evidence to support the existence of three different alternative molecular targets for developmental CPF exposure, disrupting the cholinergic (presumably M2r-mediated) and GABAergic systems (presumably GABA-A-α2 mediated) and general CNS functioning (BDNF mediated). However, the implication of these systems/molecular alterations observed in the exposed rats on the neurotoxicological effects of CPF remains unclear.

4.2. Medium and long-term behavioral effects of developmental exposure to NChEI doses of Chlorpyrifos during the preweaning window.

4.2.1. Chlorpyrifos exposure and motricity

The work described in this thesis shows that preweaning exposure to CPF alters the locomotor activity rates of the exposed rats, which develops in an interesting way throughout the life-span. That is, whilst Study 1 (Perez-Fernandez et al., 2019b) found that exposed rats increased their spontaneous locomotor activity during mid adolescence (around one month following exposure), Study 3 showed that this exposure induces a long-term in certain motor variables (velocity) in 12-months-old exposed females (Perez-Fernandez et al., 2020b). This decreased behavioral pattern was confirmed in both sexes in the empirical data obtained in Study 4, with a general reduction in different motor variables during the late-adulthood period (from 15-18 months old).

Based on the direct relationship between CPF and the cholinergic system, and the former with motor and reflex behaviors, the current literature contains numerous excellent demonstrations of alterations in motricity following CPF exposure, in humans (Steenland et al., 2000; Rauh et al., 2006; Harari et al., 2010; Malekirad et al., 2013; Rohlman et al., 2016; van Wendel de Joode et al., 2016) and preclinical models during adulthood (Pope et al., 1992; Carvajal et al., 2005; López-Crespo et al., 2007; Bockzur et al., 2010; Yan et al., 2012; López-Granero et al., 2013, 2014, 2016; Peris-Sampedro et al., 2014; Savy et al., 2015; Adedara et al., 2018), adolescence (Chen et al., 2014; Avci et al., 2018; Singh et al., 2018) and development (summarized in **Table 2**, Introduction section). Preweaning exposure has also been linked to both decreased and increased motor rates in adolescent/young adult rodents (Dam et al., 2000; Levin et al., 2001; Ricceri et al., 2003; Venerosi et al., 2008; Lee et al., 2015; Guardia-Escote et al., 2019a). However, most of these studies used dosages that were 5-fold those used here, and only two used rat models

(Dam et al., 2000; Levin et al., 2001). Thus, the results of Study 1 showing an increase in spontaneous locomotor activity complement those previously published by demonstrating that these effects can be observed even at lower doses where AChE inhibition is insignificant. This information has translational value, since different neurodevelopmental disorders include hyperactivity amongst their main clinical signs, as observed in ADHD (Cenit et al., 2017), a disorder that has been extensively linked to pesticides exposure (Sagiv et al., 2010; Mostafalou and Abdollahi, 2017; Chang et al., 2018).

Furthermore, the decreased motor rates observed in exposed animals during adulthood (Study 3) (Perez-Fernandez et al., 2020b) and late-adulthood (Study 4) are novel findings, since, to the best of our knowledge, the very long-term effects of developmental CPF exposure have not been a central focus of any previous study. The general hypoactivity observed in the different variables measured and paradigms used in Study 4 has translational significance. In particular, motor mismatching is one of the core signs of various neurodegenerative pathologies, such as ALS, AD and, eminently, PD (Scarmeas et al., 2004; Spielman et al., 2018), whilst all have been linked to pesticide exposure (Sánchez-Santed et al., 2016; Shelton et al., 2014; Sagiv et al., 2018; Chang et al., 2018). On the basis of all this information, we can conclude that preweaning exposure to NChEI doses of CPF induces selective alterations in locomotor activity defined by a time-course pattern that is characterized by a general hyperactivation at medium-term (adolescence), but a general hypoactivation as aging progresses.

Finally, we found an interesting (albeit difficult to interpret) finding concerning motricity during adolescence: the stress-induced hypermotricity observed in the exposed female rats (Perez-Fernandez et al., 2019b). The relevance of these data is unquestionable, since these findings suggest hypersensitivity to aversive stimuli, something that humans commonly face in every daily life and which could trigger maladaptive responses. Under experimental conditions used here, designed in order to habituate the animals to the intraperitoneal (i.p.) administrations prior to the different drug challenges, we found that the exposed females over-reacted to the first saline administration by increasing their motor rates by around twice than those observed in the control females. This over-reaction to aversive stimuli could be the behavioral manifestation of an altered hypothalamic-pituitary-adrenal-axis (HPA), something also proposed as a possible explanation for other behaviors described in the subsequent sections.

4.2.2. Chlorpyrifos exposure and sociability

One of our main hypothesis before starting Study 2 was that preweaning exposure to NChEI doses of CPF would alter the different social domains assessed with the 3 chambers test at both adolescence and adult ages. We expected to observe this effect, since sociability is one of the most important core signs of ASD, and pesticide exposure has been linked to the increased prevalence of this set of disorders during the last decade (World Health Organization, 2019). Although the etiology of ASD is unknown, various studies suggest that its origin could lie in the disruption of certain cellular mechanisms during perinatal periods in humans, which, in neurodevelopmental terms, is equivalent to our preweaning exposure period in rodents (Getahun et al. 2017; Martinez-Morga et al. 2018).

Although all the theoretical reasons that guided us to conduct this study were consistent and well-supported, we failed to observe this effect (Perez-Fernandez et al., 2020a). In fact, the lack of effects observed during adolescence was complemented by slight social enhancements in the adult exposed females, something that has been observed previously (Venerosi et al., 2006; De Felice et al., 2014), even with the use of preweaning exposure (Ricceri et al., 2003). We found only a subtle reaction to the decrease in social novelty in exposed males, although this was not significant when compared with their control counterparts and was nonexistent when more ethologic measures were used (e.g., sniffing), leading us to disregard these effects.

