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

Cristian Perez-Fernandez, Miguel Morales-Navas, Laia Guardia-Escote, José Antonio Garrido-Cárdenas, María Teresa Colomina, Estela Giménez, Fernando Sánchez-Santed

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; Jokanovic 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 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; Gomez-Gimenez 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; Lopez-Crespo et al., 2007; Savy et al., 2015; Peris-Sampedro et al., 2014; Yan et al., 2012; Lopez-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 & Seidler, 2007a,c; Aldridge et al., 2005c; Savy et al., 2015), serotonergic (Slotkin & Siedler 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 & Siedler 2007b; Slotkin et al., 2010) and GABAergic (Gómez-Gimenez 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 & Siedler 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 (> PND10) 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 & 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 post-natal day (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±2ºC), humidity (50±10%) and 12 light hour cycle (lights on at 8 am). 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 hours 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.

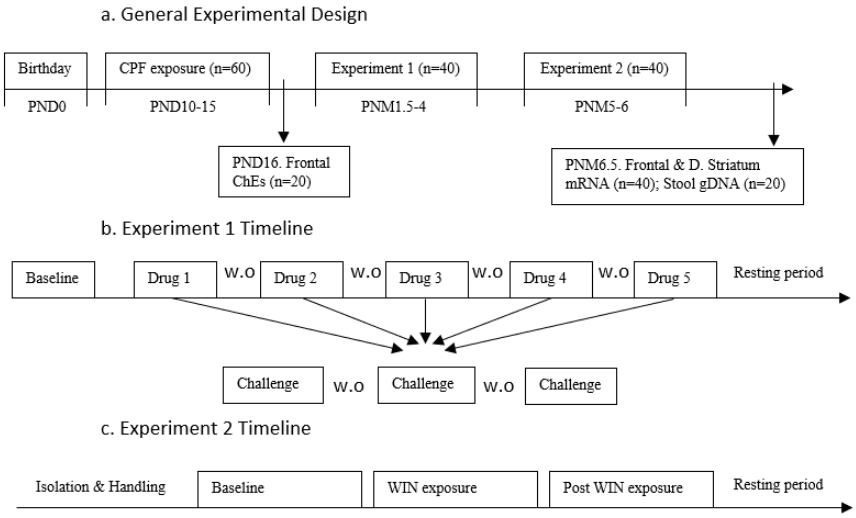

Image 1. Experimental design. a) General experimental design. Half of the male and female rats were orally exposed (gavage) to CPF from PND10 to 15. Frontal cortices from 20 rats (half females, 5 CPF exposed on per sex) were taken 24 hours after last exposure for ChEs analyses. Other 40 rats (half females, 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 hours 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.

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 1mg/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 (39x39x15cm) 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 minutes.

2.4.1.2. Procedure

The animals were driven to an experimental room for environmental habituation 1 hour 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±2ºC 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 hour per day) starting at the PND40. The first 20 minutes 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 minutes 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.

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 minutes 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 hour) 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 hours 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 -80ºC 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 hours 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 uL, in 0.1M Na

phosphate buffer; pH 8.0; final concentration = 0.33 mM) and 221 uL of sodium phosphate buffer (0.1 M; pH 8.0). After 300 seconds of incubation at 37 ºC, the reaction was initiated by the addition of 9 uL 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 ºC 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 MM, 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.

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

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

2.5.4. Metagenomic: Gut microbiota composition

The total stool material stored at -80ºC was mixed (Heidolph RZR1) and 100mg 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 Purification Kit (Invitrogen), following the manufacturer's instructions. gDNA samples were stored

until use at -80ºC. 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®.

2. 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. **Figure 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.

