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

Authors

Cristian Perez-Fernandez[a], Miguel Morales-Navas[a], Laia Guardia-Escote[b,c], Maria Teresa Colomina[b,c], Estela Giménez[d] and Fernando Sánchez-Santed[a,*]

Affiliations

[a]Department of Psychology and Health Research Center (CEINSA), Laboratory of Psychobiology, University of Almería CeiA3, 04120, Carretera de Sacramento s/n, La Cañada de San Urbano, Almería, Spain.

[b]Research in Neurobehavior and Health (NEUROLAB), Universitat Rovira I Virgili, Tarragona, Spain.

[c]Department of Psychology and Research Center for Behavior Assessment (CRAMC), Universitat Rovira i Virgili, 43007, Carretera de Valls, s/n, Tarragona, Spain.

[d]Department of Biology and Geology, University of Almería, Ctra. Sacramento, s/n, 04120 Almería, Spain.

Email addresses

Cristian Perez-Fernandez: cpf603@ual.es. Miguel Morales-Navas: miguelmoralesnavas@gmail.com. Laia Guardia-Escote: laia.guardia@urv.cat. María Teresa Colomina: mariateresa.colomina@urv.cat. Estela Giménez: estela@ual.es.

[*]Corresponding author

Professor Fernando Sánchez-Santed. fsanchez@ual.es

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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 development. Chlorpyrifos (CPF) is the most widely used organophosphate compound 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 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 inexistent 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 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 generally part of the symptomatology of the main neurodegenerative and neurodevelopmental disorders observed nowadays.

Key words

Chlorpyrifos; Locomotor activity; Learning; Attention; Inhibitory control; brain-derived neurotrophic factor.

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 PA, 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 et al., 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 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 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 &

Macphail, 1997; Terry AV, 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 PA, 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., 2005; 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 (> PND10) is equivalent to the human birthday in terms of CNS development and is an essential period for synaptogenesis (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, by far, 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; Jett 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 human 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.

Materials and Methods

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. 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, half of which were exposed to CPF) were used to determine AChE activity 24 hours after the last CPF exposure. The remaining 65 rats were included in the behavioral experiments (36 females and 39 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., unpublished). 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 20g daily and the females 18g, and thereafter the males received 18g males and the females 16g). 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 ±2ºC 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.

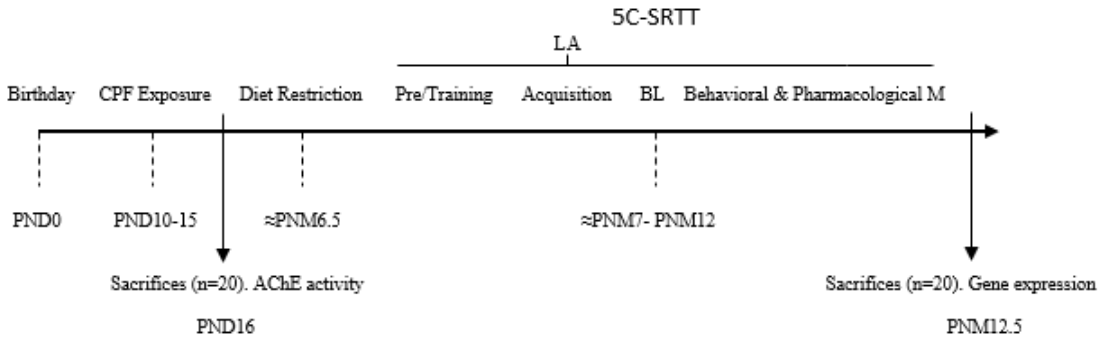

Image 1. Experimental timeline. A total of 190 rats were randomly assigned to CPF or corn oil oral exposure from PND10 to 15. 85 rats were included in this experiment. 20 were randomly selected and sacrificed 24h after the last exposure (PND16) for AChE activity analysis. The remaining 65 rats were included in the 5C-SRTT procedure. Locomotor activity (LA) was analyzed during the Acquisition phase for 30 minutes. Pre training was done around PND209. Acquisition started at PND215 and continued until the last animal reach the criteria (PND284). Behavioral manipulations started the next day following 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.

