Toxicology 303 (2013) 162–168

Contents lists available at SciVerse ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Effects of early life permethrin exposure on spatial working memory and on monoamine levels in different brain areas of pre-senescent rats

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ARTICLE INFO

Article history:
Received 13 July 2012
Received in revised form 30 August 2012
Accepted 18 September 2012

Keywords:
Rat
Permethrin
Early life exposure
3-Phenoxybenzoic acid
Spatial working memory
T-maze
Monoamines

ABSTRACT

Pesticide exposure during brain development could represent an important risk factor for the onset of neurodegenerative diseases. Previous studies investigated the effect of permethrin (PERM) administered at 34 mg/kg, a dose close to the no observable adverse effect level (NOAEL) from post natal day (PND) 6 to PND 21 in rats. Despite the PERM dose did not elicited overt signs of toxicity (i.e. normal body weight gain curve), it was able to induce striatal neurodegeneration (dopamine and Nurr1 reduction, and lipid peroxidation increase). The present study was designed to characterize the cognitive deficits in the current animal model. When during late adulthood PERM treated rats were tested for spatial working memory performances in a T-maze-rewarded alternation task they took longer to choose for the correct arm in comparison to age matched controls. No differences between groups were found in anxiety-like state, locomotor activity, feeding behavior and spatial orientation task. Our findings showing a selective effect of PERM treatment on the T-maze task point to an involvement of frontal cortico-striatal circuitry rather than to a role for the hippocampus. The predominant disturbances concern the dopamine (DA) depletion in the striatum and, the serotonin (5-HT) and noradrenaline (NE) unbalance together with a hypometabolic state in the medial prefrontal cortex area. In the hippocampus, an increase of NE and a decrease of DA were observed in PERM treated rats as compared to controls. The concentration of the most representative marker for pyrethroid exposure (3-phenoxybenzoic acid) measured in the urine of rodents 12 h after the last treatment was 41.50 μg/L and it was completely eliminated after 96 h.

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1. Introduction

Pyrethroids are among the most frequently used pesticides worldwide. They are used in agriculture, public health, and homes, as well as for the protection of textiles, such as carpets. Because these insecticides are relatively nonvolatile, the primary source of exposure is believed to be through diet. Additional exposure via ingestion of contaminated household dust may occur after the indoor application of pesticides such as permethrin that is the main pyrethroid used in the home for indoor pest control, in pet shampoos and treatment for wood furniture.

Population-based biomonitoring data for pyrethroid metabolites measured in US and German population revealed that pyrethroid insecticide exposure is widespread. The presence of its metabolites in the urine of U.S. and German residents indicates that children may have higher exposures than adolescents and adults (Barr et al., 2010; Heudorf and Angerer, 2001). A developing brain is much more susceptible to the toxic effects of chemicals than an adult brain. This vulnerability period extends from fetal development through infancy, childhood and adolescence. The biological effects of pyrethroid are in part caused by their ability to alter neuronal activity since they interact with specific binding sites of voltage-gated sodium channel (VGSC) slowing the rate of VGSC closing, prolonging the inward sodium conductance and then shifting the membrane to more polarized potentials (Narahashi, 1996). A secondary consequence to cell membrane depolarization is an increased Ca^{+2} influx into the neurons through voltage-gated calcium channel (VGCC) that contributes to impact neuronal synaptic plasticity of neurons (Imamura et al., 2006). These changes in synaptic transmission may alter neuronal function and may contribute to excitotoxicity and neurodegenerative pathology (Gomez-Villafuertes et al., 2007).

Studies conducted in our laboratory demonstrated that PERM exposure, at a dose of 34 mg/kg close to the NOAEL (25 mg/kg) during early life, induced, later in the life of rats, a significant neurodegeneration of the striatum (Str) characterized by a decrease of

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http://dx.doi.org/10.1016/j.tox.2012.09.016

Nurr1 gene and protein expression, and an increased lipid peroxidation (Carloni et al., 2012). Lower DA levels and accelerated DA turnover following early life PERM treatment was also observed (Nasuti et al., 2007). Nurr1 is a transcription factor belonging to the nuclear receptor family and it regulates the development and maintenance of dopaminergic neurons. In fact, our previous studies demonstrated that early life permethrin treatment impaired the Nurr1 expression and then the dopaminergic system.