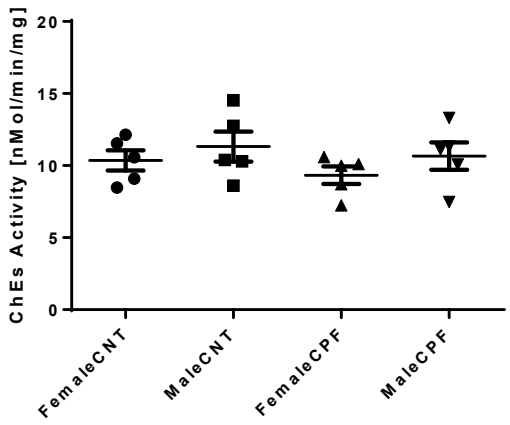

Figure 1. ChEs activity in Frontal Cortex 24h (PND16) after the last exposure to CPF. Data are expressed by means, SEM and the individual plots.

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.

3.2.2. 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 minutes 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 Figure 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 (**Figure 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 Figures 2 and 3). There was no significant effect of TREATMENT or an interaction SEXxTREATMENT for either distance or vertical activity. 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 Figure 4). However, vertical activity was differentially affected by stress, as revealed by the significant interaction SEXxTREATMENT [F(1,34)=4.999, p= 0.032] (**Figure 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).

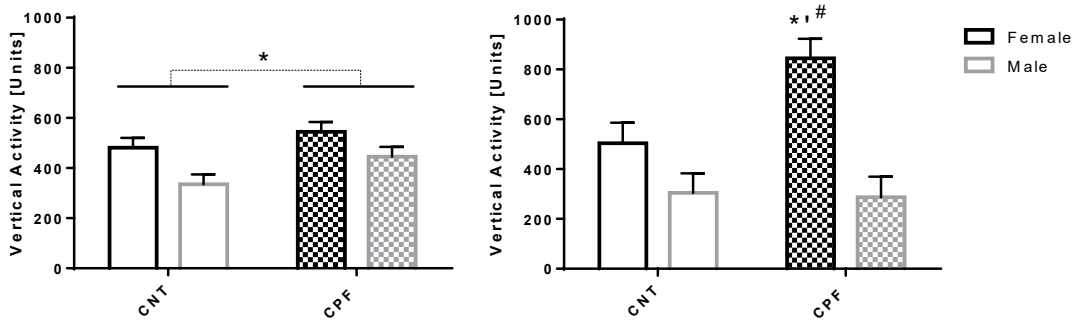

Figure 2. Locomotor activity. a) Spontaneous locomotor activity in the open field for vertical activity (Left). b) Locomotor activity alteration following acute stress (saline i.p. injection) for vertical activity (Right). Data are expressed by means and SEM. * indicates a significant (p<0.05) difference 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.

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 Figures 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

3.2.3. Drug challenges

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

3.2.3.1. 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).

3.2.3.2. 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) (**Figure 3a**). This effect was not observed in males (p= 0.717). Finally, although this same pattern of findings was observed for vertical activity (**Figure 3b**), differences between groups were not significant, with no significant interaction TREATMENTxSEX or SEXxTREATMENTxDOSE.

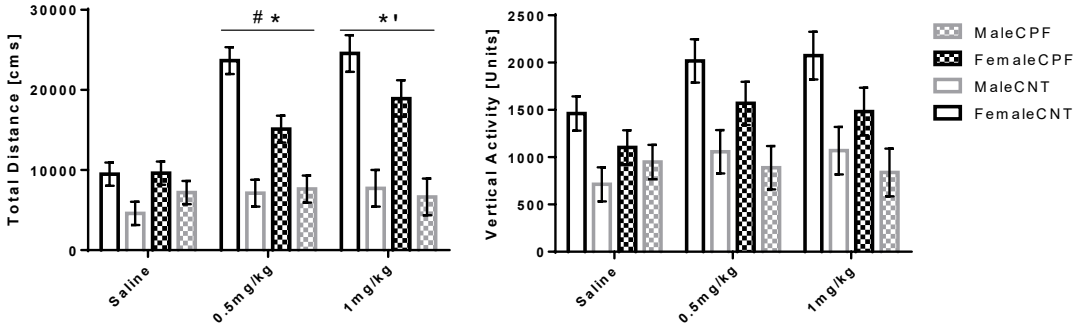

Figure 3. Scopolamine (i.p.) effects on locomotor activity. a) Total distance and b) Vertical activity. Data are expressed by means and SEM. # indicates significant ($p<0.05$) differences between groups at the lowest dose. * indicates significant differences for CNT animals at the lowest dose from saline.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

3.2.3.3. 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 0.1mg/kg, with a strong decrease at 0.5mg/kg, as in the case of exposed males (**Figures 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).