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. Synaptogenesis, myelination, and the peak in oxytocin and vasopressin systems play an important role in this stage of mammalian CNS development (Venerosi et al., 2006; Semple et al., 2013; Tait et al., 2009). Furthermore, in rats, PND10 is equivalent to around birth in humans in neurodevelopmental maturation terms, thus representing a good model to study perinatal alterations (Semple et al., 2013). Finally, we selected this developmental period since it is the least studied age range in CPF developmental toxicology. For the

experimental group, one mg/kg/ml/day of 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).

Drugs

Two different compounds were used in the 5C-SRTT procedure: (−)-Scopolamine Hydrobromide trihydrate (Sigma, lot number 106H0796, purity of > 98%) 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%), 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 both doses of 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.

Behavioral protocols

Locomotor activity

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/sec in movement) outcomes were analyzed for 30 minutes.

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 9h to 15h. The experimental room was dimly lit, and the temperature and humidity conditions were set according to those of the standard homeroom, as previously described.

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

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-second 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 minutes had elapsed. The dependent variables for study were: accuracy $[100 \text{ x } (\frac{\text{Correct Responses}}{(\text{Correct Responses}+\text{Incorrect Responses})})]$, percentage of omissions $[100 \text{ x } (\frac{\text{Omissions}}{\text{Total trials}})]$, 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).

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 13-15g/day (for males) or 10-12.5g/day (for females) in order to maintain a body weight between 85-90% 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 minutes 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 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 seconds) and the inter-stimulus interval (ITI) (2, 5 and 7 seconds) 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 hours 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 minutes 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 minutes 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 box bias controlling. The experiment was conducted between 9h and 14h once the animals had learned the procedure. Temperature and humidity were set as previously described.

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			

Table 1. 5C-SRTT phases. 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).

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 hours 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-). Blood samples were directly taken by a filter into a PYREX tube with 400uL Ethylenediaminetetraacetic acid (0.5 M, pH8) solution. Plasma samples were obtained following 20 minutes of centrifugation (3000 rpm). The brains of the rats were quickly removed from the skull and dissected. Both dorsal striatum and frontal cortex samples were collected when relevant to the 5C-SRTT derived outcomes (Robbins TW, 2002; Agnoli and Carli, 2012). All areas were directly placed into RNAse-free tubes (1.5mL) and flash-frozen immediately after extraction. All samples were stored at -80ºC until use. All utilities, spaces, and materials were autoclaved and treated with RNAse ZAP ® (Sigma Aldrich) to avoid RNA degradation.

Biochemical procedures

Acetylcholinesterase activity assessment

Frontal cortex AChE activity was assessed 24 hours 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 0.1M PBS (pH8) with 1% Triton X-100, 1/10 (w/v). After centrifugation (15,000xg, 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 seconds 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 minutes. 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 592nm (Bradford, 1976). The activity was normalized to total protein concentration.

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 ug) 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**.

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*	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*	Slc12a2	catggtgtcaggatttgcac	gatattgtccttacatagag	Cho et al., 2013
5HT2a	Htr2a	aacggtccatccacagag	aacaggaagaacacgatgc	Kindlundh-Högbergetal.,2006
5HT2c	Htr2c	ttggactgagggacgaaagc	ggatgaagaatgccacgaagg	Kindlundh-Högbergetal.,2006
BDNF	bdnf	ggtcacagcggcagataa	ccgaacatacgattgggtag	Own design

Table 2. Primers selected for the RT-qPCR study. 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. * 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).

Statistical analyses

The data from the frontal AChE activity (nmol/min/mg) were analyzed with a two-way analysis of variance (ANOVA), with *treatment* (two levels, control and CPF) and *sex* (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. 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 *dose* (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 *treatment* as the factor. The statistical error was set at $p<0.05$. 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. 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).