To further explore the impact of early life PERM exposure, in the present study, we sought to investigate cognitive functions related to spatial working memory in pre-senescent rats that were exposed to this insecticide during the neonatal period. Concomitantly with spatial working memory tasks, DA, NE, 5-HT and their major metabolites levels from different brain areas were also measured ex vivo.

2. Materials and methods

2.1. Materials

All reagents were of pure analytical grade. Technical grade (75:25, trans:cis; 94% purity) 3-phenoxybenzyl-(1R,S)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxyl-ate, PERM (NRDC 143) were generously donated by Dr. A. Stefanini of ACTIVA (Milan, Italy). Corn oil, dopamine, 3,4-dihydroxy phenylacetic acid (DOPAC), 3-methoxy-4-hydroxyphenylacetic acid (HVA), dihydroxybenzylamine (DHBA), 1-octanesulphonic acid sodium salt, sodium metabisulphite and acetonitrile were obtained from Sigma (Milan, Italy).

2.2. Animals

Male and female Wistar rats from Charles River (Calco, LC, Italy), weighing 250–270 g and about 90 days old were housed in plastic (Makrolon) cages (five rats per cage) in a temperature controlled room (21 ± 5 °C) and 60% humidity on 12 h light/dark inverted cycle (lights on at 7:00 p.m.) and maintained on a laboratory diet with water ad libitum. Rat pups born in our laboratory from primiparous dams were used in the study. The parturition day was set as PND0. On PND1, all litters were examined externally for the presence of gross abnormalities, sexed, weighed, the female pups were discarded and two male pups were assigned to each dam until weaning (PND21) for this study. No cross-fostering was employed. At 2 days of age, litters were randomly assigned to two experimental groups ($n = 11$ pups for each). Rats were weighed at 4-week intervals throughout the course of study.

At PND21, six treated and 6 control rats were housed for 4 days in metabolic cages and submitted to 3-phenoxybenzoic acid measurement in the urine.

On pre-senescent age (PND500), control and PERM treated rats ($n = 7$ per group) were subjected to a battery of behavioral tests comprising the T-maze test, water maze spatial reference memory task, open field (OF) test and elevated plus-maze. Home cage food intake of pre-senescent rats was also measured. In a subsequent experiment, 3 control and 3 PERM treated rats were trained on the T-maze task for three days. On day four, at completion of the T-maze experiment, rats were sacrificed, brains were removed and the medial prefrontal cortex (mPFC), the hippocampus (Hip) and the Str were dissected out for biochemical analysis. All procedures were conducted in adherence to the *European Community Council Directive for Care and Use of Laboratory Animals.*

2.3. Treatment

PERM was dissolved in corn oil and administered by gavage at the dose of 34.05 mg/kg/4 ml, which corresponds to 1/50 of LD_{50} as determined in adult rats (Cantalamessa, 1993). PERM or its vehicle (corn oil, 4 ml/kg) was administered once a day from PND6 to PND21. PERM dose was adjusted daily based on rat pups body weight. On PND21, the offspring were weaned and the littermates were housed together. For the experiments, the experimental groups were formed by drawing animals from different litters, so that no group contained siblings.

2.4. Urine 3-phenoxybenzoic acid levels

3-Phenoxybenzoic acid (3-PBA) is the main metabolite of the PERM and other pyrethroids resulting from the oxidation of the 3-phenoxybenzyl alcohol. Its concentration in the urine is the most representative marker for pyrethroid exposure in rodents (Nakamura et al., 2007) and humans (Leng et al., 1997).

To assess PERM levels, after the last treatment day (PND 21), 6 control and 6 PERM treated rats were housed in metabolic cages for 4 days. Every 12 h, the urine samples were collected and the volumes were measured. Urines were immediately frozen at −20 °C until assays were performed. Immediately prior to analytical detection, a hydrolysis step was introduced to convert potentially conjugated 3-PBA into free 3-PBA. The 2-PBA was added to urine samples and used as an internal standard. Briefly, urine samples were pre-treated with KOH and then, by solid-phase extraction using Strata X C cartridge, the 3- and the 2-PBA were isolated. Derivatization of 3-PBA and 2-PBA was achieved by addition of DIC and HFIP. At this point, 1 μL of upper layer was injected on a Thermo Trace-Ultra gas chromatograph coupled to an ion trap mass detector Thermo Polaris, operated in the electron impact ionization at 70 eV. The ion source temperature and the MS transfer temperature were at 250 °C. Operating in the splitless mode, helium was used as carrier gas at a constant flow rate of 1.3 mL/min. The injector was maintained at 240 °C. Final values of 3-PBA at 12, 24, 48, 72 and 96 h were expressed as μg/L and then multiplied for the corresponding urine volume so that the quantity of 3-PBA per each urine sample was obtained.

