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Dopaminergic system modulation, behavioral changes, and oxidative stress after neonatal administration of pyrethroids

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Abstract

Pyrethroids are a class of insecticides involved in different neurological disorders. They cross the blood–brain barrier and exert their effect on dopaminergic system, contributing to the burden of oxidative stress in Parkinson's disease through several pathways. The aim of the present study was to evaluate the effect of neonatal exposition to permethrin and cypermethrin (1/10 of DL_{50}) in rats from the eighth to the fifteenth day of life. Open-field studies showed increased spontaneous locomotor activity in the groups treated with permethrin and the one treated with cypermethrin, while a higher number of center entries and time spent in the center was observed for the cypermethrin-treated group. Lower dopamine and higher homovanillic acid levels were measured in the striatum from both treated groups. A reduction of blood glutathione peroxidase content was measured, while no change in blood superoxide dismutase was observed. Carbonyl group formation increased in striatum, but not in erythrocytes. Lipid peroxidation occurred in erythrocytes, but not in striatum. No changes in fluidity at different depths of plasma membrane were measured in striatum or erythrocytes. The activation of monocyte NADPH oxidase by phorbol esters (PMA) shows that superoxide anion production was reduced in the pyrethroid-treated groups compared to the control group. Our studies suggest that neonatal exposition to permethrin or cypermethrin induces long-lasting effects after developmental exposure giving changes in open-field behaviors, striatal monoamine level, and increased oxidative stress. Although the action of pyrethroids on various target cells is different, a preferential interaction with the extracellular side of plasma membrane proteins can be observed.
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1. Introduction

The pyrethroid insecticides are members of a chemical class of heavily used compounds, and hazards from exposure to insecticides exist from their manufacture, storage, and spraying, as well as through contact with insecticide-contaminated food or areas.

They can be divided into two classes. Type I has no cyano-group at the carboxyl α position (α-carboxyl), whereas Type II presents this cyano-group (Verschoyle and Aldridge, 1980). Permethrin (PERM) is claimed to be one of the most potent Type I insecticides, whereas cypermethrin (CY) belongs to Type II.

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It has long been known that exposure to pyrethroid insecticides can induce neurobehavioral effects in rodents, as well as in others species, including humans (Eriksson and Fredricksson, 1991; Ray and Cremer, 1979; Shettgen et al., 2002). Studies have documented effects of the pyrethroids on dopaminergic nerve pathways, which may be a contributory factor in the etiology of environmentally induced Parkinson's disease. There is a consistent epidemiological linkage between insecticide exposure and the incidence of Parkinson's disease (Semchuk et al., 1992; Butterfield et al., 1993; Gorell et al., 1998). Although pyrethroid neurotoxicity to adults has been well characterized, and several comprehensive reviews of pyrethroid toxicity, metabolism, and actions are available (Shafer et al., 2005; Kolaczinski and Curtis, 2004; Fry and Ray, 2006), information regarding the potential developmental neurotoxicity of this class of compounds is limited. Developmental neurotoxicity involves alterations in behavior, neurohistology, neurochemistry, and/or dysmorphology of the central nervous system occurring in the adult age, as a result of neonatal exposure to pyrethroids. Eriksson's group has reported that mice exposed to deltamethrin and bioallethrin during postnatal days (PND) 10–16 exhibited increased motor activity and changes in cholinergic muscarinic receptor after cessation of exposure (Eriksson and Fredricksson, 1991). Similar to the muscarinic cholinergic system, the dopaminergic system may be affected by developmental exposure to pyrethroids: some studies on adult rats have reported that the pyrethroid class can modulate the dopaminergic system by up-regulation of dopamine transporter (Gillette and Bloomquist, 2003) and marked increase in dopamine turnover (Brodie and Opacka, 1985; Karen et al., 2001). Both deltamethrin (Lazarini et al., 2001) and bioallethrin (Shafer et al., 2005) were reported to increase 3,4-dihydroxyphenylacetic acid (DOPAC) levels in the adult striatum after prenatal exposure of dams to a non-toxic deltamethrin dose. Nevertheless, the relationships between biochemical alterations and pyrethroid-induced developmental neurotoxicity have yet to be established by better characterization of the neurochemical mode of action.

Our previous studies on adult rats orally treated with different concentrations of CY and PERM, showed biochemical alterations, such as oxidative stress, in the plasma membrane and antioxidant enzymatic activity of erythrocytes (Nasuti et al., 2003) as well as DNA damage in lymphocytes (Gabbianelli et al., 2004).

The aim of the present work was to investigate in rats the long-lasting effects after developmental exposure (from PND6 to PND15) to Type I (PERM) or Type II (CY) pyrethroids at a dose that does not induce acute toxicity in pups. Subsequently, open-field behaviors as well as striatal monoamine levels in adulthood were examined. In addition, in an attempt to assess whether pyrethroids can cause oxidative stress in striatum, and to shed some light on the mechanisms involved in the reported neurotoxicity of pyrethroids, we have performed an exhaustive investigation of oxidative stress caused by pyrethroid exposure, examining the effect of both pyrethroid types on plasma membrane fluidity, lipid peroxidation, protein oxidation in striatum, and erythrocytes of rats.

2. Materials and methods

2.1. Materials

All reagents were of pure analytical grade. Technical grade (62.8:37.2, *trans*:*cis*; 92.4% purity) (*R,S*) α-cyano-3-phenoxybenzyl (1*R,S*)-*cis*, *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate, CY (NRDC 149) and technical grade (75:25, *trans*:*cis*; 94% purity) 3-phenoxybenzyl-(1*R,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, 5,5 dithio-bis(2 nitrobenzoic acid) (DTNB), 2,4-dinitrophenylhydrazine (DNPH), guanidine hydrochloride, 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. *o*-Phosphoric acid, perchloric acid, heptanesulphonic acid sodium salt and sodium EDTA were obtained from Fluka. 1,6-diphenyl-1,3,5-hexatriene (DPH) and 6-lauroyl-2-dimethylaminonaphtalene (Laurdan) were acquired from Molecular Probes (Eugene, OR, USA).