Results

AChE activity. Lack of brain AChE inhibition following CPF exposure

CPF exposure did not significantly inhibit frontal AChE activity 24 hours after the last exposure (**Figure 1**).

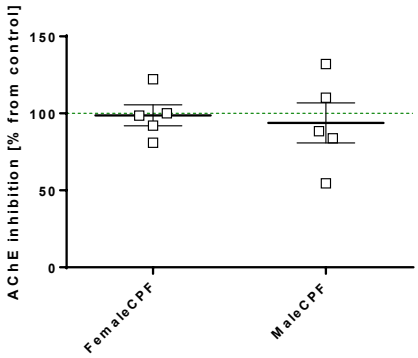

Figure 1. AChE inhibition. Percentage of inhibition (from control) of AChE activity 24h after the last exposure to CPF in Frontal cortex. Data are expressed as means and SEMs, and individual plots are depicted.

Locomotor activity. Decreased velocity in exposed females

Different locomotor activity outcomes in an open field were 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= 0.043] was found for velocity (**Figure 2**). Post hoc analyses revealed that the exposed females were generally slower than both non-exposed females (p= 0.008) and the exposed male rats (p= 0.023).

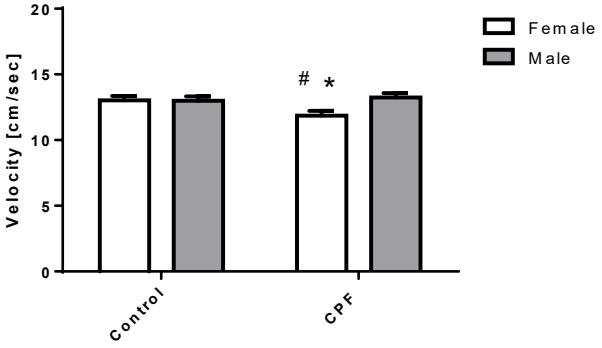

Figure 2. Locomotor activity. Long-term influences of CPF on velocity. Significant threshold was set at p< 0.05. # indicates significant differences between different exposure conditions in the same sex. * indicates significant differences between sexes within the same exposure condition. Data are expressed as means and SEMs

5 Choice serial reaction time task

Acquisition phase. CPF exposure improved females' learning rates

CPF exposure did not influence the acquisition curve of the 5C-SRTT in general terms (**Figure 3a**). However, when analyzing each of the SD criteria, CPF improved females' learning rates during the initial stages (**Figure 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= 0.007 and F(1,55)= 6.159, p= 0.016, respectively]. Post hoc analyses revealed that the usual differences observed in control animals, where males learned faster than females (p= 0.023 and p= 0.057 for the first and second stage, respectively), were abolished by exposure to CPF by improving the learning rates of exposed females in comparison with control females (p= 0.010 and p=

0.020, respectively). However, these effects of CPF disappeared throughout the learning process.