However, various rodent studies have empirically demonstrated the negative effects of CPF exposure on different social and communicative (e.g., USVs emitted by pups) outputs, effects that seem to emerge more readily with the use of gestational exposure protocols (e.g., Lan et al., 2017, 2019), although the opposite has also been found (e.g., Mullen et al., 2013; De Felice et al., 2015). Interestingly, other chemical models of ASD in rodents are commonly used during specific gestational windows, as is the case of acute doses of Valproic acid during the PND12.5 (for a comprehensive review, please see Nicolini and Fahnestock, 2018) based on the empirical evidence derived from human studies (Christensen et al., 2013). The ASD-like behavior observed in both gestational CPF and Valproic acid models suggest that this period, and not the postnatal window, is key in terms of the etiopathology of ASD or, at least, part of the symptomatology associated with this disorder. It is worth noting that a follow-up project in our laboratory used the same dose of CPF selected for the present set of experiments but administered

from GD12.5 to 15.5 to evaluate its effects on both the USVs of pups and the sociability of the adolescents. This exposure resulted in a general decrease in USVs and social domains for the exposed animals (Morales-Navas et al., under revision), giving support to the above-mentioned conclusion regarding gestational etiopathology of ASD (or, at least, part of its signs).

4.2.3. Chlorpyrifos and learning, attention, memory, and inhibitory control

Learning, attention, memory and inhibitory control alterations are commonly found in several developmental and neurodegenerative disorders such as ASD, ADHD, AD and PD (Peckham et al., 2010; Christodoulou et al., 2012; Boxhoorn et al., 2018; Picazio et al., 2018; Schmitt et al., 2018; Malhotra PA, 2019). Pesticides have been linked to these alterations (Marks et al., 2010; Suarez-López et al., 2017; Perez-Fernandez et al., 2019a), including CPF exposure in animal models (Please see the sub-section 1.4.9, Introduction section), and also during development (please see **Table 4**, Introduction section). However, inhibitory control alterations following developmental CPF are non-existent (please see Perez-Fernandez et al., 2019a). In the present thesis, sustained attention, learning and inhibitory control (both impulsive choice and perseveration) were studied in 7-12 months-old rats in Study 3, as evaluated by the 5C-SRTT (Perez-Fernandez et al., 2020b), whilst learning, spatial memory and inhibitory control (reversal stage) were analyzed with the Morris water maze test, described in Study 4.

Beginning with the performance acquisition function (learning), CPF exposure enhanced the learning rates for females, whose control counterparts showed significantly worse than control males in the initial learning stages of the 5C-SRTT (Study 3) (Perez-Fernandez et al., 2020b). This effect is novel in terms of the exposure protocol (preweaning with NChEI doses), but it is not the first time that this improved performance has been observed in females when analyzing the effects of exposure during other developmental periods (Levin et al., 2001; Aldridge et al., 2005b). That is, exposure to low doses of CPF produces a sex-specific enhancement of learning in females at a relatively wide range of neurodevelopmental stages. Although the neural basis of this enhancement is unknown, as it has not been yet explored, other authors have found that this exposure generally lead to decreased anxiety-like behaviors by using multiple paradigms in rodents (Ricceri et al., 2006; Venerosi et al., 2008; Carr et al., 2017, 2020; Peris-Sampedro et al., 2020). Thus, it is not unreasonable to suppose that the specific modulation of the HPA axis by CPF could be at the basis of these two domains (decreased

in basal anxiety that facilitates the learning of a new task). However, we did not find this pattern of results in the MWM (Study 4), which was possibly due to the age of the animals (adults vs. late-adulthood periods) or the task-specific nature of these changes (e.g., the naturally aversive nature of the water in the MWM could have increased the anxiety levels of the females, thus masking the physiological differences induced by CPF exposure during the postnatal period).

Sustained attention (accuracy and percentage of omissions) and inhibitory control (impulsive choice -premature responses- and compulsivity -perseverative responses-) were not affected at baseline levels once the animals had acquired the 5C-SRTT. However, the specific manipulation of the inter-trial interval (ITI) by increasing the time between trials revealed a greater impulsive pattern that had been hidden in the CPF exposed animals in comparison with the control rats. This is, to the best of our knowledge, the first time that the exposure to NChEI doses of CPF during development (regardless of the developmental stage) has been associated with this behavioral alteration. Interestingly, another study also found this same effect when using another OP agent during the wash-out periods (Terry et al., 2014). Although both studies used different compounds and exposure protocols, the similarity between both data sets supports the notion that different OP agents can induce the same effects on inhibitory control functioning, although the specific mechanism that regulates this behavioral mismatching is unknown. On the other hand, the changing contingencies introduced in the reversal stage of the MWM did not significantly affect the CPF exposed rats in comparison with their control counterparts. This could indicate that the influence of developmental exposure to NChEI doses of CPF is more focused on impulsive than compulsive traits. We must emphasize the term "developmental exposure to NChEI doses of CPF", since high acute doses of CPF during adulthood have been associated with selective increases in compulsive-like behaviors as opposed to impulsivity (Montes de Oca et al., 2013). However, whilst the lack of further developmental studies significantly limits the interpretations of the results found in Study 3 of the present thesis, these findings unquestionably represent an interesting starting point for future research.

Finally, CPF had no effects on the different stages of the MWM, indicating that the spatial memory of the aged exposed rats was not significantly impaired in comparison with their control counterparts, as described in Study 4. However, the commonly observed alteration in performance induced by the temporary removal of the platform (Probe stage) was not

observed in the exposed rats, contrary to the controls. This insensitivity to environmental disruptions during the learning process is surprising, something for which we have no explanation. In fact, this could be indicative of four distinct possibilities: a) the spatial memory of CPF exposed rats is more readily conserved, b) CPF exposed rats have an increased perseveration that protects them from brief disruptions, c) CPF exposed rats need more time to learn new rules, and d) CPF exposed animals used a non-guided allocentric and/or egocentric type of processing in spatial learning tasks. Although we have not enough empirical information, we think all these hypotheses should be discarded based on the performance of the animals during the acquisition and reversal learning stages.

In any case, all these data are relevant, since this is the first attempt to analyze the very long-term effects of developmental CPF exposure on spatial memory and learning in late-adult/ pre-elderly rats, ages at which it is possible to observe early signs of a decline in various cognitive functions (Goodpaster et al., 2006; Harada et al., 2013). Interestingly, other developmental studies have found that NChEI doses of CPF can alter reference and working memory, along with spatial learning (Levin et al., 2001, 2002; Icenogle et al., 2004; Aldridge et al., 2005b; Gómez-Gimenez et al., 2017; Alipour et al., 2019), whilst others have found no evidence for this link (Cole et al., 2012). Although there are some studies that have reported a lack of effects on these functions following preweaning exposure (Levin et al., 2001; Cole et al., 2012), most of the studies have found a clear negative influence of preweaning exposure on these functions (Jett et al., 2001; Guardia-Escote et al., 2018, 2019a; Wang et al., 2018; Basaure et al., 2019a), particularly concerning performance in the MWM in rats (Jett et al., 2001; Wang et al., 2018).

