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

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ABSTRACT

The molecular and behavioral effects of the developmental exposure to low doses of Chlorpyrifos (CPF) have been intensively studied in young (neonates and adolescents), and adult animals. However, no study examined influences of developmental CPF exposure in older adult or geriatric rats. This is relevant as such ages are generally linked to cognitive decline and the onset of specific neurodegenerative disorders, some of them previously associated with CPF exposure in both preclinical and human studies. 1 mg/kg/mL of CPF was orally administered to both male and female Wistar rats from Postnatal day 10 to 15. Animals' spatial memory, learning, compulsivity, motricity, and anxiety were analyzed with Morris Water Maze (15–16 months of age) and the Plus-maze (at 18 months of age). Results showed that postnatal CPF exposure did not alter either spatial memory, compulsive-like behaviors, or anxiety levels in late-adult rats. However, CPF exposed rats were hyposensitive to brief disruptions (Probe stage) following the learning phase and showed a general decrease in locomotor activity in both paradigms. These data are relevant as it is the first time that developmental exposure to CPF has been studied at such a late age, observing important effects in locomotor activity that could be linked to specific pathologies previously associated with CPF effects in people. Future studies should extend these findings to other behaviors and molecular outcomes.

1. Introduction

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

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

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The developmental profile of "normal aging" as well as the different neurodegenerative disorders, has an important genetic basis which controls the final physiological and behavioral output along with the evolution of such behaviors (Rodríguez-Rodero et al., 2011). However, as previously mentioned, the context plays an essential role in the regulation of these genetic vulnerabilities. Amongst the range of environmental factors that are presumably involved, continuous exposure to various xenobiotic agents has attracted considerable interest in the last decade (e.g. Kim et al., 2015; Paul et al., 2018). Among these, the organophosphate compounds (OP) are a group of neurotoxicological agents generally used as insecticides for multiple targets (Gupta, 2006). Chlorpyrifos (CPF) has, for several decades, been one of the most widely used OPs worldwide (Eaton et al., 2008).

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

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

The postnatal, preweaning exposure protocol [around postnatal day (PND) 10], is particularly relevant, given its translational significance and the specific neurodevelopmental benchmarks that take place within this period (defined in the Methods section). However, studies of the long-term effects of low doses (<1 mg/kg/day) of CPF during this preweaning stage are relatively scarce (for good examples, see Carr et al., 2011, 2013, 2014, 2015, 2020) and there is rather less empirical evidence of such effects during this stage in comparison with the early postnatal period, particularly the gestational stages. Finally, the effects of exposure to this dose on various behavioral and molecular outcomes during this developmental period in memory, anxiety and motor functions in late-adulthood rats (< 15 months-old) has not yet been explored, with many studies focusing on these effects in rats as old as 9 months (Guardia-Escote et al., 2018).

Thus, there is a need to specifically analyze the very long-term effects of the developmental exposure to a ubiquitous toxicological compound such as CPF. For this reason, we aimed to establish whether exposure to low doses of CPF during this preweaning stage (that is, exposure to low doses during a short period of time) could be linked to more profound and specific alterations in memory, anxiety and motricity in late-adult rats, ages at which there is evidence for early clinical features of different pathologies and dysfunctional aging. We consider that even low doses during short periods as those to be studied here will be sufficient to induce long-term subtle alterations in the above-mentioned behaviors at ages where these deficits are commonly manifest.

2. Materials and methods

2.1. Experimental animals

The present study used late-adult aged Wistar rats (> 15 months-old) — both males and females (n = 41, 22 males and 19 females) from 18 different litters. Briefly, a total of 19-timed pregnant female rats arrived at our laboratory 5 days before the expected delivery date, housed individually. The day of delivery was considered as Postnatal day 0 (PND0). At PND1, all pups were separated from their biological mothers, randomly mixed and pseudo-randomly distributed between dams (n = 10, 5 males and 5 females per dam). At PND10, the pups were randomly assigned to either the control condition or CPF, with around half of the pups of each sex from each dam assigned to one condition or the other (n = 2–3 per sex/per dam exposed to CPF). At PND21, all the pre-adolescent rats were weaned 4 per cage of the same sex, with an appropriate representation of both treatment conditions in each home cage (2 control and 2 CPF exposed animals per cage). For MWM testing, 10 out of these 41 animals were taken off the experiment at the first session of the acquisition because of experimenters' error that made them not valid for learning, thus a total of 31 rats were used for MWM (n = 16 males and 15 females). From these 15 female rats, 7 were exposed to CPF (8 control rats were exposed to the vehicle), while 8 out of the 16 males were exposed to CPF (8 control males were exposed to the vehicle). For the Plus-maze test, all the 41 original rats were included, where 11 out of the 22 males were exposed to CPF (11 exposed to vehicle) and 8 out of 19 females were exposed to CPF (11 exposed to vehicle). The animals received a maintenance diet of 17 g and 20 g (females and males, respectively) from the age of 7 months old, consuming water ad libitum. There were no significant effects of CPF exposure neither in physical appearance, nor weight or weight gain between groups at any time throughout the lifespan of the rats (data not shown). In the home room where the animals were housed, the temperature was set 22 °C±2 and humidity 50 %±10 for the entire lifespan of the rats. Animals had a 12 light hour cycle (lights on at 8:00 h). Image 1 summarizes the whole experimental protocol described in the present manuscript. This work is 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. The present study was also approved by the Animal Research Committee from the University of Almería, and comply with the ARRIVE guidelines.