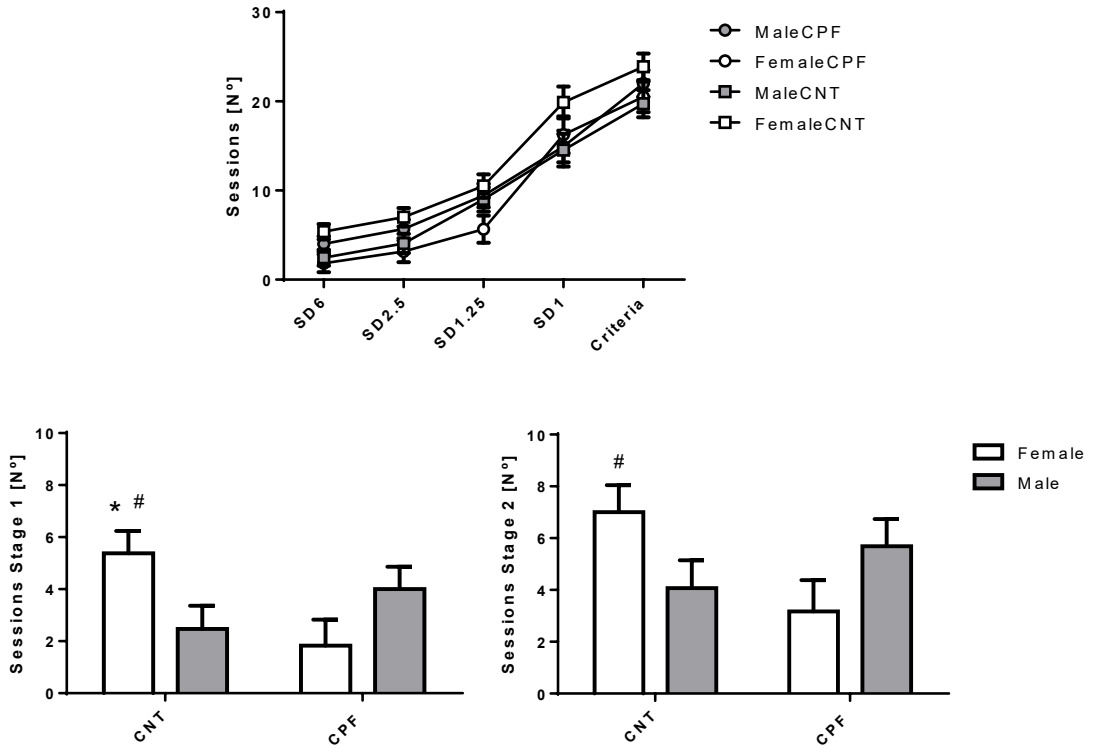

Figure 3. Acquisition curve & learning of the 5C-SRTT. Number of sessions needed to reach one stage of difficulty across all the learning stages until basal criteria was achieved (a, up). Number of sessions needed to reach the stage 1 (SD8) (b, down-left). Number of sessions needed to reach the stage 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 $p < 0.05$. Data are expressed as means and SEMs.

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 [$F(1,55) = 13.349$, $p = 0.001$], where females showed a higher percentage of omissions (**Figure 4 a-d**).