In summary, exposure to low doses of CPF during the preweaning developmental window enhances learning in females, increases impulsivity when the contextual contingencies are prone to the development of such behaviors (increased ITI), and induces a hyposensitizing state in response to temporal learning disruptions. This exposure, however, does not alter either basal sustained attention or spatial memory at adulthood and late-adult ages, respectively.

4.3. Medium and long-term molecular effects (peripheral) of developmental exposure to NChEI doses of Chlorpyrifos during the preweaning window.

In addition to the previously described CNS gene expression experiments that empirically demonstrated a link between CPF exposure and both up-regulation (M2r and GABA-A-α2 subunit) and down-regulation (BDNF) on the expression of various genes at different brain structures, we were also interested in the presumed effects of CPF exposure on peripherical targets such as the composition of gut microbiota and the metabolomic profile in both fecal and plasma samples, respectively. We chose both systems based on the relevance of both bacteria population composition and different systemic metabolites in the characterization of most of the disorders described, including neurodevelopmental, psychiatric and neurodegenerative disorders (for gut microbiota, see: Strati et al., 2017; Cenit et al., 2017; Spielman et al., 2018; Nguyen et al., 2018; Cheung et al., 2019; Molina-Torres et al., 2019. For metabolites, see: Ruggeri et al. 2014; Mussap et al. 2016; Wilkins and Trushina, 2017; Mohamadkhani 2018; Picca et al., 2019; Glaab et al., 2019; Shao and Le, 2019; Chan et al., 2020). Furthermore, the last decade has seen a growing interest in the direct effects of CPF on different components of these two systems (for gut microbiota, see: Joly-Condette et al., 2013, 2014, 2015, 2016; Reygner et al., 2016a, b; Réquillé et al., 2018; Liang et al., 2019; Fang et al., 2018; Zhao et al., 2016; Li et al., 2019; Guardia-Escote et al., 2019b. For metabolites, see: Slotkin et al., 2005; Wang et al., 2009; Xu et al., 2015; Guardia-Escote et al., 2019b).

4.3.1. Chlorpyrifos and the composition of gut microbiota

Whilst there are some good examples of the effects of developmental CPF exposure on gut microbiota, primarly affecting to the relative abundance or total counts of Enterococcus, Bacteroides, Clostridium, Bacteroidetes, Bifidobacterium, Brevundinomas, Firmicutes, and Firmicutes/Bacteroides ratio, amongst others (Joly Condette et al., 2013, 2015; Zhao et al., 2016; Reygner et al., 2016a; Fang et al., 2018), or even gut permeability modulations (Tirelli et al., 2007; Joly Condette et al., 2014), there is only one recent study of the preweaning exposure in mice (Guadia-Escote et al., 2019b). Interestingly, the authors found that CPF exposure affected the composition of the gut microbiota in an APOE-dependent manner, with a relevant decrease of the relative abundance of Verrucomicrobia at the phylum level only, in the APOE-4 mice, along with to the specific modulation of several classes of bacteria at both genus and species levels. However, the specific medium and long-term effects of preweaning CPF exposure on the composition of gut microbiota in rats remain unexplored.

Part of the work described in Studies 1 and 2 focused on analyzing the bacteria population (at phylum, genus and species taxonomic levels) following preweaning exposure to NChEI doses of CPF at medium (adolescence, PND35) and long-term (adulthood, PNM6) time-points (Perez-Fernandez et al., 2019b, 2020a). Results indicated that CPF exposure did not alter the composition of gut microbiota at the highest taxonomic level at any age (for on Firmicutes, Bacteroidetes, Verrucomicrobia, Actinobacteria, and Proteobacteria). This means that this exposure protocol does not alter large clusters of bacteria within this system. The lack of effects of this exposure protocol on the Verrucomicrobia bacteria phylum, contrary to the data found by Guardia-Escote et al., (2019b), could be due to differences in the animal model (mice vs. rats), genetic background (human APOE-4 mice vs. wild type rats) and/or time of sample collection after the last CPF exposure (4 hours vs. two weeks and 6.5 months). However, our exposure procedure produced significant gut dysbiosis at both genus and species levels in both adolescents and adults.

With regard to our analysis of adolescents (Study 2) (Perez-Fernandez et al., 2020a) and adults (Study 1) (Perez-Fernandez et al., 2019b), we found that CPF exposed rats showed, at the genus and species levels, important alterations in relative abundance of several bacteria populations, showing sexual-specific effects in some cases. In spite of the significant number of altered bacteria in exposed animals at both genus and species taxonomic levels, the relative abundance of the most important bacteria populations at phylum level was not affected by exposure. Thus, the theoretical importance of their modulatory effects on gut physiology, CNS functioning, and behavioral outcomes is, at the very least, questionable. Moreover, it is also important to point out that most of these affected bacterias have not an empirically confirmed biological function at the time of writing this thesis, and hence their regulation, even at a local physiological level, remains unclear. Among these, the most important altered bacteria were, at the genus level, Slackia, Aggregibacter, and Caldicellulosiruptor during adolescence, and Pelagicoccus, Butyrivibrio, Anaerobranca, and Vibrio (adulthood), whilst at the species level were the Moryella Indoligenes (adolescence) and Anaerobranca Zavarzinii (adulthood). Of all of these bacteria, only Brevundinomas has previously been linked to CPF exposure (Fang et al., 2018), but with opposing results, presumably due to the notable methodological differences between the studies. Thus, the data concerning the gut microbiota analyses published in both Studies 1 and 2 are mostly novel and relevant. However, we must

approach these findings with caution since most of these bacterias had a low-very low relative abundance with respect to the composition of the whole gut microbiota, and thus their impact may be limited.

4.3.2. Chlorpyrifos and the plasma metabolomic profile

Although there is a strong body of published data on the effects of developmental CPF exposure on both systemic and central metabolite composition (Slotkin et al., 2005; Wang et al., 2009; Xu et al., 2015), the specific effects of preweaning exposure to NChEI doses of CPF remain unstudied, with the exception of some studies that focused on mice models (Guardia-Escote et al., 2019b). These studies were primarily concerned with short-chain fatty acids and found that CPF generally increased the levels of Isovaleric acid and 4-Methylvaleric acid in the CNS.