2.2. Animals

Male and female Wistar rats from Charles River (Calco, LC, Italy), weighing 250–270 g, and about 90 days old were used. The animals were housed in plastic (Makrolon) cages (five rats/cage) in a temperature controlled room ($21 \pm 5\,^{\circ}$C) and maintained on a laboratory diet with water ad libitum. The light/dark cycle was from 7 a.m. to 7 p.m. Animal use in this study complied with the Italian government's guidelines for the care and use of laboratory animals (D.L. n.116 of January 27, 1992). One hundred and twenty Wistar rat pups (30L) born in our laboratory, from primiparous dams, were used in the main study. Parturition day was determined to be PND0. On PND1, all litters were examined externally for the presence of gross abnormalities, sexed, weighed and the female pups were discarded. Four male pups were assigned to each dam until weaning (PND21). No cross-fostering was employed. At 2 days of age, litters were randomly assigned to three experimental groups (10L for each). Litters were weighed at PND1, PND7, PND14 and PND21.

2.3. Treatment

CY and PERM were dissolved in corn oil and administered orally (5 ml/kg) at a dose of 1/10 of DL_{50} by intragastric tube. The DL_{50} was determined in pups aged 8 days (Cantalamessa, 1993), thus 1.49 and 34.05 mg/kg were the doses of CY and PERM, respectively. The compounds were administered once a day in the morning from PND6 to PND15. Control rats were treated with vehicle (corn oil 5 ml/kg) on a similar schedule. The volume of the compound administered was adjusted daily based on body weight measured during the dosing period.

On PND21, the offspring were weaned and the littermates were housed together.

On PND35, 10 animals from each group (PERM-treated, $n = 10$; CY-treated, $n = 10$; control rats, $n = 10$) were weighed and sacrificed by exposure to CO_2, and striatum were dissected out. Striatum were frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ until use.

For the behavioral and biochemical experiments, the groups of animals were formed by drawing animals from different litters, so that no group contained siblings.

All data were analyzed considering the litter as the smallest unit.

2.4. Open-field studies

At PND21 and PND35, 10 rats of each group (CY, PERM and control) were submitted to the open-field test. Automated locomotor activity boxes (MedAssociates, VT 05478) were used to quantify behavioral activity. Each animal was placed in the activity box, a square plastic box measuring $43\,cm \times 43\,cm \times 30\,cm$, and spontaneous locomotor activity parameters were monitored in the darkness conditions. Activity was recorded for 5 min, starting 2 min after placing the animal in the test cage. Each rat was automatically recorded by interruptions of two orthogonal light beams (3.5 and 13.0 cm above the activity box floor), which were connected to automatic softwares (Activity Monitor, MedAssociates). All tests were carried out during the same period of time (6:00–7:00 p.m.) and each rat was used only once. The behavioral parameters observed were locomotion (number of ambulatory episodes), rearings (number of rears), stereotype counts (number of grooming movements) and the number of entries into the central square of the arena. Locomotion counts were recorded when the low row of photocells was interrupted, while rearing counts were recorded by interruptions in the higher row of photocells. The open-field is divided into two squares, and we recorded the number of entries into the central area ($25\,cm \times 25\,cm$), which can be considered an unprotected area for rats. Entries into the central squares of the arena provided a measure of anxiety-like behavior (Contò et al., 2005). Rats that spend less time in the center are regarded as more "anxious".

Between each test session, the apparatus was cleaned with alcohol (10%) and dried with a cloth.

2.5. HPLC measurements

For the measurements of the DA and its metabolites DOPAC and HVA, striatum from each animal were individually homogenized for 2 min with a Dyna-Mix homogenizer (Fisher Scientific) in 500 μl of 0.05N perchloric acid solution containing (w/v) 0.064% 1-octanesulphonic acid sodium salt, 0.060% heptanesulphonic acid sodium salt, 0.004% sodium EDTA, 0.010% sodium metabisulphite and 25 ng/ml DHBA as an internal standard. The whole procedure (Alburges et al., 1993) was carried out on ice. The resulting homogenate was then centrifuged at $4500 \times g$ for 10 min and the supernatant was filtered using 0.45 μ Millipore filters. The filtrate was set in a low volume insert vial and a portion was injected directly into the liquid chromatography equipment (10 μl). The HPLC system consisted of a PU-2080 Plus pump (Jasco), a Rheodyne 7295 injector with a 10 μl loop and an ESA Coulochem III detector. Separation was achieved on a Waters Symmetry RP-C_{18} column (4.6 mm $\times$ 150 mm, 5 μm). The mobile phase consisted of 0.045 M monobasic sodium phosphate, 0.001 M 1-octanesulphonic acid sodium salt, 0.006% triethylamine, 0.015% 100 μM sodium EDTA and 6% acetonitrile. The pH of the mobile phase was adjusted to 3.0 by o-phosphoric acid. The mobile phase was filtered and degassed by vacuum. A flow rate of 1 ml/min was used in all experiments. The electrochemical detection system included a high sensitivity dual detector analytical cell: detector 1 set at +350 mV; detector 2 set at -180 mV. The signal was recorded using the response from detector 1.

Monoamine stock solutions were prepared at a concentration of 1 mg/ml (as a free base) in 0.05N perchloric acid containing 0.064% 1-octanesulphonic acid sodium salt, 0.060% heptanesulphonic acid sodium salt, 0.004% sodium EDTA, and 0.010% sodium metabisulphite. These standard solutions were freshly prepared every week and stored at $4\,^{\circ}C$ for use right away.

The monoamine and their metabolites were identified on the basis of retention time. The concentration of each compound was established from the peak area ratio using DHBA as internal standard. Final values were expressed in terms of picomoles per gram of tissue. Measurements were performed in triplicate for each original sample.

2.6. Antioxidant activities in plasma

On PND35, assay blood from six rats of each group was collected in vials containing heparin (250 I.U.), and washed three times with physiological solution before the experiments. The GPx and SOD activities were measured in the whole blood of each subject. The GPx activity was determined by the method of Paglia and Valentine (1967). The SOD activity was measured by the Bioxytech SOD-525 (Oxis International Health Products, Inc., Portland, OR). This method is based on the SOD-mediated increase in the rate of autoxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene in aqueous alkaline solution to

yield a chromophore with maximum absorbance at 525 nm (Nebot et al., 1993).