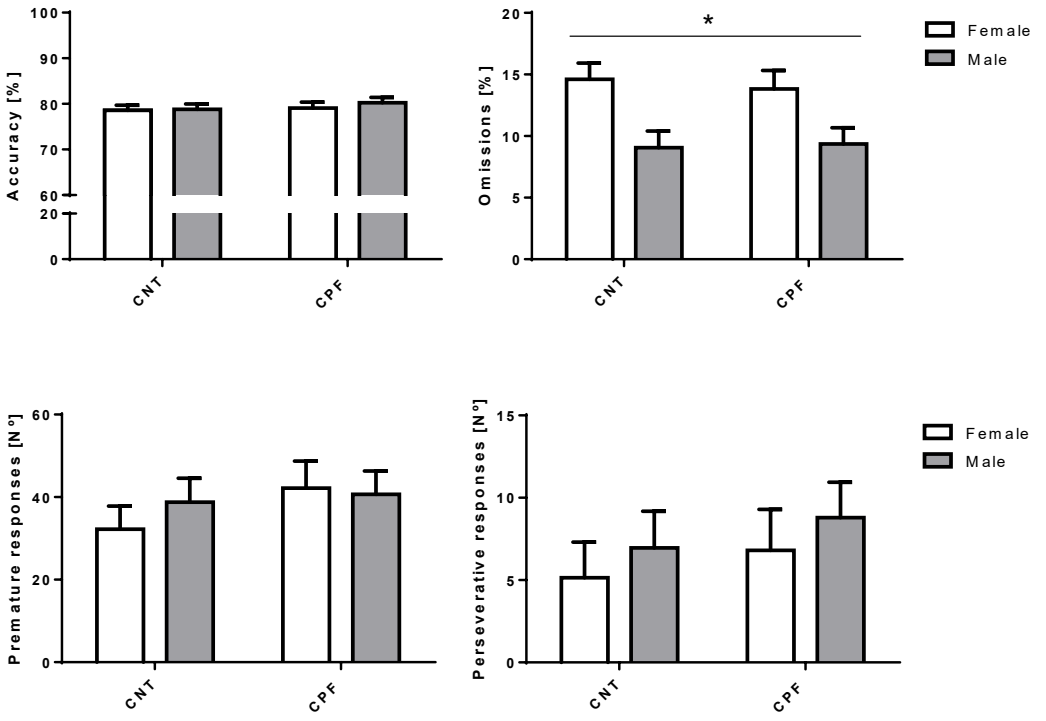

Figure 4. Baseline performance in the 5C-SRTT. Average of three consecutive days in SD1 criteria for accuracy (a, up-left), percentage of omissions (b, up-right), premature (c, down-left) and perseverative responses (d, down-right). * indicates significant differences between sexes. Significance was set at $p < 0.05$. Data are expressed as means and SEMs.

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 seconds) 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 seconds) improved the accuracy rates of all the rats except for males, as revealed by a *SD x Sex* interaction [$F(3,159)= 3.055$, $p< 0.030$]. Although the increase of SD had little effect on omissions, the significant *SD x Sex* interaction [$F(2.3, 122.1)= 4.578$, $p= 0.009$] revealed that male rats did not significantly increase their omission rates in the shorter manipulation, an effect that was observed in the females ($p= 0.401$ vs. $p< 0.001$, 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 [$F(3,159)= 3.897$, $p= 0.010$]. Post hoc analyses revealed that this manipulation did not affect the female rats but produced important increases in impulsive action in male rats ($p< 0.001$ and $p= 0.010$ for SD0.25 and 0.5 from SD1, respectively). Finally, a decrease in SD produced a significant decrease in compulsivity in terms of perseveration, without further influences on any other related factor. Thus, no significant influences were found concerning CPF exposure, in any explored interaction for any analyzed outcome.

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 [$F(1.5, 84.1)= 4.640$, $p= 0.020$]. Post hoc analyses revealed that whilst no differences were found in ITI2 and 5 between exposure conditions, an increase of the ITI to 7 seconds triggered higher rates of impulsivity in CPF exposed rats when compared with their control counterparts ($p= 0.030$). 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 [$F(2,110)= 4.854$, $p= 0.010$], and a marginally significant three-way *Sex x Treatment x ITI* interaction [$(2,110)= 2.806$, $p= 0.065$]. 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 increase in these responses in males ($p= 0.007$).

	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 (Nº)		
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 (Nº)		
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&	M > F
ITI7	12,50 ± 3,77	10,60 ± 4,12&	6,75 ± 3,56	27,88 ± 18,62&	M > F

Table 3. ITI manipulation. Influences of inter trial interval (ITI) manipulation on the different outcomes. $p < 0.05$. # means significant differences between Chlorpyrifos (CPF) and CNT rats. & means significant differences between males (M) and females (F). Data are expressed by means and SEM.

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

Although a significant main effect of *Dose* [$F(3.2, 173.6) = 7.773$, $p < 0.001$] was found for Scopolamine exposure, this drug 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 (**Figure 5a**). For CPF exposure, a significant *Treatment x Dose* interaction was found in the main analysis [$F(3.2, 173.6) = 2.930$, $p = 0.033$]. Post hoc analyses revealed that CPF exposed rats were less affected (better performance) following both 0.125mg/kg ($p = 0.043$) and 0.250mg/kg doses (approaching significance, $p = 0.081$). 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 and both the lowest dose (improved performance, $p = 0.05$) and the highest (worse performance, $p = 0.015$) as well a generally poorer performance following all doses in relation to 0.065mg/kg ($p = 0.05$, 0.02, 0.03 and < 0.001 for saline, and doses 0.125,

0.250 and 0.5mg/kg, respectively). However, in the CPF animals there were no significant effects of Scopolamine exposure at any dose.