We analyzed the effects of CPF exposure on plasma metabolites at four time points: 24 hours after the last exposure (PND16), 6 days after the last exposure (PND21), during adolescence (PND35) and at adult ages (PNM7), as shown in Study 2 (Perez-Fernandez et al., 2020a). Of all these time periods, we found that CPF exposure produced sex-specific alterations in different metabolites, affecting adult females. During this period, CPF increased the low and very low density lipoproteins (V/LDL), fatty acids (FA), N-acetylglycoprotein (NAc) and unsaturated fatty acids (UFA), whilst it decreased the levels of lactate, glucose, citrate, glutamine, alanine, leucine and serine, with all effects exclusively observed in adult female rats.

Of those metabolites that increased, we can confirm that this exposure protocol induced a long-term hyperlipidemic state in females. Hyperlipidemia is commonly linked to exposure to OP agents (Elsharkawy et al., 2013), but also following CPF exposure with NChEI doses during neonatal ages in males (Slotkin et al., 2005). However, the results of Study 2 constitute the first empirical evidence of sex-specific metabolic alterations in female animals following CPF exposure. Interestingly, other studies have also found selective lipid alterations following higher doses of CPF during adulthood (Acker and Nogueira, 2012). The increase of lipid levels in the organism is generally associated with poor health and pathologic states such as metabolic syndrome, although the decreased levels of plasma glucose observed in the exposed females are the opposite of what might be expected in this pathological profile (Brindley DN, 1995)

The different components of the lipid system are important in the regulation of glucose levels and vice versa (Parhofer KG, 2015). In this case, we found that CPF exposure decreased glucose levels during adulthood. This state of hypoglycemia refers to the decreased metabolization of glycogen to glucose (hypoglycogenolysis), or could be triggered by hypogluconeogenesis, which defines the decreased production "de novo" of glucose from different non-glucidic precursors, as in the case of different amino acids such as lactate, pyruvate, glutamine, alanine, and others (Gerich et al., 2001). Interestingly, other authors have also found the potential influence of CPF on glucose metabolism (Acker and Nogueira, 2012; Fang et al., 2018), although this is the first time that its exposure has been exclusively linked to metabolic perturbations in females. This general hypoglycemic state found in the exposed adult females commonly lead to several clinical alterations, from headache to confusion, memory loss, irritability and, in the long term, is related to cardiovascular disease (Field, 1989).

Exposure to CPF also decreased the plasma levels of different amino acids, some of which are partially required to produce glucose from the gluconeogenesis pathway, as previously described. Some of these amino acids include lactate, alanine (both were previously found to be decreased following CPF exposure in Wang et al., 2009, but in males) and citrate, which play and essential role in energy production in the Cori, Cahill and Krebs cycles, respectively. Citrate is converted from the oxaloacetate and acetyl-CoA metabolism. At the same time, high levels of glucose increase the glycolysis eventually also increases the citrate levels derived from the Krebs cycle, and this citrate can be subsequently be metabolized increasing the levels of acetyl-CoA (Storey KB, 2004). In this case, since the exposed females had low levels of glucose, all components of this process are altered, and the levels of elemental amino acids would also be expected to be modulated. Finally, leucine is the most important ketogenic amino acid in humans, the main role of which is the biosynthesis of proteins (Lynch et al., 2003) and one of its most important metabolic products is the acetyl-CoA (Kohlmeier M, 2015), linking this metabolite with the citrate cycle.

Taken together, the reduced levels of glucose, lactate, alanine, citrate and leucine could be taken to indirectly indicate a general reduction of cell energy production in the exposed female rats. In addition to the altered lipid metabolism balance, the general metabolic state of the adult female rats postnatally exposed to CPF is characterized by pathological state whose physiological and behavioral manifestations are still, for the most part,

unknown. Given that we did not conduct an in-depth analysis of the mitochondrial functioning following this exposure protocol, the apparent reduction in energy production observed in our experiments should be considered as a hypothesis.

4.4. Conclusions and future guidelines

Preweaning exposure to NChEI doses of CPF altered several behavioral domains and molecular targets at both medium and long-term developmental stages. In terms of specific behaviors, this exposure protocol induced early motor hyperactivity during adolescence and sensitized female rats to pain/stress by increasing their locomotor rates following i.p. injection (stress-induced hyperactivity, Study 1) (Perez-Fernandez et al., 2019b), but also progressively generates a general motor hypoactivity that is firstly detected during adulthood in females (Study 3) (Perez-Fernandez et al., 2020b) and later confirmed at the late-adult, pre-elderly stages in both sexes (Study 4). This exposure protocol had little effect on sociability, enhancing the reaction to social novelty in the exposed females (Study 2) (Perez-Fernandez et al., 2020a), and also improved their learning performance in the acquisition of the 5C-SRTT (Study 3). CPF also increased the rates of impulsive action in both sexes, although this effect had to be revealed by inducing an operant context that facilitates behavior of this sort (longer ITI), since performance under normal learning conditions (baseline) was similar to that observed in their control counterparts.

In terms of alternative mechanisms of action and molecular outcomes, the empirical information derived from this thesis also supports the existence of other mechanisms of CPF toxicity, involving both cholinergic (hyposensitized, as found in both Studies 1 and 3) and GABAergic systems (hypersensitized, as found in Study 1), where two specific molecules could be playing an important role, since these were modulated by CPF (up-regulation of the expression of both M2 receptors at the dorsal striatum and the GABA-A-α2 subunit genes in the frontal cortex in Study 1). This was complemented by the empirical evidence that CPF down-regulated the expression of BDNF gene at the dorsal striatum (Study 3). This exposure protocol also altered the gut microbiota composition at both adolescent and adult ages, as well as induced a general hyperlipidemic, hypoglycemic, and apparent reduced energy production state in adult female rats. To certain extent, all of these behaviors and molecular outputs form, in some degree, the basis of many of the most important neurodevelopmental (e.g., ASD and ADHD), neurodegenerative (e.g. AD and PD) and psychiatric disorders (e.g., depression and

anxiety), and thus the relevance of this data is unquestionable. However, the present thesis opens more doors than it closes. In particular, we believe that following results require further research:

