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Intranasal Administration of a Chlorpyrifos Formulation in Mice Induces Long-Term Changes in Spatial Memory, Brain Redox Balance, and in the Activity of Enzymes Belonging to the Cholinergic and Glutamatergic System †

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Abstract: The intranasal (IN) administration route represents a pathway for xenobiotics to reach the brain. The present report investigated the long-term consequences of IN administration of a chlorpyrifos formulation (fCPF) in mice. After an 8-month fCPF-free washout period, fur appearance and body injuries improved in the fCPF-treated group. Notably, spatial learning and memory enhancement was observed in the fCPF group. Changes in oxidative stress markers and the activities of enzymes from cholinergic and glutamatergic pathways were observed in specific brain areas from fCPF-treated mice. These disturbances, acting together, could be responsible for the described behavioral observations. Our results emphasize the IN-pathway's importance in accessing xenobiotics to the brain.

Keywords: xenobiotics; chlorpyrifos; intranasal administration; brain; behavior; neurochemistry; memory; oxidative stress; glutamate; acetylcholinesterase

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1. Introduction

The olfactory epithelium is accessible from the environment and is exposed to a wide variety of environmental aggressions. Through the olfactory epithelium, xenobiotics can access the brain triggering the onset of neuropathological processes [1]. Chlorpyrifos (CPF) is a widely used organophosphate pesticide (OP). Originally, the toxicity of OPs was attributed to their capacity to inhibit the enzyme acetylcholinesterase (AChE). However, the neurotoxic effects of CFP seem to involve noncholinergic mechanisms as well [2]. Recently, we demonstrated that after a 1-month fCPF-free washout period, fCPF-treated mice exhibit impairments in recognition memory and showed anxiogenic behavior. In addition, the IN fCPF administration altered several neurochemical markers in different brain areas [3].

The present study is the first report examining the long-term behavioral consequences of a commercial formulation of CPF administered intranasally to mice. In addition, we evaluate indicators of oxidative damage and the enzymatic activities of AChE and glutamate (GLU) transaminases in those brain areas relevant to the behavioral characteristics analyzed, to assess the probable central nervous system (CNS)-biochemical process involved after an 8-month fCPF-free washout period.

2. Materials and Methods

2.1. Animals and IN fCPF Administration

CF-1 mice (~65 days old) were randomly assigned to the control or the fCPF-treated groups. The fCPF-IN administration was conducted as previously described [3]. Mice received the equivalent of 10 mg/kg of CPF/day through the nostrils by using a micropipette, 3 times per week, during 2 (fCPF 2W) or 4 (fCPF 4W) weeks, respectively.

2.2. Functional Observational Battery (FOB)

This test was performed 8 months after the last IN administration, and was conducted to evaluate animals' appearance, behavior, and functional integrity, according to [4,5].

2.3. Behavioral Analysis

The behavioral tests were performed 8-months after the last IN administration following the sequence:

2.3.1. Open Field (OF) Test

Locomotor activity was analyzed using the OF test [3,6].

2.3.2. Novel Object Recognition (NOR) Test

Recognition memory was studied through the NOR test according to [3,7].

2.3.3. Plus Maze (PM) Test

The anxiety levels were evaluated through the PM test according to [3,8].

2.3.4. Barnes Maze (BM)

Spatial learning and memory were studied through a shortened Barnes maze protocol described by [9].

2.4. Evaluation of Enzyme Activities

Once behavioral tests were completed, mice were sacrificed by cervical dislocation. Brains were removed, and different brain areas were dissected: olfactory bulb (OB), prefrontal cortex (PFC), striatum (CPu), cortex (CTx), hippocampus (HPC), and midbrain (Mb). The brain areas were homogenized and processed as in [3,5].

2.4.1. AChE Activity

The activity of AChE was determined following Ellman's method [10].

2.4.2. Glutamate (GLU) Transaminases Activities

GLU oxaloacetate transaminase (GOT) and GLU pyruvate transaminase (GPT) activities were measured with a commercial kit and following the manufacturer's indications [3,5].

2.5. Thiobarbituric Acid Reactive Substances (TBARS) Analysis

Lipid peroxidation was quantified as malondialdehyde (MDA), according to [3,11].