2.7. Determination of protein oxidation in striatum and erythrocytes

The samples of striatum were homogenized in 50 mM phosphate buffer pH 7.4 at 4 °C. Samples were normalized by the method of Lowry et al. (1951) and a modification of the method of Lentz (1989) was used for measurement of protein carbonyls. To summarize, 1 ml of 10 mM DNPH in 2.5 M HCl was added to 1.1 ml of each sample; blank reactions lacked only DNPH. Following 1 h of incubation with continuous shaking, the protein was precipitated by adding 2 ml of 20% TCA and centrifuged at $3000 \times g$ for 10 min. The protein was washed three times with ethanol:ethyl acetate (1:1) and dissolved in 0.5 ml of 6 M guanidine HCl (pH 7.4). Protein carbonyls were then read at 370 nm.

2.8. Lipid peroxidation in erythrocytes and striatum

The "oxidation index" in erythrocytes and striatum was used as a relative measurement for conjugated dienes on lipids extracted according to the method of Folch et al. (1957). Dried lipids were re-suspended in ethanol and the absorbance ratio A233/A215 was measured on a Carry 1 Varian spectrophotometer at 25 °C (Konings, 1984).

2.9. Striatum and erythrocyte membrane preparation

Cells from striatum were incubated with 100 µl of PBS containing 1.25 mg/ml collagenase for 30 min at 37 °C. After removal of plasma and buffer coat, the erythrocytes were washed three times with isotonic buffer. Erythrocyte membranes were prepared by hypotonic haemolysis in 10 mM Tris–HCl pH 7.4 according to Bramley et al. (1971). Samples were normalized by the method of Lowry et al. (1951). Lipids were extracted from striatum and from erythrocyte membranes by chloroform/methanol according to Folch et al. (1957), in order to perform fluorescent study and lipid peroxidation.

2.10. Fluorescence measurement

Fluorescence measurements on striatum, erythrocyte membranes, and lipid extracts were performed on a Hitachi 4500 spectrofluorometer. Steady-state fluorescence anisotropy (r) measurements for DPH was obtained using the excitation and emission wavelengths at 360 and 430 nm, respectively. The degree of fluorescence anisotropy (r) was calculated according to Shinitzky and Barenholz (1978) from equation:

$$r = \frac{(I_{\parallel} - I_{\perp})g}{(I_{\parallel} + 2\,I_{\perp})g}$$

where g is an instrumental correction factor, and $I_{\parallel}$ and $I_{\perp}$ are, respectively, the intensities measured with the polarization plane parallel and perpendicular to that of the exciting beam.

Generalized polarization of Laurdan (GP$_{340}$) ($\lambda_{ex} = 340$) was calculated according to Parasassi et al. (1990) using equation:

$$GP = \frac{I_B - I_R}{I_B + I_R}$$

where I_B and I_R are the intensities at the blue (440 nm) and red (490 nm) edges of the emission spectrum and correspond to the fluorescence emission maximum in the gel and liquid-crystalline phases (Parasassi et al., 1991) of the bilayer, respectively. The final protein concentration in the assay was 0.4 mg/ml, while the probe concentration was 10^{-6} M. Samples were suspended in 10 mM Tris pH 7.4 and the measurements were performed at 25 °C.

2.11. GSH content

The GSH content in cells from striatum was determined spectrophotometrically by the method of Butler et al. (1994) using the 5,5 dithio-bis(2 nitrobenzoic acid after normalization of samples with the method of Lowry et al. (1951).

2.12. Respiratory burst of monocyte

Monocyte separation was performed by using Ficol density gradient. Whole blood diluted with PBS (1:1) was stratified on a solution of lymphoprep and then centrifuged for 15 min at 2500 rpm. Monocytes, obtained by Ficol density gradient, were washed three times with PBS and counted.

Monocytes from the three groups (control, PERM and CY) were joined in order to obtain three samples, each containing 1×10^6 cells. In every sample, 150 µM lucigenin was added to 1 ml of Krebs–Ringer phosphate solution plus glucose pH 7.4. Monocyte suspensions were activated by 3×10^{-4} M PMA and the chemiluminescence was measured for 50 min. The measurements were performed in duplicate twice.

2.13. Statistical analysis

Basal concentrations were determined as the mean of at least three measurements with ≤5% variation obtained at the beginning of the experiment. One-way ANOVA was used to evaluate the effect of procedures on each group of animals. If a general effect was determined by ANOVA, post hoc analysis was performed with the Newman–Keuls test with $P < 0.05$ used as the level of significance.

3. Results

3.1. General findings

Rats treated daily from PND8 to PND15 with 1/10 of DL$_{50}$ of CY and PERM by intragastric tubing showed no signs of pyrethroid poisoning or gross behavioral abnormalities throughout the experimental period.
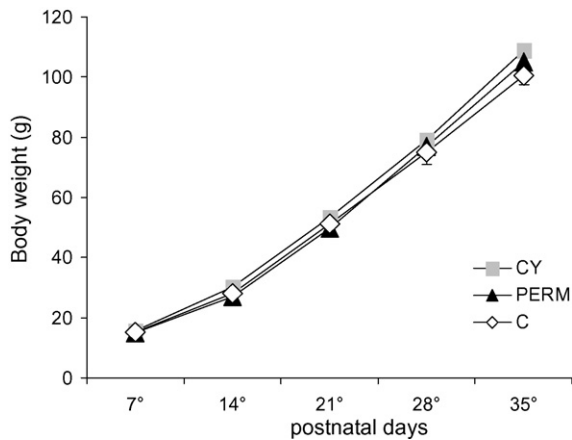
Fig. 1. Body weight in CY-treated (■), PERM-treated (▲) and control rats (◊) during 35 postnatal days. Data are presented as mean ± S.D. [*]P < 0.05 vs. control (C).