2.2. Neurotoxic agent and exposure protocol

Chlorpyrifos (CPF) [O, O-dietil O-3,5,6-trichloropyridin-2-il fosforotioato (Pestanal, Sigma Aldrich)] was orally administered from PND10 to PND15 inclusive. Animals from both sexes were randomly assigned to vehicle (corn oil) or CPF, using a dose of 1 mg/kg/mL per day. The importance of this stage in neurodevelopmental terms has previously been defined (Perez-Fernandez et al., 2019, 2020a,b). Briefly, during this period, various neurological processes reach their developmental peak (e.g., synaptogenesis, myelination, glial cell growth, maturation of oxytocin and vasopressin systems) and PND7-10 in rats is, to a certain degree, approximately equivalent to the birth day in humans in neurodevelopmental terms (Venerosi et al., 2006; Semple et al., 2013; Tait et al., 2009; Tau and Peterson, 2010). Moreover, this preweaning period is the least studied in comparison with other gestational and early neonatal periods. CPF was diluted in corn oil since this vehicle facilitates the absorption rates of the OP (Timchalk et al., 2002), and this was used at the same volume for the control condition (1 mL/Kg). This dose was chosen because it does not induce systemic toxicity but temporal and limited ChE inhibition during this developmental period, which seems to be stabilized 24 h after the last exposure (Carr et al., 2013; Perez-Fernandez et al., 2019, 2020b).

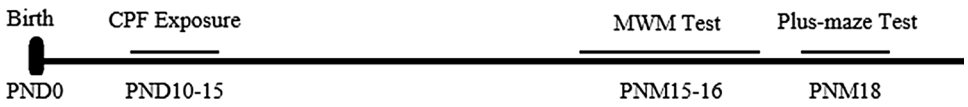

Image 1. Experimental procedure. Rats' day of birth was considered as postnatal day (PND) 0. Both male and female pups were randomly assigned to CPF or vehicle exposure from PND10 to 15. At the age of 15 months (PNM15), rats began the Morris Water maze testing (MWM) and completed the final phase at around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

2.3. Behavioral procedures

2.3.1. The Morris Water Maze task

2.3.1.1. Description of the apparatus. A black pool (50 cm height, 150 cm diameter) was used in the present study. Briefly, the pool was filled to around 1.5 cm above a black platform. The pool was digitally divided into 4 quadrants. The black platform was always placed in the same position for each individual animal, although 2 different (opposite) positions were used in the present study (half of the animals of each sex and exposure condition were trained in one of these positions and the remainder in the other) in order to control for position bias. Different external stimuli were distributed around the pool in order to facilitate contextual cues. The water temperature was always maintained at 22 °C. All animals were driven to the testing room 1 h before the beginning of the test. The testing was always conducted between 9 h and 13 h. The temperature and humidity of the testing room was the same as that described for the home room living conditions. Illumination of the testing room was set as dim-light (20 lx at the paradigm level).

2.3.1.2. Experimental procedure. Animals were around 15 months-old at the beginning of the test and were approximately 16 months-old upon completion of the test. The MWM protocol described here follows the guidelines defined in de Bruin et al. (1994), with some minor changes. Animals were transferred to the testing room 1 h before the beginning of the test. The experiment was divided into 5 phases: *Acquisition, Probe, Reinstatement, Reversal learning* and *Visual control*. Feces were removed after each trial. The order in which the animals received the exposures was counterbalanced in order to avoid hour of day bias. The *Acquisition* (original spatial learning) phase was completed after 8 consecutive sessions of 4 trial per session, the *Probe* (reference memory) phase consisted of a single trial lasting 30 s, the Reinstatement phase consisted of a session of 4 trials and the *Reversal* (perseveration, inflexibility, and new learning) and *Visual* (visual function integrity) phases were characterized by 3 sessions of 4 trials each session. Each trial consisted of leaving the rat in some of the 4 quadrants (pseudo-randomly selected and different depending on the session, but the same within each session) and allowing it to freely explore the pool. Since water is an aversive context for rats, the animal will attempt to escape, with the hidden platform providing the only opportunity for this escape. The time between the animal being dropped into the pool and reaching the platform was taken as the measure of escape latency. Each trial had a time limit of 90 s. If the animal did not reach the platform within this period, it was placed on the platform for 30 s. Once the animal escaped (or was placed on the platform) and the 30 s period had elapsed, the animal was briefly dried and left to rest for a further 30 s and once again placed into the pool for a new trial, completing 4 trials per day.