Scopolamine [$F(2.6, 140.4)= 65.943$, $p< 0.001$] significantly increased the percentage of omissions made by the rats in a dose-dependent manner ($p< 0.001$ from saline), except for the lowest dose where rats even showed a reduced omission rate ($p= 1.000$ from saline) (**Figure 5b**). Interestingly, a significant *Sex x Dose* interaction was found in the main analysis. Post hoc analyses revealed that the usual higher rate of omissions observed in females following saline administration ($p= 0.02$) on baseline performance is abolished following both 0.125 ($p= 0.575$) and 0.250 ($p= 0.059$) mg/kg doses of Scopolamine, whereas both the lowest and the highest doses significantly maintained this pattern of results ($p= 0.01$ and 0.003, respectively). However, no significant effects were found concerning *Treatment* condition.

However, Scopolamine exposure generally increased impulsivity except at the lowest dose, although no significant main effect of *Dose* was found in the main analyses [$F(3.5,192.1)= 2.329$, $p= 0.066$] (**Figure 5c**). A significant *Treatment x Dose* interaction was also found in the main analysis [$F(3.5,192.1)= 2.585$, $p= 0.046$]. Post hoc analyses revealed that the control rats were more impulsive than their exposed counterparts, but only following the largest dose of Scopolamine ($p= 0.008$). 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 ($p= 0.003$), a finding that was not observed in the CPF treated animals ($p=0.999$). Finally, Scopolamine reduced perseveration in a dose-dependent manner [$F(1.3,70.1)= 7.384$, $p= 0.005$], with increased rates following the two largest doses, which was not affected by either the exposure condition or the sex of the animals (**Figure 5d**).