Data on body weight in rats treated with CY, PERM and control are reported in Fig. 1. As shown, body weight values increased as a function of age and no significant differences were observed in the body weight of exposed versus control rats during treatment.

3.2. Open-field behavior

In order to evaluate the dopaminergic activity of CY and PERM, we studied the spontaneous loco-motor activity of rats in comparison with control animals. On PND21, no significant modifications were revealed in behavioral activities between treated and control groups (Fig. 2A and B). As can be observed, on PND35 (Fig. 3A), pyrethoid treatment increased (738 ± 46.57 PERM; 797.38 ± 50.87 CY) the spontaneous locomotor activity of rats in comparison with control group (463.7 ± 49.83) ($F(2,27) = 133.4$; $P < 0.05$). In addition, a significant ($F(2,27) = 52.37$; $P < 0.05$) increase of rearing was observed after treatment with CY on PND35 (160.15 ± 12.37 PERM; 183.08 ± 12.07 CY; 131.17 ± 9.43 control). With regard to the anxiety-like behavior, the CY-treated rats had more entries ($F(2,27) = 44.24$; $P < 0.05$) and time spent ($F(2,27) = 57.82$; $P < 0.05$) in the center than control animals, as shown in the Fig. 3B and C.
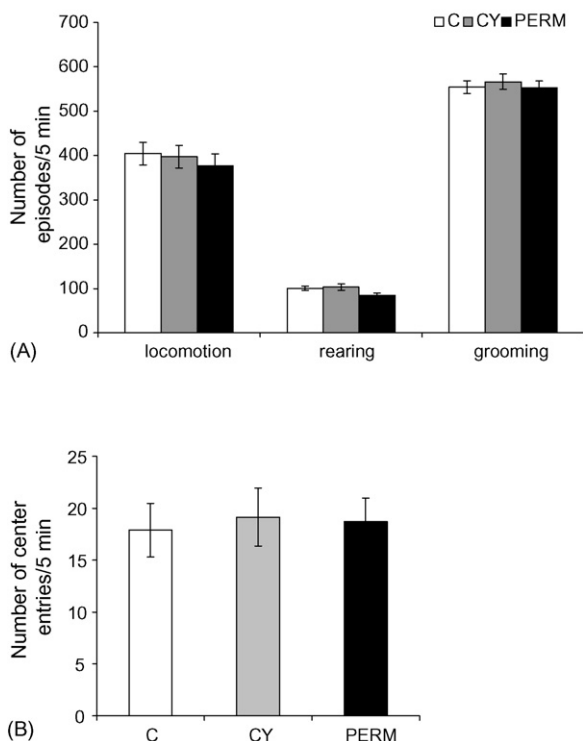

Fig. 2. Effects of neonatal cypermethrin (CY) and permethrin (PERM) exposure on locomotion, rearing and grooming (A) and anxiety test (B) in rats observed at 21 days of age. Data are expressed as mean ± S.D. for 10 rats ($n = 10L$). [*]P < 0.05 vs. control (C).
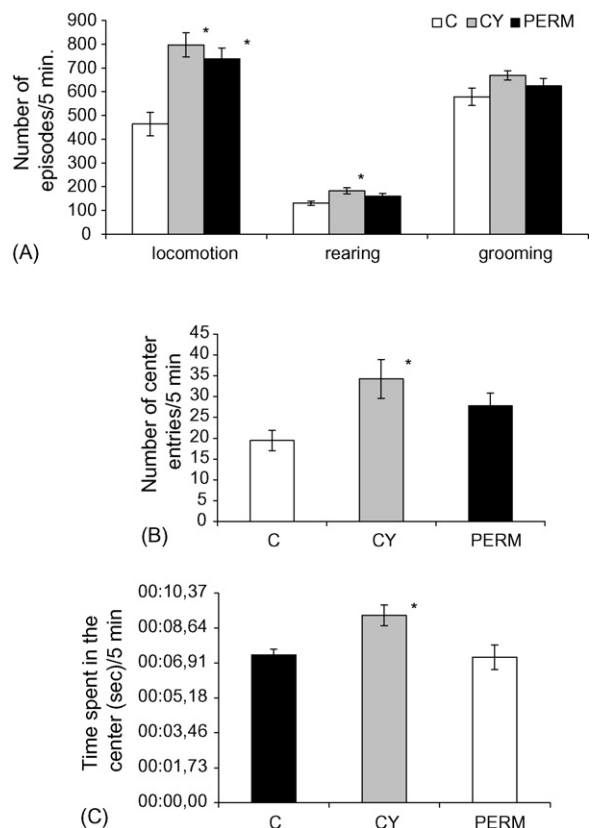

Fig. 3. Effects of neonatal cypermethrin (CY) and permethrin (PERM) exposure on locomotion, rearing and grooming (A) and anxiety test (B and C) in rats observed at 35 days of age. Data are expressed as mean ± S.D. for 10 rats ($n = 10L$). [*]P < 0.05 vs. control (C).
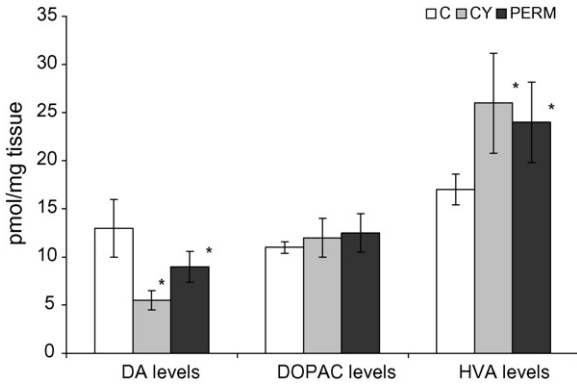
Fig. 4. Levels of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and 3-methoxy-4-hydroxyphenylacetic acid (HVA) in striatum cell of rats from control group (□), CY-treated (▨) and PERM-treated (■) groups. Data are presented as mean ± S.D. for four rats. Measurements were performed in triplicate for each sample. *$P < 0.05$ vs. control (C).