2.5. T-maze apparatus

Spatial non-matching-to-place testing was conducted on an enclosed-T maze made of clear plexiglas T-shaped platform with a start arm (70 cm long and 11 cm wide) and two identical goal arms (51 cm long and 11 cm wide), all arms were bordered with 11-cm-high walls. At the distal end of each arm a barrier (3 cm high) concealed a food well to contain the food reward (0.1 g pellet). A small grid compartment containing inaccessible fresh food was located at the end of both arms to mask the food odor (coming from the well) to prevent the use of olfactory cues to locate the food. Apparatus arms were wiped with 10% alcohol solution and dried with a paper towel between trials to eliminate the use of local odor cues. The room was surrounded by extramaze spatial cues and was illuminated by a dim light to reduce rat anxiety.

2.6. T-maze testing

The T-maze test is classically used for spatial working memory studies in the rat. In the T maze, rats are trained to choose between two goal arms alternatively rewarded with food and the rat is trained to visit the rewarded arm to obtain it. Daily, training session is composed of several choice trials. Animals' behavior is scored for accuracy (% correct arm choice), perseveration (number of consecutive incorrect trials) and choice reaction time (CRT) for each trial.

In the present study, one group of control ($N = 7$) and one group of PERM ($N = 7$) treated rats were used. One week prior to the beginning of the experiment, rats were housed in individual cages and mildly food deprived (85% of their voluntary daily food intake). T-maze training started by moving the animals into the testing room where they were kept for 10 min prior to initiate the performance. Animals were then positioned on the maze where they had free access to food in both arms for 10 min a day for 2 consecutive days. Starting from day 3, rats were subjected to 11 daily consecutive choice trials. In the first daily trial, both goal arms were rewarded and the rat was asked to choose one of the two arms. In the second trial, only the arm opposite to the first choice was baited. The test continued for other 9 daily trials in which the two goal arms were rewarded with an alternated sequence. At the beginning of each trial, the rat was placed on the start arm of the T make where it was confined with a wood block for 5 s. The block was then removed and the animal allowed to run toward the two goal arms and to choose one of them. After the choice, the rat was confined in the goal arm (using a wood block) and allowed to eat the food until finished. Rats were subjected this training for 7 consecutive days (10 choice trials per day) for a total of 70 trials.

The experiment was replicated in a new group of rats ($N = 6$). Specifically, 3 controls and 3 PERM treated animals were subjected to the same experimental sequence described above but the choice tests were stopped on day 4 when, immediately after completion of the behavioral task, the rats were sacrificed and the brains were removed for neurochemical analysis. Day 4 was when the maximal difference in the T maze performance between controls and PERM treated rats was observed.

2.7. Morris Water maze testing

One day after completion of the T-maze testing, controls ($N = 7$) and PERM ($N = 7$) treated rats were trained in the hidden-platform water maze task. The maze consisted of a large, circular tank (diameter 150 cm; wall height 60 cm) filled with water (23–25 °C) and placed in the center of a large room with extramaze cues. An escape platform was located 1 cm beneath the water surface in a fixed position and a nontoxic black paint was added to obscure the platform and to aid tracking of the animals' swim paths. In order to secure them from the water, the rats had to find a hidden escape platform located in the same position across all days of training. Animal performance was tracked by video camera using EthoVision 7.0 software (Noldus Information Technology, The Netherlands). Briefly, rats received four trials per day for five consecutive days using a 30 sec intertrial interval. On each training trial, rats were placed in the water and allowed to swim until finding the platform or for 90 s at which time they were placed on the platform by the experimenter. Once on the platform, rats were allowed stay for 30 s to acquire memory of its position using the surrounding cues. The start position for each trial was semi-randomly varied among four equally spaced positions around the perimeter of the pool. Latency to escape platform was the dependent measure, with shorter latency time indicating better performance. Mean proximity to escape platform was recorded as the primary measure of the animals' cognitive ability based on demonstration of its superior sensitivity compared to alternative measures (Gallagher et al., 1993). Lower scores, on

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