a) What are the dynamics of the locomotor alterations throughout the life-span? This could be addressed by periodically checking motricity in an open field at different ages and using different animals in each age (animals at adolescence, early-adulthood, late-adulthood, and older ages).
b) How does the CPF exposure modify CNS expression levels? Although mRNA information derived from the present thesis is reliable, relevant, and informative, multiple molecular mechanisms could be regulating the translation process from mRNA to protein, as it is the role proposed to the micro RNAs (Wahid et al., 2010). New experiments would allow to determinate regulatory mechanisms implicated in the gene miss-regulation due to CPF exposure. As the protein is the essential functional molecule in terms of cell activity/sensitivity, its activity might be also analyzed and confirmed by using enzymatic activity assays (e.g., enzyme-linked immunosorbent assay).
c) Does preweaning exposure to CPF directly or indirectly alter CB1 receptor functioning? Other types of experimental procedures should be employed in the specific study of the endocannabinoid system and motor and feeding behavior concerning preweaning CPF exposure, based on the empirical evidence derived from the studies by Carr and colleagues regarding different endogenous agonists and enzymes from this system. This could be addressed by changing the route of administration of the drug (oral instead of i.p., which increases the basal stress levels), studying rats of different ages (younger instead of 5-6 months old) or using knock out models for the CB1 receptor, amongst others.
d) What is the physiological/molecular basis of the stress-induced hyperactivity observed in the exposed female rats? Although this phenomenon has been largely overlooked in the present thesis, its statistical power is unquestionable and it is relevant in a translational sense, given that humans are required to face multitude of uncomfortable situations on a daily basis. One logical, preliminary approach could be to specifically analyze the HPA axis (as previously proposed), as well as other components of the neuroendocrine system, due to the sex-specificity of the effects found. To the best of our knowledge, no study has analyzed the effects of

CPF during preweaning stages on such systems. Another interesting approach could be to establish whether this behavior can also be observed in instrumental behaviors (e.g. operant skinner box related tasks), and not only in free-exploratory contexts.

e) Would CPF exposure during another developmental period, particularly during gestation, induce alterations in sociability? Our failure to find a link between preweaning exposure to CPF and decreased levels of sociability could be related to the age of exposure. Based on the previous literature, it appears that the detrimental effects of CPF and other chemical agents (e.g., Valproic acid) are stronger when exposed during gestation. These gestational models are currently being tested in our facilities and point towards a significant link between gestational CPF exposure and an ASD-like phenotype (Morales-Navas et al., under review).

f) What is the physiological/molecular basis of the enhanced learning found in exposed females? Does this vary according to age? Is it task/domain-dependent? Our results suggest that the enhanced learning observed in exposed females is more strongly linked to sustained attention than spatial memory functions (5C-SRTT vs. MWM, respectively), and is more likely to be found during adulthood but not at late-adult ages. However, our conclusions are restricted by the fact that we limited, and also used animals in the MWM that had previous experience in learning a task. To address this, a new experiment would be needed, which reverses the order of these tasks (MWM during adulthood and 5C-SRTT during late-adult ages) or doing both experiments separately with naïve rats on each condition. Given that improved learning performance was sex-specific in favor of female rats, the physiological/molecular substrate could be strongly linked to the HPA axis and neuroendocrine system.

g) What is the physiological/molecular substrate of the increased (but hidden) impulsive action of the exposed animals? Although in Study 3 we analyzed the expression of several genes, we did not cover all the molecular options required to solve this question. Indeed, the lack of significant alterations of the mRNA levels of both 5-HT2a and c receptors in the dorsal striatum is relevant concerning impulsivity, but there are several regions and genes/proteins that also play an essential role in the regulation of inhibitory control (Bevilacqua and Goldman, 2013).

h) What is the physiological/molecular substrate of the hyposensitized cholinergic and hypersensitized GABAergic systems? Although we found two altered genes regarding exposure condition, one of each system and brain area, we are aware that there are additional components from both systems that remain unexplored in our analyses, whilst this selective study could be extended to relevant areas concerning motor regulation, such as the case of Cerebellum.

i) Could this exposure protocol induce a more general gut dysbiosis at an earlier after the exposure is finished? As mentioned, we did not find alterations in any explored bacteria at the phylum level (Studies 1 and 2). However, other studies using this exposure protocol found such alterations, albeit in mice models (Guardia-Escote et al., 2019b). The main limitation here could be the age of exploration, since we analyzed both medium (adolescence) and long-term (adulthood) but not short-term (e.g., few hours after the latest exposure) effects of the exposure condition. A new experiment could focus on this issue, even collecting samples at different times during the exposure protocol (e.g., four hours after the first, the third, and the final administration), in order to explore in detail the evolution of the CPF-induced dysbiosis.

j) Does this preweaning exposure to NChEI doses of CPF generally decrease cell energy production in the exposed females? Although this appears to the case, as observed in our experiment (Study 2), this question could only be answered by deeply analyzing the functioning of the mitochondria at the systemic system. Furthermore, we would extend this relevant information by using brain samples to specifically cover different tissues of exceptional relevance in behavior regulation.

k) There is one important point that was not addressed in the present thesis but is essentially based on the theoretical assumptions that guided our different studies: does CPF exposure during preweaning stages alter the neurodevelopmental processes that specifically reach maturity around these ages (e.g., synaptogenesis, glial cells growth, oxytocin, and vasopressin systems, GABAergic system shifting, amongst others). If so, how are these changes in brain maturation related to the observed behavioral and molecular modulations presented in this thesis?

Final conclusions

Study 1.

- Preweaning exposure to CPF increased spontaneous locomotor activity in adolescent rats, a behavior associated with developmental pathologies such as ADHD. This exposure also increased the sensitivity of the female rats to stress following an i.p. injection of saline, as evaluated by motricity. Since humans have to daily face stressful situations, this hypersensitized state to external aversive factors induced by CPF could trigger maladaptive responses in humans, particularly in women.
- CPF induced a long-term hypo and hyper sensitization of both the cholinergic and GABAergic systems, respectively, without affecting others neurotransmitter systems, with the former being particularly relevant since various disorders such as AD are known to involve a progressive cholinergic degradation. Based on this notion, CPF could be, at least in part, a relevant environmental factor in AD progression, as proposed in the current literature. CPF also up-regulated the expression of both M2r (dorsal striatum) and GABA-A-α2 subunit receptor (frontal cortex) genes. The over-expression of this latter gene is of interest due to its association with the abuse of alcohol and other drugs, indirectly associating the CPF exposure to these pathological behaviors, at least at molecular level.
- CPF induced gut dysbiosis concerning several bacteria populations at both genus and species levels. This is relevant, since the gut microbiome is known to have physiological and functional inter-relationships with the CNS and the immune systems, thus the CPF-induced changes in the microbiome could be also indirectly affecting the biological centers that regulate behavior.