3.3. Monoamines levels

On PND35, pyrethroid-treated animals exhibited lower striatal DA levels (5.5 ± 2 pmol/mg tissue PERM; 9.0 ± 2 pmol/mg tissue CY) than control animals (13 ± 0.6 pmol/mg tissue) ($F_{(2,33)} = 60.65$; $P < 0.05$), as in the Fig. 4. No significant changes were observed in DOPAC levels, while HVA concentration increased significantly ($F_{(2,33)} = 17.02$; $P < 0.05$) in rats treated with CY (26 ± 5.2 pmol/mg tissue) and PERM (24 ± 4.2 pmol/mg tissue) compared with control animals (17 ± 1.6 pmol/mg tissue, Fig. 4).
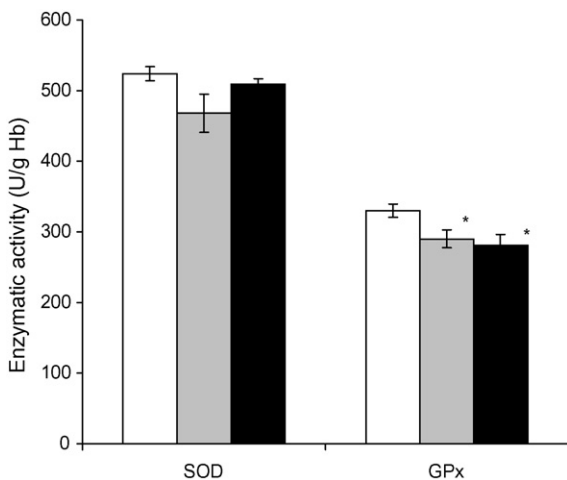

Fig. 5. SOD and GPx blood levels measured in rats, at 35 days of age, from control group (□), CY-treated (▨) and PERM-treated (■) groups. Data are presented as mean ± S.D. for six rats. *$P < 0.05$ vs. control (C).

3.4. Superoxide dismutase and glutathione peroxidase activities

Antioxidant enzymes such as SOD and GPx may play an important role in the protective mechanism against oxidative stress. The evaluation of their activities in rats treated with pyrethroids showed a significant ($F_{(2,5)} = 25.75$; $P < 0.05$) decrease of plasmatic GPx activity (CY: 290.25 ± 12.59; PERM: 281.4 ± 9.5) compared with control group (C: 330.2 ± 14.96) on PND35 (Fig. 5). On the contrary, no change was measured in SOD activity among the three groups.
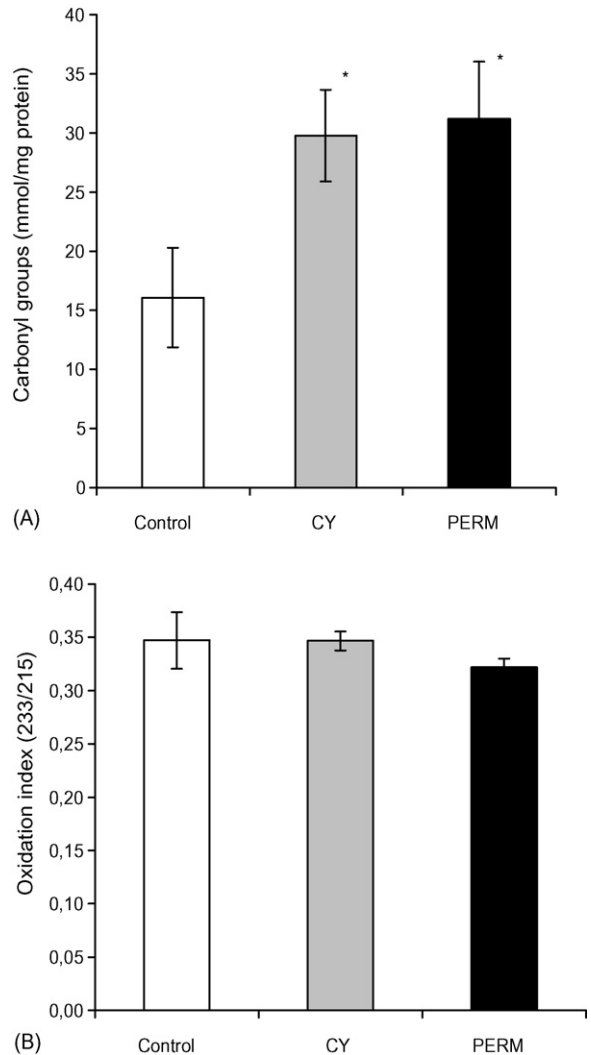

Fig. 6. Effect of pyrethoids on protein carbonyl formation (A) and oxidation index measured on lipids extracted (B) in striatum cells of rats from control group (□), CY-treated (▨) and PERM-treated (■) groups. The results are indicated as mean values ± S.D. for six rats. *$P < 0.05$ vs. control.