2.6. Protein Concentration Was Measured Using the Method of Bradford [12]

2.7. Statistical Analysis

Results represent the mean ± SEM. A value of $p < 0.05$ was considered statistically significant. Statistical analysis was performed with GraphPad Prism software.

3. Results and Discussion

To investigate the long-term consequences of IN administration of an fCPF, mice were treated with fCPF, 10 mg/kg, every other day, for 2 (fCPF 2W) and 4 (fCPF 4W) weeks, respectively.

After an 8-month fCPF-free washout period, we observed that fur appearance improved, and the number of body injuries decreased in the fCPF-treated group as evaluated by the FOB (Table 1).

Table 1. Functional observational battery (FOB) parameters evaluated after 8 months from the last IN fCPF administration.

Endpoints	Control	fCPF 2W	fCPF 4W
Hand-held observations			
Fur appearance (D)	3.10 ± 0.41	1.00 ± 0.00 ***	1.15 ± 0.08 **
Body injury (D)	2.05 ± 0.28	1.00 ± 0.00 **	1.10 ± 0.07 *
Open field observations			
Activity level (R)	2.55 ± 0.20	2.50 ± 0.13	2.75 ± 0.17
Rearing (R)	1.45 ± 0.19	1.10 ± 0.07	1.90 ± 0.26 #
Arousal (R)	3.30 ± 0.25	3.05 ± 0.25	3.10 ± 0.27
Pelvic elevation (R)	2.70 ± 0.21	2.80 ± 0.20	2.75 ± 0.17
Tail position (D)	2.00 ± 0.00	2.65 ± 0.13 ***	2.05 ± 0.05 #
Fecal Depositions (C)	2.10 ± 0.38	2.30 ± 0.40	1.70 ± 0.40
Urine stains (C)	0.20 ± 0.13	0.20 ± 0.13	020 ± 0.13

Descriptive (D) and binary (B) data are expressed as a percentage of incidence; Ranked (R) data expressed as the mean score of the scale used ± SE (Kruskal–Wallis test followed by Mann-Whitney U tests); Continuous (C) data expressed as mean value ± SE (one-way ANOVA test followed by DMS post hoc test). * $p < 0.01$; ** $p < 0.005$; *** $p < 0.001$ compared to control group. # $p < 0.005$ compared to CPF 2W group. $n = 10$ per group.

The experimental groups had similar locomotion, recognition memory, and anxiety levels (data not shown). The Barnes maze test was performed to assess control mice's cognitive status in learning and memory compared to the fCPF-treated group.

The analysis of the training trials did not show differences in target hole primary latency between control and fCPF-treated groups (Figure 1).

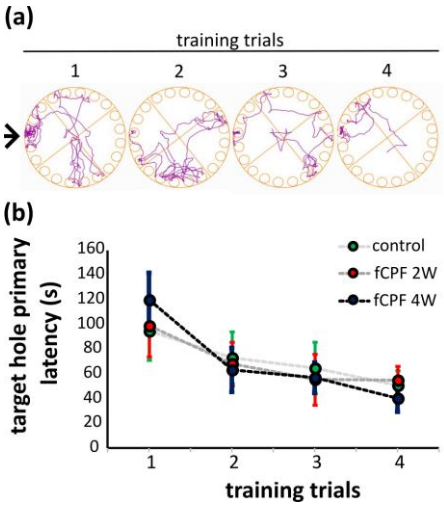

Figure 1. (**a**) Representative traces of paths to target hole (arrow) tested in the Barnes maze. (**b**) Primary latency, out of 180 s, for control and fCPF-treated mice.

During the test trial, the number of holes searched and time spent in the target quadrant were measured to assess the capacity of the mice to retain the location of the escape device. A mouse whose memory is unimpaired will spend more than 25% (chance level) of the test time in the target quadrant [9]. The percentage (%) of holes searched in the target quadrant was well above a chance level of 25% for the three groups tested (Figure 2a). However, the time spent in the target quadrant was significantly different between control and fCPF2W groups (Figure 2b). The time spent in the target hole was above the chance level for all the groups analyzed, however, this parameter was significantly higher in the fCPF 2W group (Figure 2c). A similar observation was obtained when the number of entries in the target hole was analyzed (Figure 2d). These results could indicate that although the learning and long-term memory remain intact in the three experimental groups, albeit more efficient in the fCPF 2W group.