Twenty-four hours after the animals had acquired the task following 8 consecutive days of *Acquisition*, the platform was removed and the time spent engaged in exploratory behavior on each quadrant was assessed for 30 s, which was termed the *Probe* phase. Following this, another session composed of 4 trials was completed using the same platform localization as the *Acquisition* phase, namely the *Reinstatement* phase. Once the animals had mostly recovered their learning rates, the position of the platform was changed to the opposite quadrant with respect to the original position in the *Acquisition stage,* forcing a new learning process (*Reversal stage*) for 3 consecutive sessions, 4 trials per session. Finally, the visual performance of the animals was analyzed in order to rule out any effects of CPF-induced alterations in this level, namely the *Visual control* phase. The level of the water was reduced, leaving around 1.5 cm of the platform visible. The external border of the platform was marked with a clear grey adhesive to facilitate object visualization. The animals completed this phase after 3 consecutive sessions, 4 trials per session. Unlike the other phases, the position of the platform was switched from trial to trial.

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

2.3.2. The Plus-maze test

2.3.2.1. Description of the apparatus. A standard Plus-maze apparatus was employed in the present experiment. Briefly, the apparatus consisted of a central square surrounded by a structure with 4 arms, 2 of which were enclosed within a wall (closed arms), while the remaining 2 were only composed of the floor (open arms). Within this test, a stress situation is defined by the conflict triggered by the pro-exploratory instinct of the rat and the aversive situation generated by the lack of physical protection in the open arms as opposed to the closed arms. That is, the anxiety state of the rat is linked to the time that it spends in a lesser-protected, open space (open arms) as opposed to the time spent in the protected space (closed arms). The longer the animal spends in the open arms, the lower the level of anxiety. All animals were driven to the experimental room 1 h before the beginning of the test. Temperature and humidity were set according to the home room conditions. Illumination was set as Dim-light (20 lx at paradigm level).

2.3.2.2. Experimental procedure. The rats were 18 months-old at the time of the Plus-maze assessment. The animals were transferred to the testing room and allowed to habituate to the room for 1 h. They were then introduced to the center of the experimental apparatus and their behavior was recorded only once for 5 min. The order in which the animals were exposed was counterbalanced in order to avoid hour of day bias. The anxiety-related dependent variables were the total time spent in the open arms and the number of entries into the open arms. The motor control variables were the number of entries into the closed arms (frequencies), the total distance covered (cm), immobility (frequencies), velocity (cm/s) and, primarily as a general motor factor, the general mobility rate (sec). All these variables were automatically recorded and analyzed using Ethovision® version 3.1. (Noldus).

2.4. Statistical analyses

For the WMW experiment, both the escape latency and the motor variables (total distance covered, velocity and immobility) were equally analyzed, depending on the specific stage. A repeated measures Analysis of Variance (rmANOVA) was conducted for the *Acquisition* phase, with *Session* and *Trial* as the within-subject variables (with 8 and 4 levels, respectively) and *Sex* and *Treatment* as the between-subject variables

(with 2 levels each). These between-subject variables were subsequently used in all the analyses. A two-way ANOVA was conducted for the *Probe* phase, with the above-mentioned factors. A further rmANOVA was conducted for the *Reinstatement* phase, as this session was compared with the latest session of the *Acquisition* phase, with a within-subject variable *Session* (with 2 levels, Session 8 and reinstatement) and *Trial* (4 levels) and the mentioned between-subject factors. Both the *Reversal* learning phase and the Visual stage were separately analyzed with a rmANOVA, with *Session* and *Trial* as the within-subject variables (3 and 4 levels, respectively). For the Plus-maze experiment, both anxiety and motor-related behaviors were equally analyzed with individual two-way ANOVAs for each individual variable (time spent in open arms, entries into open arms, entries into closed arms, total distance covered, velocity and general mobility), with the two above-mentioned variables as the between-subject factors. As there were few animals that were from the same dam, sex and treatment condition, dam was included as covariable in the Plus-maze test analyses. All analyses were conducted with the SPSS® software version 24 (IBM). The significance level was set at $p < 0.05$. Figures were designed with GraphPad Prism® software version 6.1. A description of the statistically significant results is provided within the text. Mean and SEMs are displayed in the figures.

3. Results

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

3.1.1. MWM. Cognitive variables

All animals learned the position of the platform by the end of the sessions, needing around 76 s in the first session to 18 in the last (Fig. 1). The ANOVA revealed a significant main effect of *Session* [$F(7,189) = 49.788$, $p < 0.001$]. Post hoc analyses revealed a significant improvement (reduced escape latencies) across all sessions, from Session 1 (always $p < 0.001$). However, there was no significant *Treatment x Session* or *Treatment x Sex x Session* interaction.

In addition to the lack of effects on learning performance, spatial memory consolidation was not affected by the treatment condition when assessed in the Probe phase (Fig. 2). There were no significant effects of exposure condition, or of the interactions *Treatment x Session,* or *Treatment x Sex x Session.*

Although all the rats mostly recovered the learning rates achieved at the last session of the acquisition stage during reinstatement (Fig. 3), a significant *Treatment x Session* interaction was found [$F(1,27) = 4.342$, $p = 0.047$] in relation to reinstatement performance from Session 8 of acquisition. Post hoc analyses revealed that the control rats' performance during reinstatement was significatively affected in the Probe stage ($p = 0.019$), an effect that was not observed in the exposed animals ($p = 0.638$).

