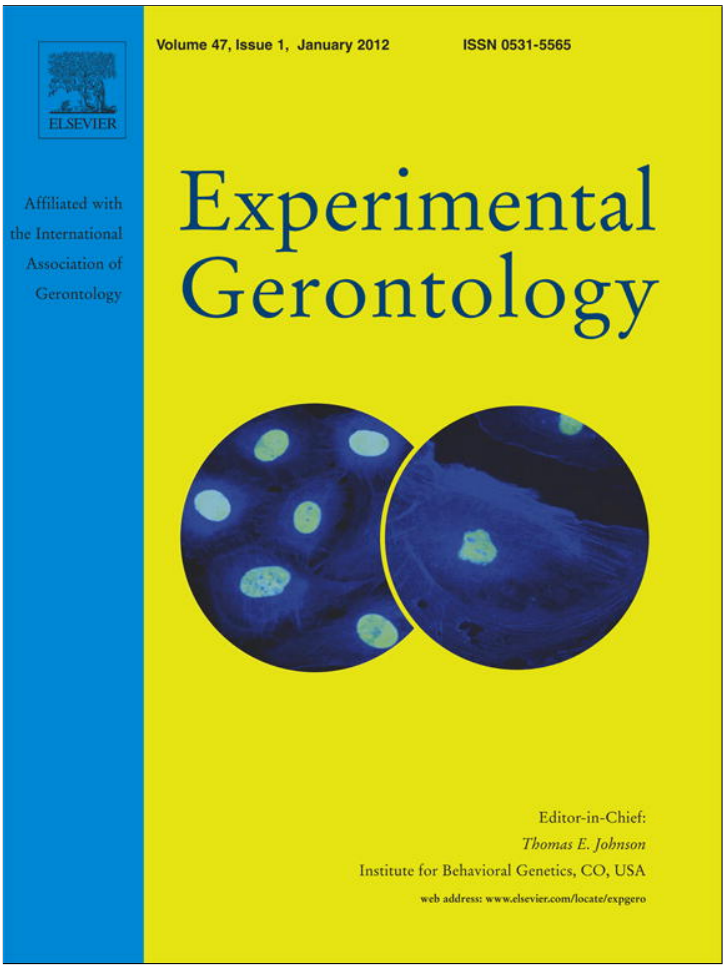

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The impact of early life permethrin exposure on development of neurodegeneration in adulthood

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ABSTRACT

Early life environmental exposure to pesticides could play a critical role in the onset of age-related diseases. The present study aims to evaluate in brain, plasma and leukocytes of 300 day-old rats, the effect of a low dose of the insecticide permethrin administered during early life (1/50 LD_{50}, from 6th to 21st day of life).
The outcomes show that Nurr1, mRNA and protein expression, as well as calcium and NO levels are decreased in striatum. Moreover, the pesticide induces an imbalance in glutamate, calcium and NO in hippocampus. Low calcium concentrations in leukocytes and in plasma were observed, while increased NO and decreased SOD plasma levels were measured.
The results suggest that permethrin intake at a dose close to the NOAEL (25 mg/kg) during the perinatal period can interact with Nurr1 by reducing its expression on striatum nucleus. Consequently, the maintenance of dopaminergic neurons as well as Nurr1 inhibitory effect on the production of proinflammatory mediators fails.
The changes in biological markers found in our animal model could represent the basis to study neurodegenerative diseases whose development depends on individual gene signature and life style.

1. Introduction

Epidemiologic evidence suggests that environmental factors are an important cause of neurodegenerative diseases and psychiatric disorders (Parrón et al., 2011; Zhang et al., 2006; Hatcher et al., 2008; Giasson and Lee, 2000). Contaminants accumulated through the food chain and environmental exposure can modify gene expression changing protein synthesis in different tissues (Relton and Smith, 2010). Pesticides are identified as one of the risk factors involved, through epigenetic mechanisms, in the regulatory processes controlling gene expression leading to the initiation and progression of age-related diseases (Relton and Smith, 2010). Low levels of pesticides may negatively affect the brain inducing loss of neurons that leads to cognitive decline, impaired memory and attention, and motor function (Parrón et al., 2011). These neurobehavioral disturbances may be associated with neurological disorders such as Alzheimer's disease, Parkinson's disease or dementia in old age (Parrón et al., 2011).

Previous studies have shown that pesticides such as rotenone, paraquat and dieldrin are able to induce Parkinson's disease in animal models (Song et al., 2010), moreover other insecticides, fungicides and herbicides have been correlated with neurological disorders (Parrón et al., 2011). Permethrin, a member of the family of synthetic pyrethroids is largely used as insecticide for indoor residential treatment (i.e. carpets, kitchen worktops and other treated wood furniture), outdoor applications on the lawn, mosquito control, occupational take-home exposures, pets, personal care products (Bradberry et al., 2005). The environmental exposure to this pesticide is frequent since it reaches roughly 25% of global insecticide sales together with the other components of the pyrethroid family (Williamson et al., 1996). The diet and indirect exposure through house dust represents the major routes of entrance into the human body for permethrin; however it can also be absorbed through inhalation, oral and dermal routes. It is metabolized in mammals and its metabolites are excreted in urine in humans (Saieva et al., 2004; Barr et al., 2010; Nakamura et al., 2007). Permethrin is able to induce impairment of striatal mitochondrial function, changes in the immune system and oxidative stress in adult rats treated sub-chronically with a low dose of this pesticide (Gabbianelli et al., 2002; Gabbianelli et al., 2003; Gabbianelli et al., 2004; Gabbianelli et al., 2009a; Gabbianelli et al., 2009b; Nasuti et al., 2007; Nasuti et al., 2008; Falcioni et al., 2010). Recently, we showed that when permethrin was administered during early life