3.5. Protein oxidation and lipid peroxidation in striatum

In order to evaluate the possible implication of dopamine metabolism on protein and lipid oxidation, we have measured carbonyl group formation and the "oxidation index", used as a relative measurement for
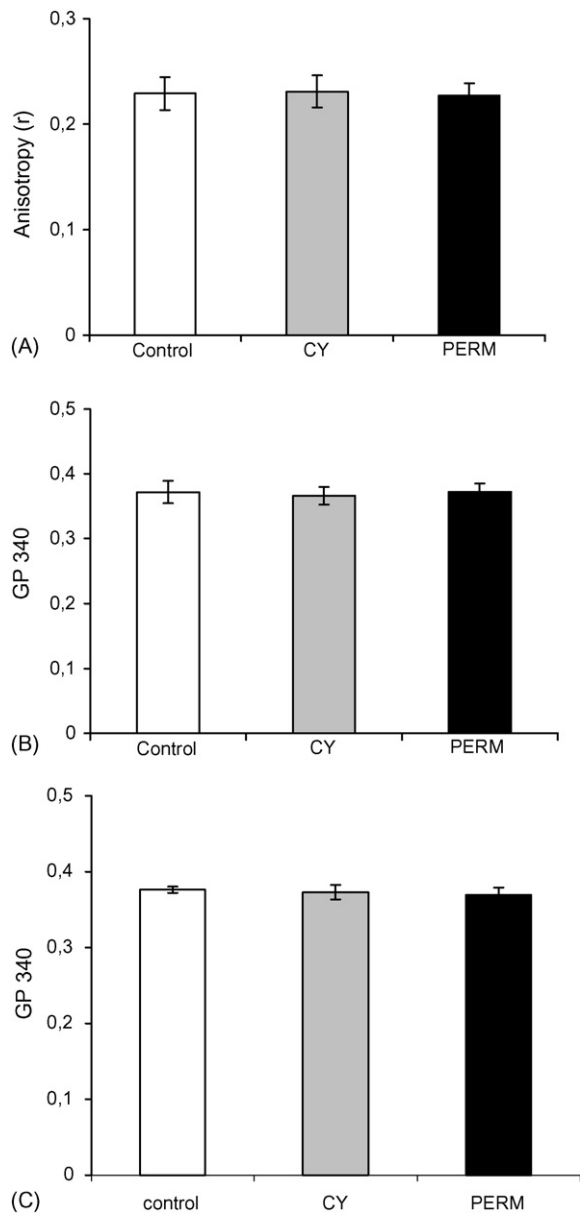
conjugated dienes in lipid extracts. In Fig. 6A a significant increase ($F(2,15) = 22.44$; $P < 0.05$) of carbonyl group content could be observed in both pyrethroid treated groups compared with the control group. No change in oxidation index could be detected among the three groups (Fig. 6B).

3.6. Effect on plasma membrane fluidity of striatum

Steady-state fluorescence anisotropy of DPH reflects the perturbations induced by pyrethroids in the bilayer structure at the hydrophobic core of the bilayer (Wang et al., 1991; Lentz, 1989). Fig. 7A showed no changes in DPH fluorescence anisotropy (r) values obtained in striatum cells from rats exposed to pyrethroids.

Laurdan has been used to monitor membrane fluidity and polarity at the hydrophilic–hydrophobic region of the bilayer, where it localizes (Parasassi et al., 1990, 1991). Fig. 7B showed that the generalized polarization by Laurdan was not changed following pyrethroid treatment. Since we measured an increase in protein oxidation and no change in lipid peroxidation on striatum cells, we focused our study on the plasma membrane outer layer of the striatum in order to determine if it could be damaged by insecticide treatment. Results shown in Fig. 7C indicated that no modification in the physicochemical state of phospholipids can be observed on lipids extracted.

3.7. GSH level in striatum

GSH is among one of the most abundant soluble antioxidant molecules in the brain. Depletion of GSH could significantly affect the survival of dopamine neurons, particularly if they are under oxidative stress.

Fig. 7. Steady-state fluorescence anisotropy (r) of DPH (A), generalized polarization (GP_{340}) of Laurdan (B) in striatum cells and generalized polarization (GP_{340}) of Laurdan (C) in lipid extract of striatum cells from control group (☐), CY-treated (▨) and PERM-treated (■) groups. Data represent the means ± S.D. for six rats. For experimental details, see Section 2.
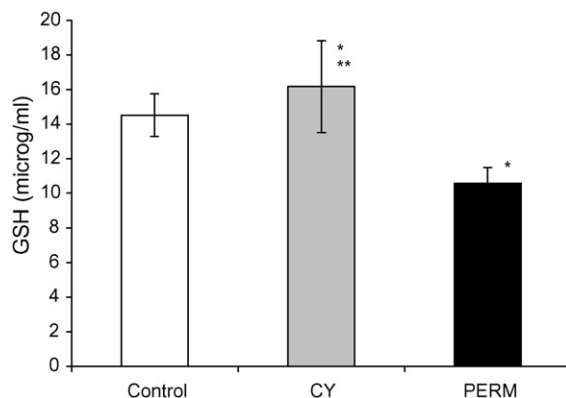

Fig. 8. GSH level in striatum cells of rats from control group (☐), CY-treated (▨) and PERM-treated (■) groups. Data represent the means ± S.D. for six rats. [*]$P < 0.05$ vs. control, [**]$P < 0.05$ vs. PERM. For experimental details, see Section 2.

In the light of this knowledge, we measured the GSH level on striatum cells. A significant increase ($F(2,15) = 15.74$; $P < 0.05$) of GSH level was observed in the CY group (16.17 ± 2.65 μg/mgPr) compared with the control (14.51 ± 1.24 μg/mgPr). A significant reduction ($F(2,15) = 15.74$; $P < 0.05$) was measured in PERM group (10.57 ± 0.92 μg/mgPr) (Fig. 8).

3.8. Protein oxidation and lipid peroxidation in erythrocytes

Since pyrethroid treatment induced an increase in protein oxidation on striatum cells, and previous studies on erythrocytes from adult rats treated consistently with the same pyrethroid types showed an increase of lipid peroxidation, we decided to evaluate the possible damage on both proteins and lipids of erythrocytes. No change in carbonyl group formation was measured (Fig. 9A), while the rats treated with PERM (1.00 ± 0.037) and CY (0.97 ± 0.047) presented a significant increase ($F(2,15) = 10.16$; $P < 0.05$) of the oxidation index compared to the control group (0.90 ± 0.029) (Fig. 9B).
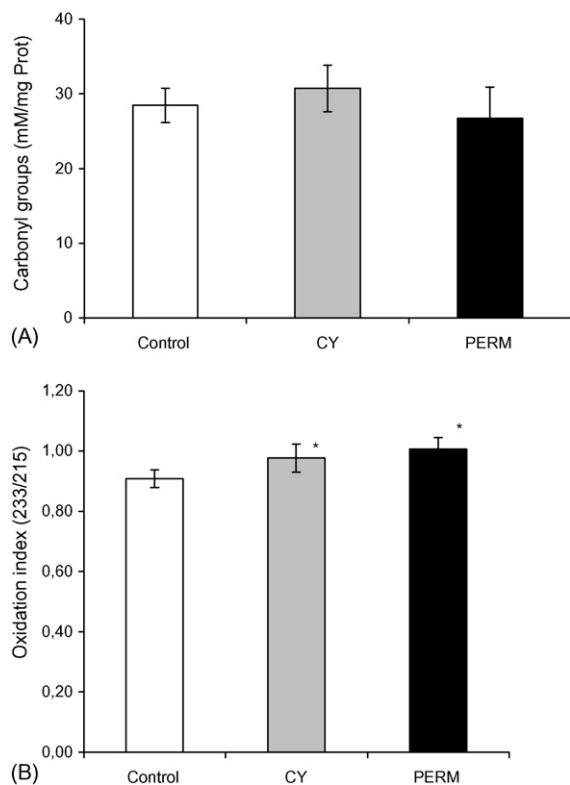