Study 2.

- Preweaning exposure to NChEI doses of CPF had no effect on either sociability or reaction to social novelty and dominance in both adolescent and adult rats, and thus it cannot be related to ASD. However, it induced significant gut dysbiosis at both genus and species levels, driving to similar conclusions described in Study 1 concerning microbiome.
- CPF triggered long-term hyperlipidemia, hypoglycemia and an apparent generally altered cell energy production as assessed in the plasma metabolites,

exclusively in adult females. This is a relevant finding, since some of the most common psychiatric, neurodevelopmental and neurodegenerative disorders are characterized by selective alterations in some of these components, whilst hyperlipidemia is also associated with a general states of ill health, indicating that women could be more sensitive to these states when living in contexts with higher rates of OP exposure.

Study 3.

- Preweaning exposure to NChEI doses of CPF induced a long-term learning enhancement in females, but it failed to alter either attentional or inhibitory control functioning at baseline in either sex. However, CPF increased motor impulsivity, but only when the ITI was manipulated. This unmasked impulsivity is relevant, since external stressors are commonly face by humans on a daily basis, and an increase in impulsive tendencies is, in many contexts, maladaptive.
- CPF induced long-term hyposensitization of the cholinergic system, which was measured with attentional and inhibitory control outcomes. This is relevant since it extends and complements the previous findings in Study 1 to other relevant behaviors and later life stages, linking CPF exposure to one of the most important physiological features at the basis of AD.
- CPF produced a significant decrease in the gene expression of BDNF in the dorsal striatum, thus CPF could be affecting the integrity of the CNS since this neurotrophic factor is essential for the adequate functioning of the CNS in terms of neuronal growth and consolidation, and its alteration has been systematically associated with several disorders.

Study 4.

- Preweaning exposure to NChEI doses of CPF failed to alter performance on either learning or spatial memory in aged rats. Exposed animals, however, were less sensitive to learning disruptions (temporary platform removal in the Probe phase). Further, this exposure failed to induce either compulsive-like patterns (Reversal phase) or visual functions alterations (Visual phase). It did not affect anxiety rates either.

- CPF exposure generally decreased motricity in aged rats, which is of interest since various neurodegenerative disorders (e.g. AD and PD) as well as normal aging are characterized by a progressive decline in physical capacity. Thus, CPF exposure could accelerate the degradation of various physical capacities by the aging process.

Conclusiones Finales

Estudio 1.

- La exposición pre-deteste a CPF aumentó la conducta locomotora espontánea en ratas adolescentes, un comportamiento asociado con patologías del desarrollo como el déficit de atención con hiperactividad. También incrementó la sensibilidad de las ratas hembra al estrés tras una inyección i.p. de salino, evaluado mediante conductas motoras. Dado que los seres humanos tienen que lidiar con situaciones estresantes todos los días, este estado hipersensibilizado a factores externos aversivos inducido por CPF podría facilitar respuestas desadaptativas en los seres humanos, especialmente en las mujeres.
- La exposición a CPF indujo una hipo y una hiper sensibilización de los sistemas colinérgico y GABAérgico a largo plazo, respectivamente, sin afectar a otros sistemas de neurotransmisión, siendo el primero relevante ya que diferente patologías, como la enfermedad de Alzhéimer, son conocidos por tener una degradación progresiva del sistema colinérgico. Basándonos en esto, el CPF podría ser, al menos en parte, un factor ambiental relevante en la progresión de la enfermedad de Alzhéimer. La exposición a CPF también reguló al alza tanto los receptores muscarínicos 2 (estriado dorsal) como la subunidad α2 del receptor GABA-A (corteza frontal). La sobre expresión de este último gen es de especial interés ya que se ha asociado con el consumo de alcohol y otro tipo de drogas, lo que asocia de manera indirecta la exposición a CPF con esos comportamientos patológicos, al menos a nivel molecular.
- La exposición a CPF indujo una disbiosis intestinal en relación con multitud de poblaciones bacterianas tanto a nivel de género como de especie. Esto es relevante, ya que la microbiota intestinal es conocida por tener interrelaciones fisiológicas y funcionales con el CNS y el sistema inmune, por lo que los cambios intestinales inducidos por CPF podrían afectar indirectamente a los centros biológicos que subyacen a la conducta.

Estudio 2.

- La exposición pre-deteste a dosis NChEI de CPF no afectó ni a la sociabilidad ni a la reacción ante la novedad social, así como tampoco afectó a la dominancia tanto en ratas adolescentes como adultas, por lo que no se puede asociar a los trastornos del espectro autista. Sin embargo, indujo una importante disbiosis

intestinal a nivel de género y especies, llevando a conclusiones similares descritas en el Estudio 1 en relación a la microbiota.

- La exposición a CPF indujo un estado hiperlipídico, hipoglicémico, así como un aparente estado general de reducción de la producción de energía celular, evaluado en la concentración de diferentes metabolitos plasmáticos y exclusivamente en las hembras adultas. Esto es relevante ya que algunas de las patologías psiquiátricas, del neurodesarrollo y neurodegenerativas más comunes presenta alteraciones selectivas en alguno de esos componentes, y el estado hiperlipídico general se suele relacionar con un estado general insano, indicando que las mujeres podrían ser más sensible a esos estados cuando presentan mayores índices de exposición a CPF.

Estudio 3.

- La exposición pre-destete a NChEI dosis de CPF indujo una mejora a largo plazo del aprendizaje en las hembras, pero no alteró ni el funcionamiento atencional ni el control inhibitorio a nivel basal en ningún sexo. Sin embargo, la exposición a CPF aumento los niveles de impulsividad motora, algo sólo observado tras la manipulación del intervalo inter-estímulo. Esta impulsividad oculta es relevante ya que los estresores externos son habituales en la vida diaria del ser humano, y un aumento de la tendencia a comportamientos impulsivos es, en muchos contextos, desadaptativo.
- La exposición a CPF indujo una hipo sensibilización a largo plazo del sistema colinérgico evaluado mediante variables atencionales y de control inhibitorio, siendo relevante ya que esto extiende y complementa hallazgos previos observados en el Estudio 1 a otros comportamientos relevantes y más tarde en la vida, relacionando la exposición a CPF con una de las características fisiológicas más importantes asociadas a la enfermedad de Alzhéimer.
- La exposición a CPF disminuyó significativamente la expresión del gen para el factor neurotrófico derivado del cerebro en el estriado dorsal, por lo que el CPF podría estar afectando a la integridad del CNS, ya que este factor neurotrófico es esencial para el adecuado funcionamiento del CNS en términos de crecimiento y consolidación neuronal, y a que su alteración se ha asociado sistemáticamente con multitud de patologías.