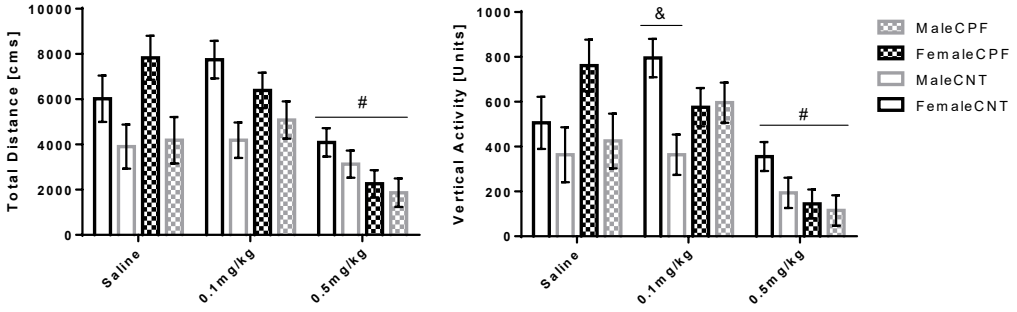

Figure 4. Alprazolam (i.p.) effects on locomotor activity. a) Total distance and b) Vertical activity. Data are expressed by means and SEM. # indicates significant ($p<0.05$) differences between CPF-CNT groups at the largest doses. & indicates significant differences ($p<0.05$) between both control groups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

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 (*Figures 9)* regions. However, we found up-regulation of both the frontal GABA-A-α2 subunit and M2 receptor 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] (**Figures 5a and b**). The remaining non-significant genes are displayed in the Supplementary materials (Figures 7-20).

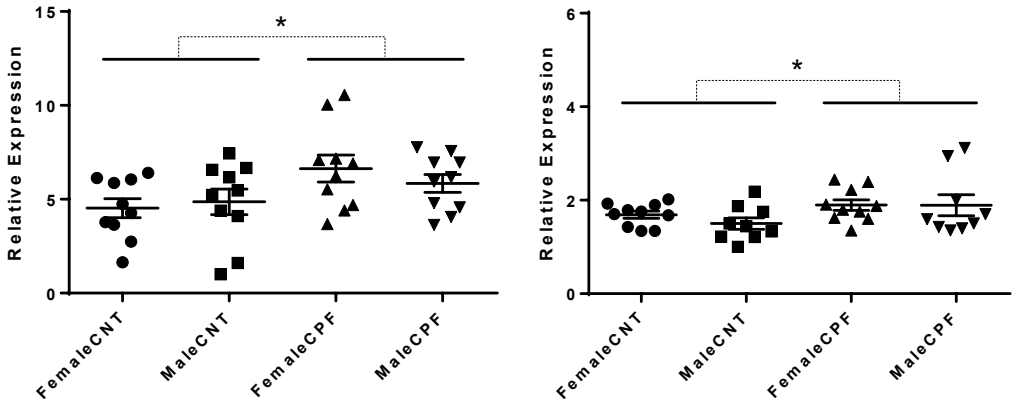

Figure 5. Relative expression of different genes. a) dorsal striatum (Muscarinic 2 receptor) and b) frontal cortex (GABA-A-α2) at PNM6. Data are expressed by means, SEM and the individual plots. * indicates significant ($p<0.05$) differences between CPF-CNT groups.

3.4. Experiment 2. WIN Challenge for Weight and Consumption

3.4.3. 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 1mg/kg of WIN 55, 212-2 did not affect body weight but reduced consumption to baseline recovery level a few days after exposure (**Figures 6a & 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 nonexistent 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 (**Figure 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.