Fig. 9. Effect of pyrethoids on protein carbonyl formation (A) and oxidation index measured on lipids extracted (B) in erythrocytes of rats from control group (□), CY-treated (▨) and PERM-treated (■) groups. The results are indicated as mean values ± S.D. for six rats. *$P < 0.05$ vs. control.

Table 1
Steady-state fluorescence anisotropy (r) of DPH (A) and generalized polarization (GP_{340}) of Laurdan (B) in erythrocyte plasma membranes from controls, CY-treated and PERM-treated rats after pyrethroid treatment

A	
Anisotropy (r)	
Control	0.2190 ± 0.017
CY	0.2189 ± 0.0152
PERM	0.2159 ± 0.01218
B	
Generalized polarization (GP_{340})	
Control	0.3760 ± 0.025
CY	0.3871 ± 0.0181
PERM	0.3978 ± 0.0125

Data represent the means ± S.D. for four rats. Measurements were performed in triplicate for each sample. For experimental details, see Section 2.

3.9. Effect on erythrocyte plasma membrane fluidity

Both DPH and Laurdan fluorescent probes were employed to evaluate the fluidity changes in erythrocyte plasma membranes from rats treated with pyrethroids. Neither probes revealed changes in the bilayer (Table 1A and B).

3.10. Respiratory burst of monocyte

Fig. 10 shows the respiratory burst behavior in monocytes from rats treated with PERM and CY. The cells were activated with PMA and the superoxide anion was revealed by the presence of lucigenin in the reaction
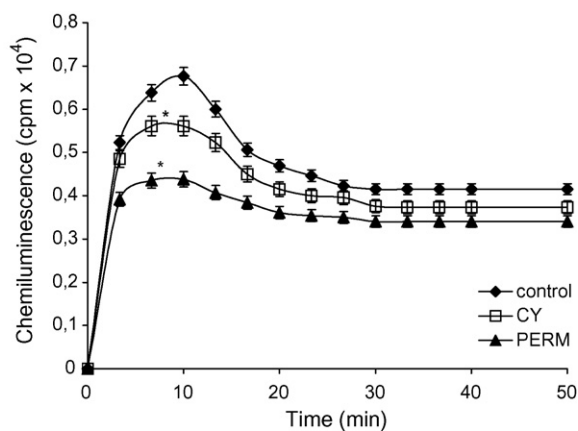

Fig. 10. Time course of lucigenin-amplified chemiluminescence of monocytes in rats from control group (♦), CY-treated (□), and PERM-treated (▲) groups. Monocytes were stimulated with 3×10^{-4} mol/L phorbol myristate acetate. Chemiluminescence was measured as counts per minute (cpm). The results are indicated as mean values ± S.D. for six rats. *$P < 0.05$ vs. control.

system. The data obtained indicate superoxide anion production in the three groups following the activation of the NADPH oxidase system by PMA. As can be observed in Fig. 10, superoxide anion production was lower ($F(2,15) = 112.17$; $P < 0.05$) in the groups treated with pyrethroids compared to the control group. In particular, the superoxide anion production decreased as follows in the three groups: control > CY > PERM.

4. Discussion

It is known that DA is the major neurotransmitter involved in the control of the motor system. Some authors (Bernardi et al., 1981) showed that locomotion and rearing frequencies observed in an open-field might be used to detect drug-induced dopaminergic interference. Since the pyrethroid acute neurotoxicity to adults has been well characterized, but the information regarding the potential neurotoxic development of this class of compounds is limited, we decided to investigate the potential neurotoxicity caused by pyrethroid exposure (from PND6 to PND15) in developing rats. During this critical period of brain development, animals acquire many new motor and sensory abilities, associated with numerous biochemical changes that transform the feto-neonatal brain into that of the mature adult (Dobbing, 1975). This is also a time during development when the synthesis of brain lipids and the turnover of proteins are at their highest levels (Lajtha and Dunlop, 1981; Eriksson et al., 2002).

As illustrated in Fig. 2A and B, no difference was observed in behavioral activities of both control and treated male pups at weaning. The lack of pyrethroid effects on open-field behaviors observed at 21 days of age is important in the interpretation of possible behavioral alterations observed later in the life of the animal. Many of these effects in newborns may be reflected in adult age as subtle modifications of specific behaviors. At PND35, neonatal pyrethroid exposure of rats increased the number of entries and time spent in the center, but this behavior is not an indication of their lower anxiety, because we observed also an increase of locomotion. As reported from some authors, only an increase in central locomotion or in time spent in the center without modification of total locomotion can be interpreted as an anxiolytic-like effect (Prut and Belzung, 2003). At PND35, neonatal pyrethroid exposure of rats increased the locomotion activity, and treatment with CY also increased rearing episodes. This trend indicated a dopaminergic impairment. These findings are confirmed by the higher HVA and the lower DA levels measured in the striatum. Thus, it is possible to

suggest that the increase of the locomotion activity observed here are the consequence of a marked reduction of DA induced by an above normal increase in its turnover. This action is consistent with previous studies in which an increase in locomotion (Husain et al.,1994) and dopamine turnover (Karen et al., 2001) of rats could be related to acceleration of cellular DA uptake. This process can be related to up-regulation of the transcription factor Nurr1, which leads to increased expression of dopamine transporter (Elwan et al., 2006). Elwan et al. (2006) report that the transcription factor Nurr1 is enhanced by pyrethoids, which block sodium channels.