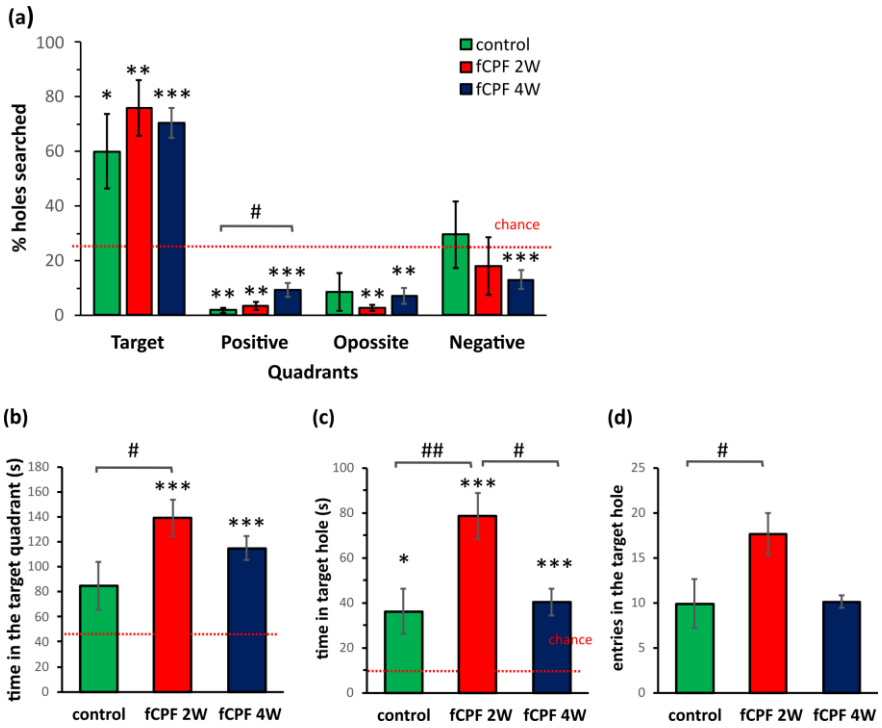

Figure 2. (**a**) Percent (%) of holes searched, on test day, in each of the four quadrants by control and fCPF-treated mice. The chance level of holes searched in each quadrant is 25%. (**b**) Time (s) spent in the target quadrant. The chance amount of time spent per quadrant is 45 s out of 180 s. (**c**) Time (s) spent in the target hole. The chance amount of time spent in the target hole is 9 s out of 180 s. (**d**) Number of entries into the target hole. Data represent mean ± SEM, n = 9–10 per group. * and # denote statistically significant differences from chance, or mice group, respectively. #, *p< 0.05, #, **p < 0.01, ***p < 0.001.

In the CNS, acetylcholine signaling is involved in learning, memory, neuronal plasticity, neurodegeneration, and neuroprotection [13]. The activity of AChE- was analyzed in different brain regions of control and fCPF-groups. We observed altered levels in the activity of this enzyme in the CPu and Mb from fCPF-treated mice, even after 8 months from the last IN fCPF administration (Figure 3).

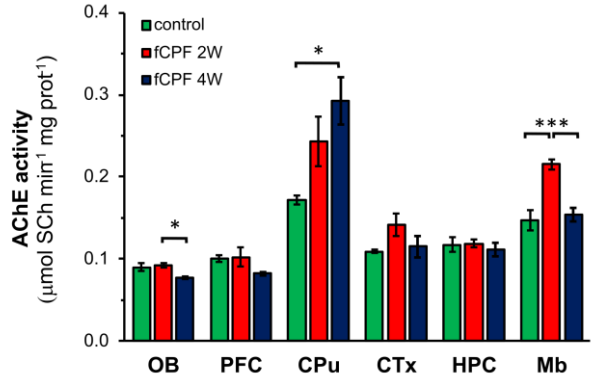

Figure 3. Activity of the enzyme AChE in the studied brain areas from mice under control and fCPF-treated conditions. Data describe mean ± SEM, n = 4–5 per group. * $p < 0.05$; *** $p < 0.001$.

GOT and GPT enzymes regulate GLU metabolism [14]. Altered GLU transaminases enzyme activities were observed in the OB, CPu, Ctx, HPC, and Mb from the fCPF group (Figure 4). Since the time between the last IN fCPF exposure and AChE and GLU transaminases activities determinations was ~8 months, the alterations observed in the enzyme's activity could be more the result of different factors (i.e gene expression, protein transduction or enzyme recycling), rather than the direct effect of the pesticide on the enzyme activity.