Concerning the reversal phase, animals performed as expected by increasing their escape latency rates from the first session, showing a rapid improvement of these rates on the second and third session (Fig. 4). An ANOVA revealed this effect according to *Session* [$F(1.6,44.3) = 7.059$, $p = 0.004$]. Post hoc analyses revealed that all rats improved their performance on Day 3 compared with the first and second days of reversal ($p = 0.006$ and 0.024, respectively). Once again, there were no significant interactions between *Treatment x Session* or *Treatment x Sex x Session.*

A visual task was conducted in order to control for the possible effects of CPF on vision (Fig. 5). An ANOVA revealed a significant effect of *Session* [$F(2,54) = 5.134$, $p = 0.009$], where animals improved their performance by repetition of the task in the last session compared with

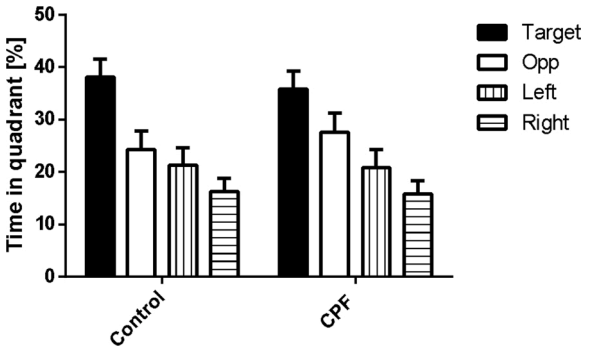

Fig. 2. Probe phase. Percentage of time exploring each quadrant once the platform was removed for a 30-sec period. The target quadrant represents the section where the platform had been placed during acquisition. The Opp quadrant is the section placed in the opposite direction to the target area. Both sexes are merged (n = 16 control and 15 CPF exposed rats). Data are expressed with means and SEM.

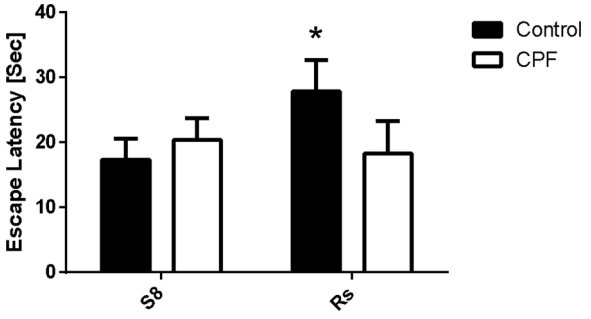

Fig. 3. Reinstatement phase. Effects of platform removal (Probe) on the learning profile during the reinstatement stage (Rs) from the last session (S8) of acquisition. * indicates significant differences concerning the performance of the control animals in both sessions. Both sexes are merged (n = 16 control and 15 CPF exposed rats). Data are expressed with means and SEM.

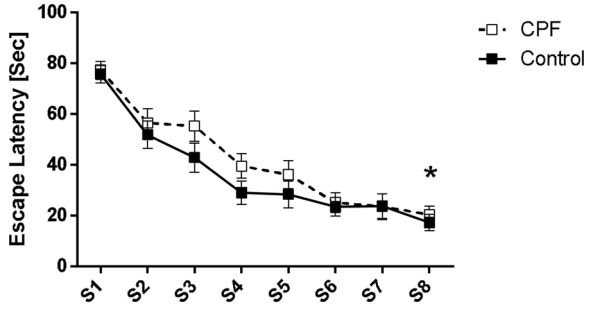

Fig. 1. Acquisition of the MWM task. S1-8 denotes sessions 1-8. * indicates that all rats needed less time to reach the platform on S8 compared with the first session. Both sexes are merged (n = 16 control and 15 CPF exposed rats). Data are expressed with means and SEM.

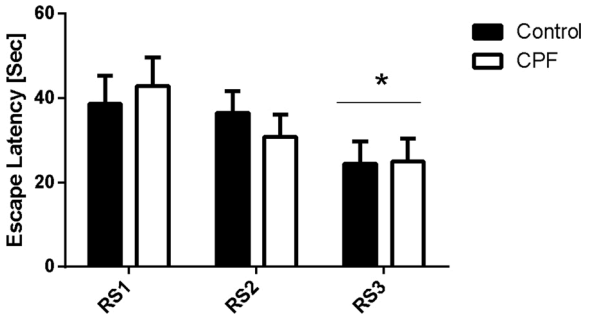

Fig. 4. Reversal phase. Effects of the change of the platform position on the animals' performance for three sessions. RS denotes reversal session. * indicates significant differences compared with RS1. Both sexes are merged (n = 16 control and 15 CPF exposed rats). Data are expressed with means and SEM.