Moreover, it might be hypothesized that the lower DA level could be correlated with an inhibition of biosynthesis of DA following a decrease in tyrosine hydroxylase and aromatic L-amino acid decarboxylase synthesis as observed after deltamethrin exposition (Liu and Shi, 2006). However, since DOPAC levels were not changed, while HVA levels increased, it might be suggested that the catabolic pathway of DA by catecol-o-methyltransferase might be influenced by pyrethoids more than monoamino oxidase (MAO) and aldehyde dehydrogenase (AD) (Nakazato, 2002), leading to increased DA degradation.

Accelerated dopamine turnover leads to the formation of reactive species such as hydrogen peroxide and reactive quinine and semi-quinone species produced by DA autoxidation (Makes et al., 1981). In the light of this fact, we investigated oxidative stress parameters. Blood glutathione peroxidase levels decreased significantly in the groups treated with CY and PERM and increased protein oxidation was measured in striatum cells from both treated groups. On the contrary, no change in lipid peroxidation and blood SOD content was measured. This behavior might suggest that brain antioxidants can partially protect from free radicals produced by DA degradation pathways. Since the oxidation process at protein and lipid levels can modify the physical–chemical state of plasma membrane, we measured the plasma membrane fluidity in order to quantify the entity of the oxidative event. Fluorescence measurements on striatum cell membranes indicated no change of fluidity in the two regions tested by Laurdan and DPH. The values of generalized polarization revealed by Laurdan and the absence of conjugated dienes indicated that the oxidation process did not involve lipids; anyway the increase of carbonyl group formation could be related to oxidized proteins that are on the outside of plasma membranes, so their changes do not modify cell fluidity.

Of the various antioxidants in the brain, glutathione is particularly important in controlling cellular redox

states and as a primary defence mechanism for peroxide removal from brain (Rabinovic et al., 2000). The measurement of the GSH level in striatum cells shows an unexpected behavior in the two treated groups: a reduction and an increase of GSH were obtained in PERM and CY groups, respectively. The different GSH levels in the two groups do not correlate with the other variations measured, so this effect could be related to the differences in structure of the two pyrethroids. In terms of the presence of the cyano-group, CY is more hydrophilic than PERM, which crosses the plasma membrane more readily and can induce oxidative stress harding on antioxidant defense system (i.e. GPx and GSH).

Our previous studies on adult rats showed that the treatment with PERM and CY induces alteration in erythrocyte plasma membrane fluidity and in lipid peroxidation (Gabbianelli et al., 2002; Nasuti et al., 2003). In this work, we evaluated whether treatment of rat pups with pyrethroids produces alterations in the erythrocytes. No change in carbonyl group formation and membrane fluidity was measured through DPH and Laurdan. Alteration in lipid peroxidation was the only damage assessed in erythrocytes in our experimental conditions. This effect was also present in adult rats following pyrethroid treatment, although changes in plasma membrane fluidity were measured when adult rats were treated for a longer time (60 days) with the same dose (1/10 of DL_{50} determined in adult rats) (Nasuti et al., 2003). It might be possible that the lower toxicity measured in pup erythrocytes could be related with the shorter treatment time. The absence of protein oxidation in the erythrocyte membrane was in accordance with our previous paper where rats were treated with CY (Gabbianelli et al., 2002). The different behavior of protein and lipid oxidation observed in striatum and erythrocytes could be related to a different distribution of pyrethroids in these two types of cells. We have to consider that pyrethroids show a high affinity to GABA receptors present in the brain (Gammon et al., 1982; Lawrence and Casida, 1983). For this reason, they could mainly bind the proteins located outside plasma membrane. In addition, the sole effect of DA oxidation products on striatum could explain the protein damage, since DA quinines bind to free cysteine and GSH or to proteins (Hasting et al., 1996).

There is an increasing body of evidence pointing towards a relevant relationship among stressors and the occurrence of variations in immune function (Fonseca et al., 2002; Palermo-Neto et al., 2003). Since high doses of CY, supercypermethrin, and deltametrin induce immunosuppressive effects on humoral and cell-mediated immune response in mice, rats and goats, we

evaluated the respiratory burst of monocytes (Stelzer and Gordon, 1984; Desi et al., 1985, 1986; Tamang et al., 1988). Our results show that CY and PERM reduce the production of superoxide anion in rat monocytes activated with PMA, and this effect seems to be more consistent in the rat group treated with PERM. As is known, during the respiratory burst, granulocytes and monocytes produce reactive oxygen species (superoxide anion ($O_2^{\bullet-}$), hydroxyl radical) and oxidant compounds (hydrogen peroxide, HOCl) that are useful for their phagocytic activity. The production of $O_2^{\bullet-}$ depends on the NADPH oxidase complex which catalyzes the reduction of oxygen to $O_2^{\bullet-}$ (Babior, 1984, 1987). As known, the NADPH oxidase complex is composed of various membranes and cytoplasmatic subunits that interact together when the cells are activated. The lack of one subunit or an alteration in the formation of the complex produces a low level of $O_2^{\bullet-}$ (Babior, 1984, 1987). In our study, the reduced production of superoxide anion negatively influences the efficiency of phagocytosis, since other steps of respiratory burst depend on its production. It might be suggested that pyrethoids alter the signal transduction or interfere in NADPH oxidase complex formation, thus reducing the amount of superoxide anion produced.

These studies suggest that the action of pyrethroids on various target cells is different, although a preferential interaction with the extracellular side of plasma membrane proteins can be observed (i.e. GABA receptors on striatum, NADPH oxidase complex in monocytes).

In summary, the above data indicate that neonatal exposure to pyrethroids during the critical period of growth has long-term effects on the biochemistry and behavior of male rats. Since neonatal exposure to a low dose of pyrethroids alters dopaminergic activity at PN35, insecticide exposure could play a role in PD by predisposing individuals to the irreversible cell loss that occurs in this disease. Further studies will be performed in order to better characterize the mechanisms linking dopaminergic alterations with the molecular modifications induced by pyrethroid during the lifetime of rats.

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