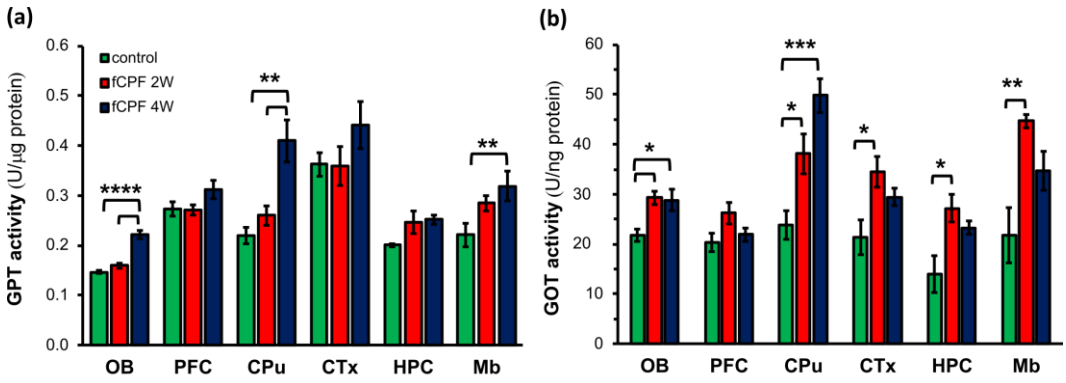

Figure 4. Activities of transaminases in mice´s brain regions under the different experimental conditions. (**a**) GPT and (**b**) GOT activities at the denoted brain areas in control and fCPF groups. Data indicate mean ± SEM, n = 4–5 per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

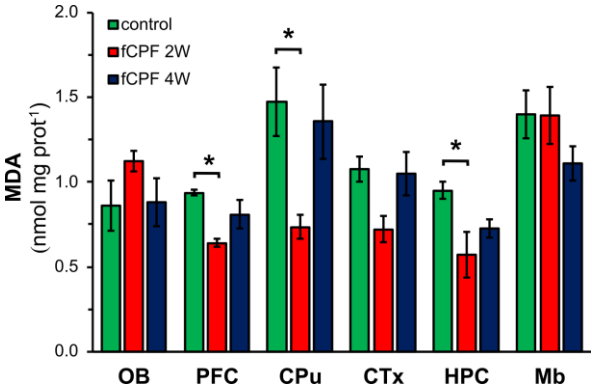

Figure 5. MDA content in different brain areas from control and fCPF-treated groups. Data depict mean ± SEM, n = 4–5 per group. * $p < 0.05$ compared to the control group.

One of the main targets of free radical damage is Lipid peroxidation affecting neurons by direct damage of neuronal membranes or indirectly by the production of

secondary deleterious compounds. Indicators of lipid peroxidation (such as MDA) increase in the CNS in many neurodegenerative diseases [15]. Interestingly, TBARs-content evaluation revealed a significant decrease in MDA levels in the PFC, CPu, and HPC of fCPF 2W-IN treated group. In previous work we described that after 1-month from the last IN fCPF administration, MDA levels increase in several brain regions. Possibly, an overstimulation of the antioxidant defenses as a consequence of initial fCPF exposure could explain the TBARs-results observed 8 months later [16].

The neurochemical disturbances observed in the intranasally fCPF group were reproduced using the human neuroblastoma cell line SH-SY5Y, treated with fCPF and CPF (data not shown).

4. Conclusions

The present study shows that IN exposure to fCPF induces long-term changes in spatial learning and memory. Moreover, still after an 8-month fCPF-free washout period, modifications regarding the activity of enzymes from glutamatergic and cholinergic systems, as well as, in the redox status, in different brain regions from the fCPF-treated group have been observed. The neurochemical changes described above could be involved for the memory improvements observed in intranasal-ly-fCPF 2W-exposed mice. The aforementioned biochemical alterations were described even in brain areas distal from the nose, such as Mb, 8 months after the last IN administration of fCPF. These results highlight the significance of the IN route on the access of xenobiotics to the brain.

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Conflicts of Interest: The authors declare no conflict of interest.

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