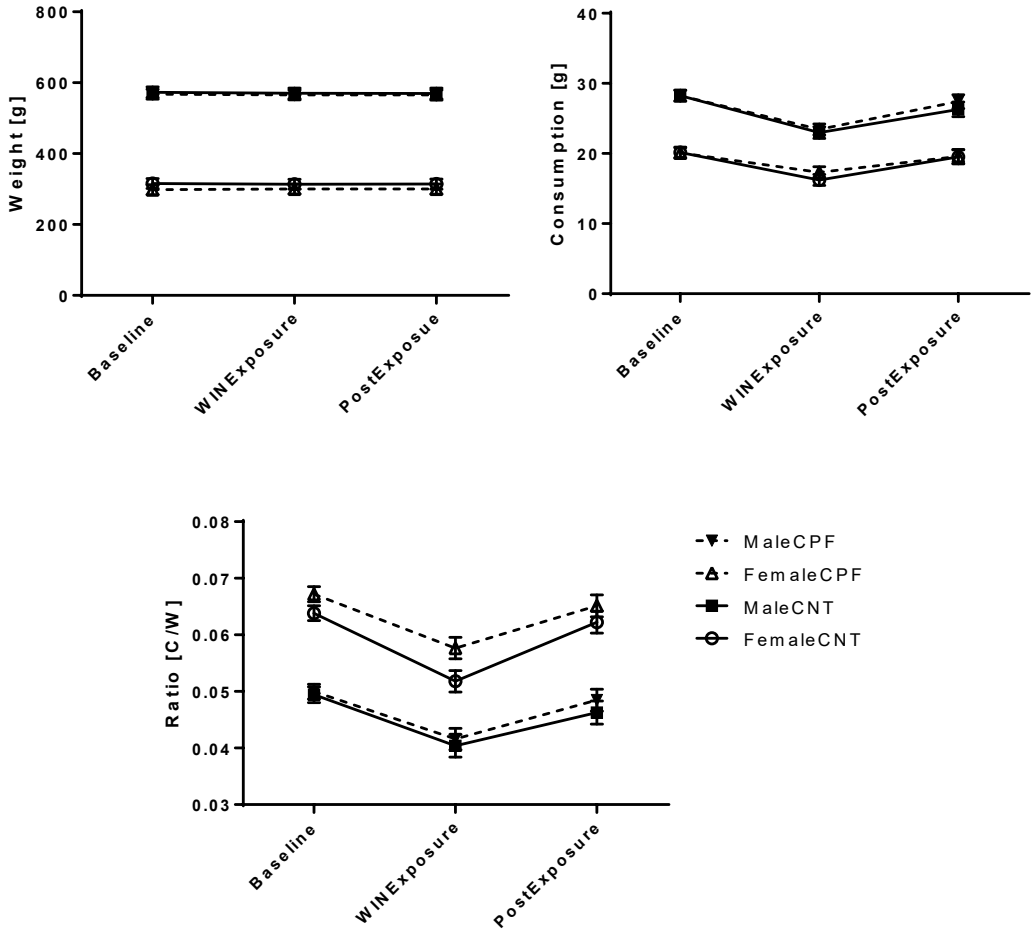

Figure 6. Effects of 4 consecutive days (i.p.) of WIN 55-212,2 administration. a) weight, b) food consumption, and c) feeding efficiency ratio. Data are expressed by means and SEM.

3.4.4. 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 (**Figure 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).