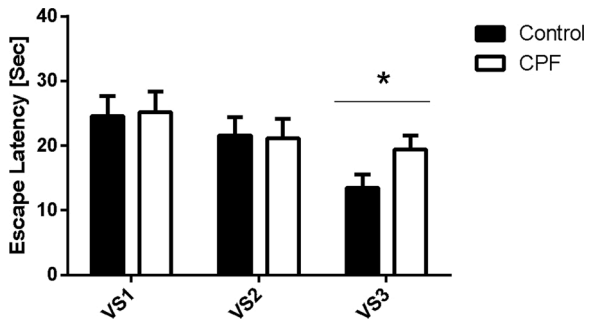

Fig. 5. Visual stage. Testing the integrity of the rats' visual system by unmasking and highlighting the platform for three consecutive sessions. VS denotes visual session. * indicates significant differences compared with VS1. Both sexes are merged (n = 16 control and 15 CPF exposed rats). Data are expressed with means and SEM.

the first session (p = 0.005). Again, the main analyses revealed no effects of exposure condition or any other complex interaction, allowing us to rule out the possibility that the results previously described could be partially due to visual alterations.

3.1.2. MWM. Motor variables

Total distance covered, velocity and immobility frequencies were analyzed in order to ensure that the spatial memory modulations induced by CPF were due to specific cognitive alterations and not a general behavioral mismatching. During acquisition, CPF exposed rats showed a hyper-activated motor state based on the total distance covered compared with the control animals in the two first sessions, which was progressively corrected throughout the learning stage (Fig. 6a). An ANOVA revealed a significant effect of *Session* [$F(7,189) = 23.980$, $p < 0.001$] and the interaction *Treatment x Session* [$F(7,189) = 3.144$, $p = 0.004$]. Post hoc analyses revealed that the rats significantly decreased their total distance covered from Session 4 onwards in comparison with the first day of training (p = 0.035 -session 4-, 0.003 -session 5- and <0.001 -sessions 6, 7 and 8-). CPF exposed animals covered a greater distance than their control counterparts in the two first sessions (p < 0.001 and = 0.015, respectively), whilst also showing a rapid decline in their activity (from Session 4 onwards, p = 0.001 -session 4- and <0.001 -remaining sessions-) compared with first day of training, although this was only observed in the last two sessions in the control rats (p = 0.033 and p = 0.002, respectively). Similarly, CPF exposed rats were faster in the earlier sessions compared with control animals, as shown by a significant *Treatment x Session* interaction [F