Estudio 4.

- La exposición pre-destete a dosis NChEI de CPF no alteró ni el aprendizaje ni el rendimiento en tareas de memoria espacial en ratas mayores. Sin embargo, los animales expuestos tendieron a ser menos sensibles a la presencia de disruptores del aprendizaje (la sustracción de la plataforma en la fase de exploración). No indujo ni comportamientos compulsivos (fase de aprendizaje inverso), ni alteraciones en la función visual (fase visual). Tampoco afecto los niveles de ansiedad.
- La exposición a CPF llevó generalmente a un descenso de la actividad motora en dichas edades, siendo relevante ya que diferentes enfermedades neurodegenerativas (enfermedad de Alzhéimer o Parkinson), así como el envejecimiento normal, se caracterizan por un declive progresivo en las capacidades físicas. Por lo tanto, la exposición a CPF podría llevar a una aceleración de la degradación de diferentes capacidades físicas con la edad.

Scientific production

Articles that compose the present thesis.

Perez-Fernandez, C., Morales-Navas, M., Aguilera-Sáez, L. M., Abreu, A. C., Guardia-Escote, L., Fernández, I., … 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. *Environmental Research*, *184*, 109341. https://doi.org/10.1016/j.envres.2020.109341

Perez-Fernandez, C., Morales-Navas, M., Guardia-Escote, L., Colomina, M. T., Giménez, E., & Sánchez-Santed, F. (2020b). Postnatal exposure to low doses of Chlorpyrifos induces long-term effects on 5C-SRTT learning and performance, cholinergic and GABAergic systems and BDNF expression. *Experimental Neurology*, 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. (2019b). 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 and Chemical Toxicology*. https://doi.org/10.1016/j.fct.2019.110865

Perez-Fernandez, C., Morales-Navas, M., Guardia-Escote, L., Colomina, M. T., Giménez, E., & Sánchez-Santed, F. Preweaning exposure to low doses of Chlorpyrifos induces a general hypomotricity in aged rats. In preparation.

Other published articles related to this topic.

Guardia-Escote, L., Basaure, P., Biosca-Brull, J., Cabré, M., Blanco, J., Pérez-Fernández, C., … Colomina, M. T. (2019b). APOE genotype and postnatal chlorpyrifos exposure modulate gut microbiota and cerebral short-chain fatty acids in preweaning mice. *Food and Chemical Toxicology*, *135*. https://doi.org/10.1016/j.fct.2019.110872

Guardia-Escote, L., Basaure, P., Blanco, J., Cabré, M., Pérez-Fernández, C., Sánchez-Santed, F., … Colomina, M. T. (2018). Postnatal exposure to chlorpyrifos produces long-term effects on spatial memory and the cholinergic system in mice in a sex- and APOE genotype-dependent manner. *Food and Chemical Toxicology*, *122*, 1–10. https://doi.org/10.1016/j.fct.2018.09.069

Perez-Fernandez, C., Flores, P., & Sánchez-Santed, F. (2019a). A Systematic Review on the Influences of Neurotoxicological Xenobiotic Compounds on Inhibitory Control. *Frontiers in Behavioral Neuroscience*, *13*, 139. https://doi.org/10.3389/fnbeh.2019.00139

Other published articles related with other topics.

Nazari, A., Perez-Fernandez, C., Flores, P., Moreno, M., & Sánchez-Santed, F. (2020). Age-dependent effects of repeated methamphetamine exposure on locomotor activity and attentional function in rats. *Pharmacology Biochemistry and Behavior*, *191*. https://doi.org/10.1016/j.pbb.2020.172879

Pérez-Fernández, C., Cánovas, R., Moreno, M., Sánchez-Santed, F., & Flores, P. (2017). Go/NoGo training improves executive functions in an 8-year-old child born preterm. In *Revista de Psicología Clínica con Niños y Adolescentes, Monográfico Aplicaciones de las TICs a la evaluación y terapia infanto-juvenil. 4(3), 60-66*

Sánchez-Kuhn, A., León, J. J., Gôngora, K., Pérez-Fernández, C., Sánchez-Santed, F., Moreno, M., & Flores, P. (2017). Go/No-Go task performance predicts differences in compulsivity but not in impulsivity personality traits. *Psychiatry Research*, *257*, 270–275. https://doi.org/10.1016/j.psychres.2017.07.064

Sánchez-Kuhn, A., Pérez-Fernández, C., Cánovas, R., Flores, P., & Sánchez-Santed, F. (2017). Transcranial direct current stimulation as a motor neurorehabilitation tool: An empirical review. *BioMedical Engineering Online*, *16*(1). https://doi.org/10.1186/s12938-017-0361-8

Sánchez-Kuhn, A., Pérez-Fernández, C., Moreno, M., Flores, P., & Sánchez-Santed, F. (2018). Differential effects of transcranial direct current stimulation (tDCS) depending on previous musical training. *Frontiers in Psychology*, *9*(SEP). https://doi.org/10.3389/fpsyg.2018.01465

Published Book chapters

Pérez-Fernández, C., Sánchez-Kuhn, A., Cánovas, R., Flores, P., & Sánchez-Santed, F. (2016). The effect of transcranial direct current stimulation (tDCS) over human motor function. *Lecture Notes in Computer Science (Including Subseries Lecture Notes in Artificial Intelligence and Lecture Notes in Bioinformatics)*, *9656*, 478–494. https://doi.org/10.1007/978-3-319-31744-1_43

Congress contributions (*those studies which concern neurotoxicology field)

1. *Title of the work: Ultrasonic calls of rat pups prenatally exposed to acid valproic and chlorpyrifos
Name of the conference: III International Congress of Psychobiology
City of event: Granada, Andalusia, Spain
Date of event: 2019
Organising entity: University of Granada Type of entity: University
City organizing entity: Granada
Authors: Ainhoa Sáncehz-Gil; Miguel Morales-Navas; Cristian Perez-Fernandez; Fernando Sánchez-Santed.