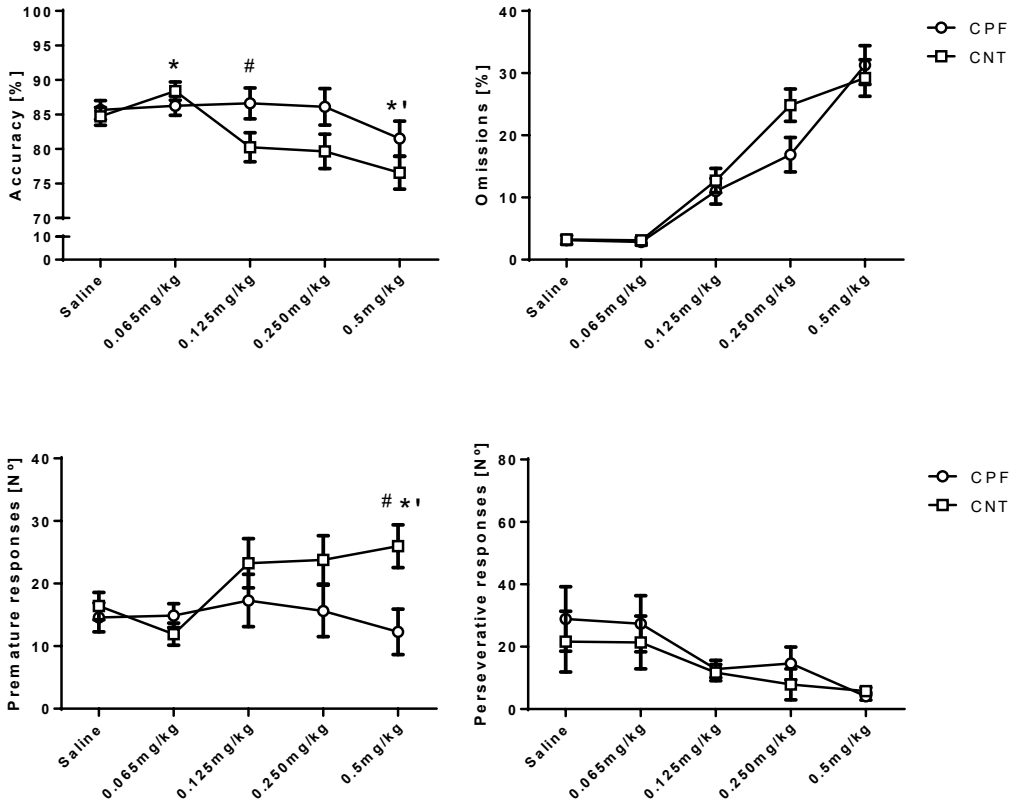

Figure 5. Scopolamine. Effects of Scopolamine hydrobromide exposure on accuracy (a, up-left), percentage of omissions (b, up-right), premature (c, down-left) and perseverative responses (d, down-right). # indicates significant differences between CPF and vehicle treated rats. * indicates that the control 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 $p< 0.05$. Data are expressed as means and SEMs.

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 (**Figures 6a, b, and d**). However, exposure to this drug altered premature responding (**Figure 6c**), where a significant *Sex x Treatment x Dose* interaction was found in the main analysis [$F(3.2,163.4)= 2.775$, $p= 0.040$]. Post hoc analyses revealed that CPF exposed males were more impulsive than their exposed female counterparts following 0.2 mg/kg dose of benzodiazepine ($p= 0.047$), an effect that was not observed in the control rats at that dose ($p= 0.744$). In addition, exposed males also showed a decrease in premature responding rates following a 0.1 mg/kg dose when compared with the lowest dose ($p= 0.048$), an effect that was not observed in any of the other groups ($p= 1.000$ in all cases).