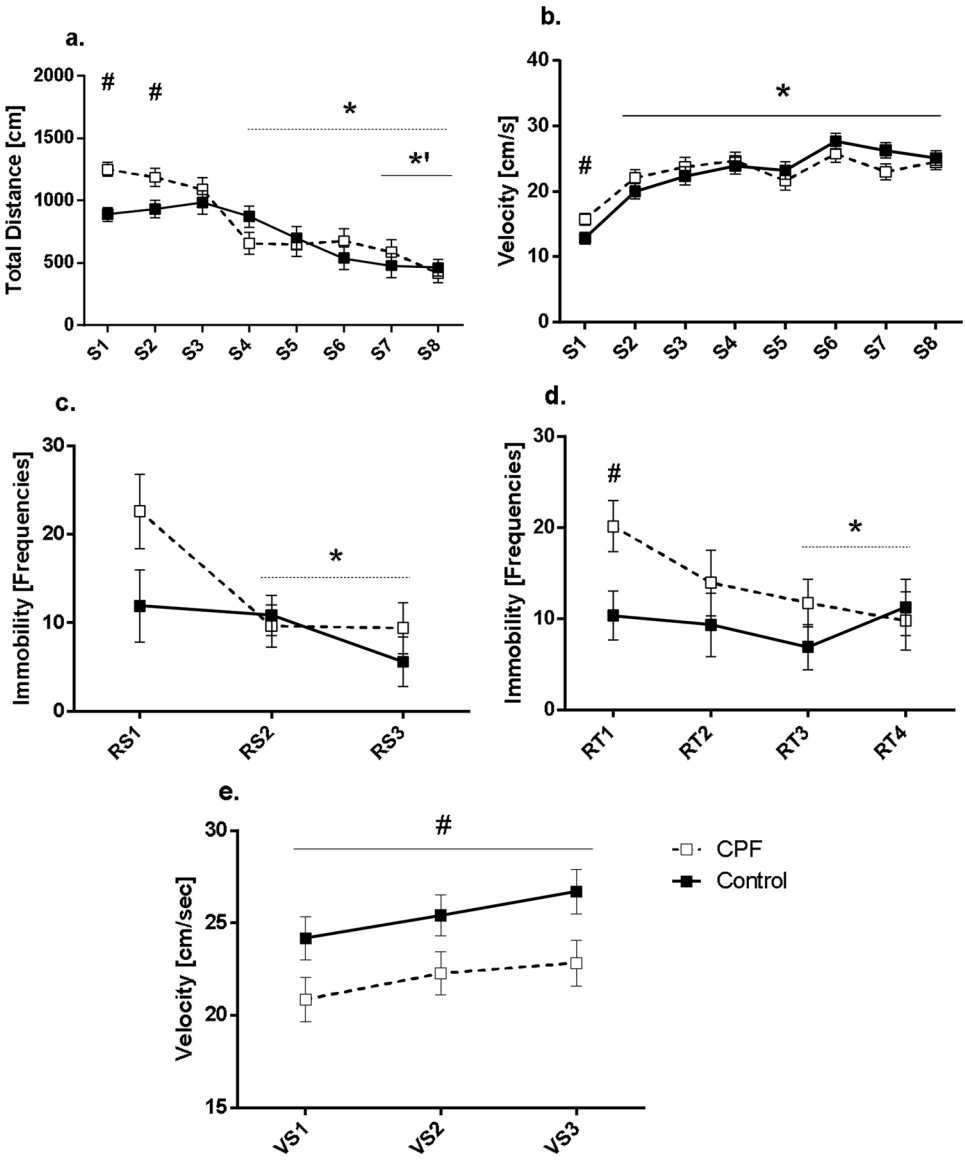

Fig. 6. Motricity in the MWM task. Locomotor variables significantly affected by CPF exposure during the acquisition (a and b, upper left and right), reversal (c and d, middle left and right) and visual (e, lower) phases of the MWM. RT refers to reversal trial. –*– (asterisk and dashed lines) indicates significant differences compared with the first session (or trial) of the phase, only for the exposed rats.-*- (asterisk and solid line) indicates significant differences compared with the first session of the phase for both groups. -*'- (asterisk and apostrophe and solid line) indicates significant differences compared with the first session of the phase only for the control rats. # indicates significant differences between control and CPF rats. Both sexes are merged (n = 16 control and 15 CPF exposed rats). Data are expressed with means and SEM.

(7,189) = 3.330, p = 0.002] (Fig. 6b). Post hoc analyses revealed that the exposed rats were significantly faster than the controls but only during the first session (p = 0.028), showing a significant increase in velocity throughout the sessions (from the first session, p < 0.001, p = 0.011 in Session 5), a finding that was also observed in their control counterparts (from the first session, p < 0.001 in all cases). Finally, there were no significant main effects on immobility concerning *Treatment, Treatment x Session or Treatment x Sex x Session* interactions (Supplementary Fig. 1).

During the Probe and reinstatement test phases, there were no differences between groups based on the different locomotor variables analyzed, and no effect of *Treatment* or *Treatment x Sex* or *Trial* interactions, with the exception of a *Treatment x Trial* interaction for immobility frequencies during the reinstatement phase [F(2.2,59.5) = 3.318, p = 0.039]. However, the post hoc analysis revealed no significant effects (Supplementary Figs. 2 and 3).

The animals covered less distance throughout the sessions in the reversal stage, with no significant effects of *Treatment*. Velocity was also not affected (Supplementary Fig. 4). However, immobility frequencies were progressively reduced throughout *Sessions* [F(2,54) = 11.520, p < 0.001] (Fig. 6c). This was affected by the treatment condition, with a significant interaction *Treatment x Session* [F(2,54) = 4.048, p = 0.023]. Post hoc analysis revealed that the high rate of immobility was observed in Session 1 in the exposed animals, which significantly reduced their immobility throughout the sessions compared with the first day of reversal (p = 0.003 and 0.001, respectively), something not observed in the control rats (p = 0.985 and 0.132, respectively). Interestingly, a significant interaction *Treatment x Trial* was also found [F(2.1,57.9) = 4.468, p = 0.014] (Fig. 6d). Post hoc analyses revealed that the exposed rats showed significantly more episodes of immobility on each first trial throughout sessions in the reversal stage when compared with their control counterparts (p = 0.018). This higher rate of immobility state was progressively normalized to match that of the control animals' performance on subsequent trials, with exposed rats showing a significant reduction in immobility by the third and fourth trial compared with the first trial (p = 0.006 and 0.007, respectively), an effect that was not observed in the control rats (p = 0.557 and 1.000, respectively).

Finally, the motor behavior of the rats during the visual test stage also followed the expected pattern in which higher activity rates were observed on the first session compared with the last one in terms of total distance and immobility, but with no effect of treatment condition. Interestingly, exposed animals showed a general decrease in velocity compared with their control counterparts when taking all three "visual" sessions together [F(1,27) = 5.083, p = 0.032] (Fig. 6e). The remaining non-significant outcomes are shown in Supplementary Fig. 5.

3.2. CPF and anxiety in pre-old age rats

3.2.1. Plus-maze test. Anxiety and motor variables

All rats spent more time exploring the closed arms than the open arms, as expected. Fig. 7 shows both the total time in (**7a**) and the number of entries into (**7b**) the open arms. CPF exposure did not affect the anxiety-related behaviors of the rats in terms of any of these variables. CPF exposure led to a general decrease in motor activity. All the locomotor variables analyzed (total distance, velocity, entries into closed arms and general mobility) showed this pattern, although only general mobility was found to be significantly affected by *Treatment* [F(1,37) = 5.044 p = 0.031] (Fig. 7c). The remaining motor-related outcomes are described in the Supplementary Fig. 6.