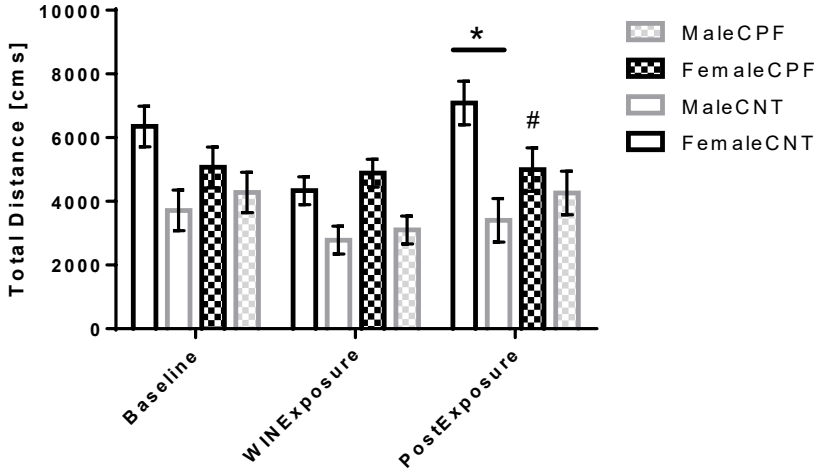

Figure 7. Effects of 4 consecutive days (i.p.) of WIN 55-212,2 administration on total distance. Data are expressed by means and SEM.* indicates significant (p<0.05) differences from the other stages. # indicates significant differences between groups.

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 (**Figure 8**). No significant differences were found neither TREATMENT nor SEXxTREATMENT interaction.

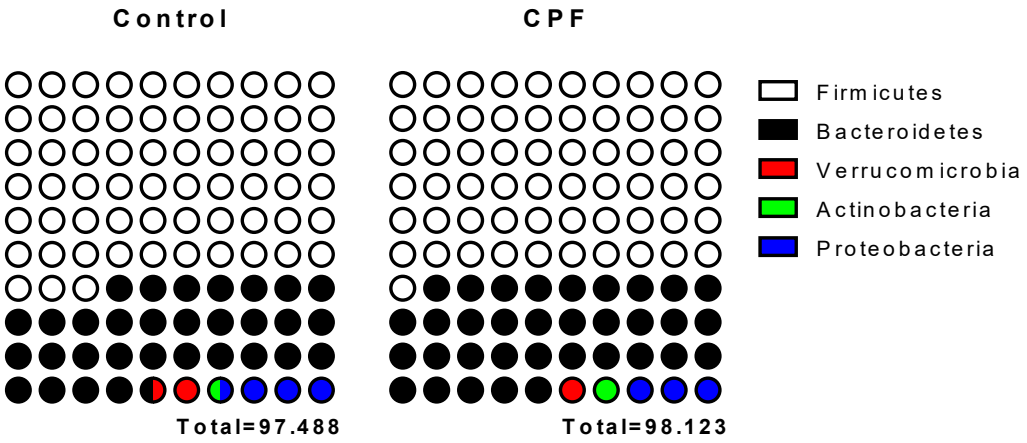

Figure 8. The relative abundance of the 5 most important bacteria in the Phylum taxonomic category in both control and CPF animals. The percentage from the total gut microbiota of the 5 bacteria is displayed (bottom-right).

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 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.

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

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.

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.

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

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.

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 1mg/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 to 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).

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 & 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 & 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 & 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 & 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 hours after exposure that

ended at the late postnatal preweaning stage (5mg/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 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; Sanchez-Amate et al., 2002; Cardona et al., 2006) or low but chronic exposure protocols in adulthood (Lopez-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 (Gomez-Gimenez et al., 2018). The authors found increased extracellular GABA levels in the cerebellum only in male rats following very low exposure (0.3mg/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 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 & 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 NC, 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 et al., 2003). Added to this, around one third of the cortical GABAergic

neurons also express M2 receptors (Disney & 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 1mg/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

1mg/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), up-regulated 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.

Acknowledgments

The authors would like to thank Dra. María Cabré, Dr. Jordi Blanco, Dra. Pia Basaure and Mr. José Mata their initial help in both molecular and behavior understanding. This study was supported by two grants from the Ministerio de Economía y Competitividad, Spanish Government and the Fondo Europeo de Desarrollo Regional (MINECO-FEDER). Grant numbers: PSI2017-86847-C2-1-RMINECO-FEDER and PSI2017-86847-C2-2-.

Conflict of interests

The authors declare no conflict of interest.

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