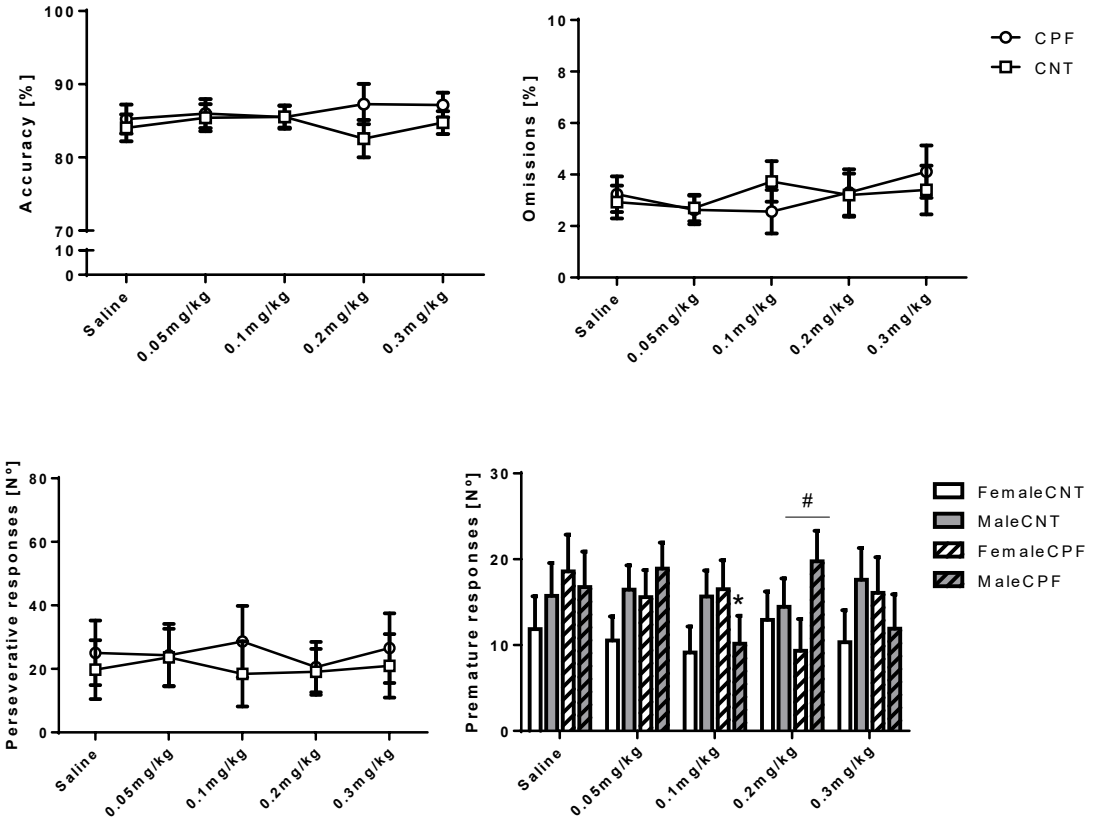

Figure 6. Alprazolam. Effects of Alprazolam exposure on accuracy (a, up-left), percentage of omissions (b, up-right), premature (c, down-right) and perseverative responses (d, down-left). # indicates significant differences between CPF treated rats following 0.2 mg/kg of Alprazolam. * indicates significant decreased premature responses of the CPF treated males compared with their own rates at the lowest dose of the benzodiazepine. Significance was set at $p < 0.05$. Data are expressed as means and SEMs.

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 brain derived neurotrophic factor (BDNF) mRNA levels in the dorsal striatum [*Treatment*, $F(7)= 14.721$, $p= 0.006$], an effect that was not observed in females [$F(8)= 0.369$, $p= 0.560$] (**Figure 7**). However, CPF exposure had little influence on the remaining genes studied here (Supplementary Figures 1-14).

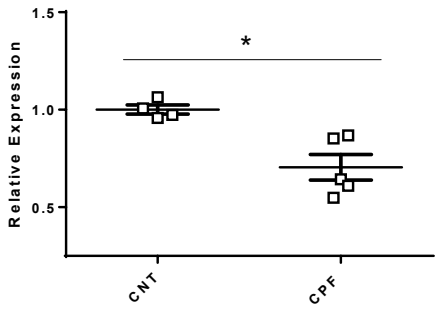

Figure 7. BDNF mRNA relative expression. Effects of postnatal CPF exposure on dorsal striatum BDNF mRNA relative expression in 1-year old male rats. * indicates significant differences between CNT and CPF rats. Significance was at set $p < 0.05$. All data are expressed as means and SEMs and individual plots are depicted.

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, which is essential for the adequate functioning of the CNS. 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 administration of Diphenyl diselenide (an antioxidant and neuroprotective compound) blocked these motoric alterations induced by CPF, pointing toward 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.

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. (2005) 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 modulate learning abilities, which is a fundamental characteristic of 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 seconds 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 of other neurotransmitter systems (Levin et al., 2001). 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) (Tin Wong et al., 2003). Furthermore, it has been linked to different neurodegenerative disorders (Calvo-Flores et al., 2018; Blaszczyk JW, 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; Sanchez-Amate et al., 2002; Cardona et al., 2006; Gómez-Gimenez 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 males compared to females following a dose of 0.2 mg/kg, whilst the control groups behaved similarly at that dose. 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 brain plasticity and cell differentiation (Lykissas et al., 2007). 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 & Carr, 2004; Slotkin et al., 2008), including preweaning stages (Betancourt et al., 2007). With regard to the developmental studies, Betancourt & Carr (2004) exposed the rats to 1.5 or 3 mg/kg/day 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 (1mg/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.

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.

Acknowledgments

The authors are grateful to Dra María Cabré, Dr Jordi Blanco, Dra Pia Basaure and Mr José Mata for their initial help in both molecular and behavioral protocols. The present 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-R MINECO-FEDER and PSI2017-86847-C2-2-R.

Declaration of interest

None

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.

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