4. Discussion

Preweaning exposure to low doses of CPF did not alter spatial and reference memory functioning in late-adult rats. Rather, this treatment set a hyposensitive reaction to temporal disruptions in memory flexibility that were induced by the *Probe test*. Added to this, anxiety-like

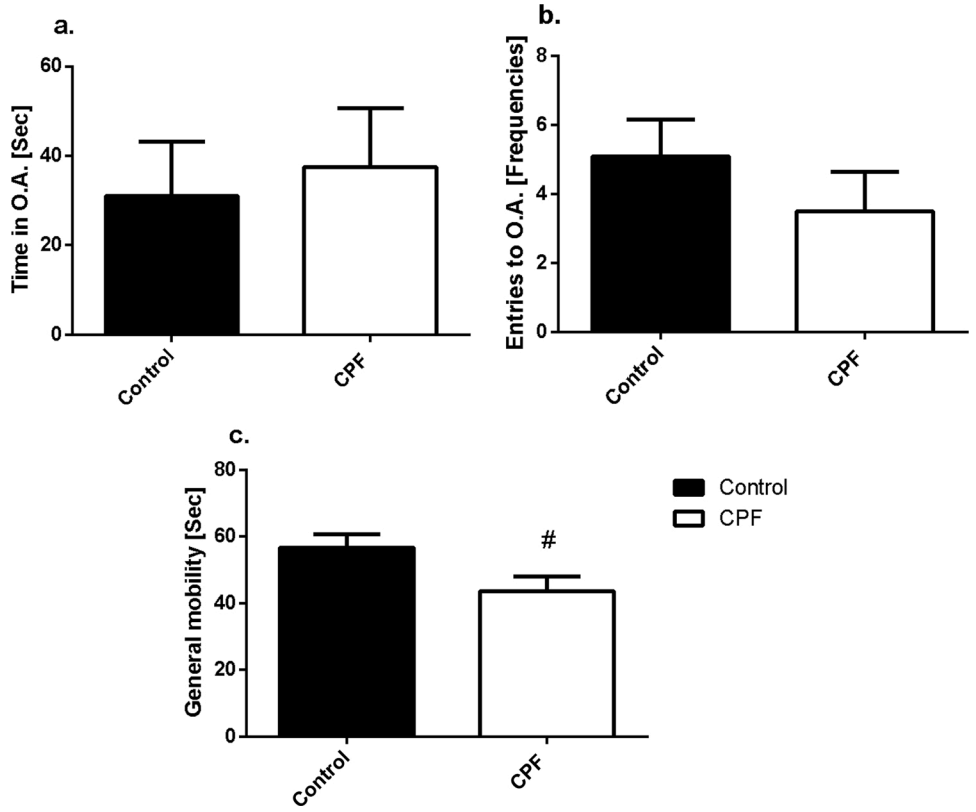

Fig. 7. Plus-maze. Anxiety (a and b, upper left and right, respectively) and motor-related (c, lower) variables. O.A. refers to open arms. # indicates significant differences between control and CPF exposed rats. Both sexes are merged (n = 22 control and 19 CPF exposed rats). Data are expressed with means and SEM.

behaviors did not differ between the control and exposed animals at these ages. However, the exposed rats were generally hypoactive in terms of motricity. To the best of our knowledge, this is the first time that the long-term effects of developmental CPF exposure have been analyzed at such late ages for these behaviors, which feature centrally in several neurodegenerative disorders.

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

In relation to preweaning exposure, although various studies did not find the effects of CPF exposure on learning, attention and/or memory (Levin et al., 2001; Cole et al., 2012) found here, most of these found the expected depauperated performance (Jett et al., 2001; Guardia-Escote et al., 2018, 2019; Wang et al., 2018; Basaure et al., 2019). With regard to rat models, these deficits appear to be task-specific as they have been found following a MWM task (Jett et al., 2001; Wang et al., 2018) but not the Radial-arm maze protocol (Levin et al., 2001). However, the differences between the studies of Jett et al. (2001); Wang et al. (2018) and the present study make it impossible to extract firm conclusions since they used higher doses and evaluated the rats' behavior at very young ages when compared with our late age model.

Following the *Probe* phase, animals completed a *Reinstatement* session in order to recover the presumable depauperated performance to baseline levels. The control rats were significantly affected by removal of the platform during the *Probe* phase, as observed in their performance during the *Reinstatement* phase, with longer escape latencies compared with the last session of the *Acquisition* phase. However, this was not the case for the CPF exposed animals. These rats were not affected by this interference. This hyposensitivity to specific disruptions in a previously learned task is surprising, and we have found no previous demonstrations of this finding in the existing literature. This effect could be considered as a compulsive-like, perseverative behavior, as control animals were sensitive to the new conditions (*Probe phase*) and their performance was affected during the *Reinstating phase* (they could expect new contingencies), while CPF rats were insensitive to this new rule. However, we found no effects during the *Reversal learning stage*, thus this compulsivity/inhibitory control hypothesis could be initially discarded.