2. Title of the work: Does the Go/NoGo task measure Impulsivity or Compulsivity?
Name of the conference: II Ibero-American Neuropsychology Congress
Geographical area: European Union
Type of participation: 'Participatory - poster
City of event: Almeria,
Date of event: 2018
Authors: Sánchez-kuhn, Ana; Leon, Jose Juan; Gongora, Karinna; Cristian Antonio Pérez Fernández; Fernando

Sánchez Santed; Margarita Moreno Montoya; María Pilar Flores Cubos.

3. *Title of the work: Low doses of sub-chronic Chlorpyrifos (CPF) exposure in late developmental stages trigger both long term hyposensitized cholinergic and hypersensitized GABAergic system in female Wistar rats: a behavioral and genetic analysis
Name of the conference: II Ibero-American Neuropsychology Congress
Geographical area: European Union
Type of participation: 'Participatory - poster
City of event: Almeria
Date of event: 2018
Authors: Cristian Antonio Pérez Fernández; Miguel Morales Navas; Estela Gimenez Caminero; Fernando Sánchez-Santed.

4. *Title of the work: Prenatal Chlorpyrifos and Valproic Acid in relation to development of ASD
Name of the conference: II Ibero-American Neuropsychology Congress
Geographical area: European Union
Type of participation: 'Participatory - poster
City of event: Almeria
Date of event: 2018
Author: Miguel Morales Navas; Sergio Castaño Castaño; Cristian Antonio Pérez Fernández; Leinekugel, Xavier; Fernando Sánchez Santed.

5. Title of the work: Total and long-lasting visual function recovery of an adult patient with amblyopia after 4 consecutive weeks of perceptual learning training: brief temporal ocular occlusion as plasticity facilitator
Name of the conference: II Ibero-American Neuropsychology Congress
Geographical area: European Union
Type of participation: 'Participatory - poster
City of event: Almeria
Date of event: 2018
Authors: Cristian Antonio Pérez-Fernández; Aragonés, Cristina; Salvestrini, Patrizia; Francisco Antonio Nieto-Escámez; Fernando Sánchez-Santed.

6. *Title of the work: Early Postnatal Exposure to NOAEL doses of Chlorpyrifos in rats: Cholinergic and GABAergic effects and its behavioral implications
Name of the conference: II International Congress in Psychobiology
Geographical area: European Union
Type of participation: 'Participatory - poster
City of event: ÁVILA
Date of event: 2017
Authors: Mata, Jose; Cristian Antonio Pérez-Fernández; Miguel Morales-Navas; Fernando Sánchez-Santed.

7. *Title of the work: Prenatal Chlorpyrifos and Valproic acid in relation to development of ASD
Name of the conference: II International Congress in Psychobiology
Geographical area: European Union
Type of participation: Participatory - oral communication

City of event: ÁVILA
Date of event: 2017
Authors: Miguel Morales Navas; Sergio Castaño Castaño; Cristian Antonio Pérez Fernández; El Omari, Saloua; Mata, Jose; Leinekugel, Xavier; Sánchez-Santed, Fernando.

8. *Title of the work: The effects of postnatal exposure to Chlorpyrifos are sex-dependent
Name of the conference: II International Congress in Psychobiology
Geographical area: European Union
Type of participation: Participatory - oral communication
City of event: AVILA
Date of event: 2017
Authors: Cristian Antonio Pérez-Fernández; Mata, Jose; Miguel Morales-Navas; Fernando Sánchez-Santed.

9. Title of the work: Amblyopia and its recovery in adulthood: Safety considerations and long-lasting effects of brief temporal ocular occlusion in complex visual functions
Name of the conference: 10th FENS Forum of Neuroscience 2016
Geographical area: European Union
Type of participation: 'Participatory - poster
City of event: Copenhagen, Denmark,
Date of event: 2016
Authors: Cristian Antonio Pérez-Fernández; Salvestrini, Patrizia; Jiménez-velázquez, Juan Antonio; Francisco Antonio Nieto-Eescámez; Fernando Sánchez-Sánted.

10. Title of the work: tDCS anodal stimulation on M1 improves motor learning: influences of music training
Name of the conference: 1st International Congress in Psychobiology
Type of event: Conference
City of event: Oviedo,
Date of event: 2015
Authors: Sanchez-kuhn, Ana; Cristian Antonio Pérez-Fernández; Margarita Moreno Montoya; María Pilar Flores, Cubos; Fernando Sánchez-Santed.

11. Title of the work: Effects of transcranial direct current stimulation on the right motor cortex: differences concerning previous musical training.
Name of the conference: EBBS EBPS Joint Meeting 12/15 Sep 2015
Geographical area: European Union
Type of participation: 'Participatory - poster
Date of event: 2015
Auhtors: Sanchez-kuhn, Ana; Cristian Antonio Pérez-Fernández; Margarita Moreno Montoya; María Pilar Flores Cubos; Fernando Sánchez Santed.

12. Title of the work: The Effect of Transcranial Direct Current Stimulation (tDCS) over Human the Motor Function
Name of the conference: 4th International Work-Conference on Bioinformatics and Biomedical Engineering, IWBBIO 2016
Geographical area: European Union
Type of participation: Participatory - oral communication

City of event: Granada
Date of event: 2015
Authors: Cristian Antonio Pérez-Fernández; Sánchez-kuhn, Ana; María Rosa Cánovas López; María Pilar Flores Cubos; Fernando Sánchez Santed.

Supervision of Final Year Project (Degree of Psychology)

Francisco Esteban Pérez Milán. Rehabilitación de la ambliopía en adultos mediante videojuegos de Realidad Virtual. University of Almería. 21/06/2019.

Antonio Martínez Herrada. Efecto del clorpirifós en las vocalizaciones ultrasónicas en crías de rata expuestas prenatalmente. University of Almería. 20/06/2019

Pablo Alcántara Vázquez. Nuevas tecnologías y neurorrehabilitación: Propuesta de la plataforma Visionary Tool como entrenamiento de los procesos atencionales. University of Almería. 19/06/2019

Scientific divulgation

Participation in both European Researchers' night (approved by European Commission within the Marie Sklodowska-Curie Actions and coordinated by the Research Results Transfer Office -OTRI-, UAL) and the Week of Science (UAL) yearly from 2015 to 2019.

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Cerebro.Brain.Tru.Gehirn.Mo3r.Mozak.Mo3bk.Cervell.Mozek.Hjerne.Mozog.Mozgani.Aju.Garuna.

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