Otherwise, the *Probe test* could be also considered as a "soft version" of the forced-swimming test. This stage could have increased the natural distress/fear of the animals in this context, observed during the *Reinstating stage* by affecting their performance. Our data would reflect reduced hypothalamic-pituitary-adrenal axis activation in CPF exposed rats in comparison with their control counterparts. This disruption can be also conceptualized as an instance of brief extinction (Vorhees and Williams, 2006). Further, it has been proposed that the cholinergic system plays an essential role in extinction learning, at least with regard to the fear component (Wilson and Fadel, 2017). We have previously demonstrated that other CPF exposed rats following this same exposure protocol had a hyposensitized cholinergic system at both young adulthood (Perez-Fernandez et al., 2019) and adulthood (Perez-Fernandez et al., 2020b) following pharmacological challenges in different motor and attentional paradigms. It is possible that the malfunctioning of the cholinergic system in those animals postnatally exposed to the OP agent could be at the basis of this abnormal reaction following the *Probe* test. However, the lack of empirical evidence and theoretical models makes it hard to draw any firm conclusions with regard to this issue.

Exposure to CPF had no effect on anxiety-like behaviors assessed by the time in and number of entries to the open arms in a Plus-maze test. We were able to find eight preclinical studies that analyzed the influences of developmental CPF on anxiety-like behaviors (Peris-Sampedro et al., 2020; Carr et al., 2015, 2020; Silva et al., 2017; Venerosi et al., 2008, 2010; Braquenier et al., 2010; Ricceri et al., 2006). Of these, five studies used a preweaning exposure protocol to low doses of CPF, which generally resulted in decreased anxiety in the exposed rodents. However, the ages at which anxiety-like behaviors were evaluated in these studies ranged from pre-adolescence to five-month-old adults. This essential difference could underlie the lack of concordance between the results of the present study and the consensus observed in the empirical literature.

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

Of particular interest for our purposes, preweaning exposure has also been linked to these types of motor alterations, both increasing (Ricceri et al., 2003; Dam et al., 2000; Levin et al., 2001; Guardia-Escote et al., 2019) and decreasing (Lee et al., 2015; Venerosi et al., 2008) the locomotor rates of the rodents. Of these, only two studies (Dam et al., 2000 and Levin et al., 2001) used rats as experimental animals, both of which observed an hyperactivated profile during adolescence and adulthood in the exposed rats, although these studies used five times the dose of CPF used in the present research. However -and in support of this CPF-induced hyperactivity in young ages following a preweaning protocol- we have previously found this increased spontaneous activity in adolescent rats, "siblings" of those used in this study, which followed the same exposure protocol (Perez-Fernandez et al., 2019). More recently, other authors have also provided support for using the same dose chosen in the present work (Carr et al., 2020).

The main limiting factor concerning the comparisons of our data with all these studies is, once again, the age of assessment. In these studies, the age range at which subjects were evaluated ranged from a few days after birth to as old as 4 months, somewhat different from the ages used in the present research. Thus, according to the data obtained in our laboratory following this exposure protocol, it appears that CPF exposure during preweaning stages induces a motor hyperactivation that progressively declines with age and is observed at pre-old age. A further source of support for this hypothesis is that we also found a decrease in velocity in the exposed females at around 9 months old (Perez-Fernandez et al., 2020b), which can presumably be taken as an initial indicator of the later general decline found in the present study.

5. Conclusions and future guidelines

Postnatal, preweaning exposure to low doses of CPF induced a long-term general hypomotricity in pre-old age rats, without affecting anxiety and spatial memory functions. This long-term exposure desensitized the rats to brief memory flexibility disruptions induced by the Probe test, something that has not been observed previously. Based on empirical data obtained in previous studies in our laboratory and others demonstrating that this exposure regime induces hyper motricity during the adolescence and early adulthood in the rat, we propose that the effects of low doses of developmental CPF on motricity are age-dependent, characterized by an initial motor excitation that progressively turns into a general degradation of motricity, primarily observed in pre-old age and,

presumably, in old age. Since motor function alteration is a core sign in most of the currently known neurodegenerative disorders, and the aging process is also characterized by a variable decline of this function, the present study supports the notion that exposure to these types of contaminants could be an environmental factor for the development of these pathologies and a poorer aging profile, which is of particular concern for the aging developed nations. Future research should focus on validating this hypothesized biphasic effect of the CPF exposure concerning age by regularly testing motricity in various paradigms during the lifespan of the rat. Finally, the lack of sensitivity shown by the Probe test to disruptions in memory flexibility could be further explored following different learning procedures including extinction in order to establish whether this effect is consistent or merely an isolated observation.

Authors' contribution

Perez-Fernandez C. Original conceptualization, animal maintenance, behavioral tasks, initial statistical analyses, figures design, first version of the manuscript. **Morales-Navas M.** Animal maintenance, behavioral tasks (Plus-maze), manuscript review. **Guardia-Escote L.** Original conceptualization, statistical analyses supervision, manuscript review. **Colomina MT.** Original conceptualization, continuous supervision, economical and material resources, manuscript review. **Giménez E.** Original conceptualization, continuous supervision, manuscript review. **Sánchez-Santed F.** Original conceptualization, continuous supervision, economical and material resources, manuscript review.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neuro.2021.07.002.

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