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(from 6th to 21st day), alterations in striatum (Nasuti et al., 2007), in heart and in plasma (Vadhana et al., 2011), were observed during adult age.

The present work attempts to comprehend if the increased dopamine turnover and oxidative stress measured in adult rats treated with a low dose of permethrin during early life (1/50 LD_{50}, from 6th to 21st day of life) (Nasuti et al., 2007), can be associated with other signs characteristic of neurological disorders. With this aim, the levels of Nurr1, a transcription factor essential for the maintenance of dopaminergic neurons, and other markers correlated with neurodegeneration such as glutamate, calcium, nitric oxide (NO), superoxide dismutase (SOD) were evaluated. Our outcomes show that early life permethrin treatment reduces Nurr1 mRNA and protein expression as well as calcium and NO levels in striatum from adult rats. An imbalance in glutamate, calcium and NO levels was measured in hippocampus from treated rats, while no changes in prefrontal cortex were detected. Calcium levels measured in leukocytes from treated rats resulted decreased, as well as calcium and SOD in plasma, in which increased NO levels were found. Since we hypothesize that the intake of this pesticide could be one of the factors involved in the onset of neurodegenerative diseases, the results are discussed considering the variations in the levels of neurodegenerative markers in cerebral areas, in plasma and in leukocytes.

2. Materials and methods

2.1. Materials

All reagents were of pure and analytical grade and were obtained from Sigma Chemical Co. (USA). Technical grade (75:25, trans:cis; 94% purity) 3-phenoxybenzyl-(1R,S)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxyl-ate, Permethrin (PERM) was generously donated by Dr. A. Stefanini of ACTIVA, 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 used. The animals were housed in plastic (Makrolon) cages (five rats/cage) in a temperature controlled room (21 ± 5 °C) and maintained on a laboratory diet with water ad libitum. The light/dark cycle was from 7 p.m. to 7 a.m. Animals used 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). Rat pups born in our laboratory from primiparous dams were used in the study. The parturition day was set as Post Natal Day 0 (PND0). On PND1, all litters were examined externally for the presence of gross abnormalities, sexed, weighed and the female pups were discarded.

As already reported in a previous study (Nasuti et al., 2007), only male rats were chosen in order to avoid any possible involvement of hormonal changes in adult female rats. Two 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 two experimental groups (n = 6 rats for each).

2.3. Treatment

PERM was dissolved in corn oil and administered orally by intragastric tubing (4 ml/kg) at a dose of 1/50 of DL50 corresponding to 34.05 mg/kg (Cantalamessa, 1993). The dosage was chosen considering the “no observed adverse effect level” (NOAEL) which for PERM is 25 mg/kg.

The insecticide was administered once a day in the morning from PND6 to PND21. Control rats were treated with vehicle (corn oil 4 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. At adult age (PND300), six rats from each group (PERM treated and control groups) were sacrificed by exposure to CO_2, and their blood and brain tissue (striatum, prefrontal cortex and hippocampus) were collected for analysis. For the experiments, the groups of animals were formed by drawing them from different litters so that no group contained siblings. All data were analyzed considering the litter as the smallest unit.

2.4. RNA extraction and cDNA preparation

Total RNA was extracted from pool of striatum obtained from control and treated rats by using a RNA Isolation kit (NucleoSpin RNA II Purification Kit, Clontech Laboratories, Inc., USA). RNA quality was checked by gel visualization and spectrophotometric analysis ($OD_{260/280}$), while its quantity was measured using the OD_{260} by Nano-Drop spectrophotometer. The RNA was transcribed in vitro to cDNA using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Fermentas, Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions.

2.5. Gene expression analysis

RT-PCR and qPCR were employed to evaluate mRNA expression of genes of interest. The following specific sense and antisense primers were designed on the basis of gene and mRNA sequences available online (http://www.ncbi.nlm.nih.gov/gene) and purchased from Sigma Chemical Co. (USA): β-Actin, TAAAGACCTCTATGCCAACACAGTGC (forward) and AGAGTACTTGCGCTCAGGAGGAG (reverse); Nurr-1, GGTTTCTTTAAGCGCACGGTG (forward) and TTCTTTAACCATCCCAA CAGCCAG (reverse). These primer sets specifically recognized only the genes of interest, as indicated by the PCR amplification products (141 bp for Nurr-1 and 145 bp for β-actin). Amplification of β-actin, a relatively invariant internal reference RNA, was performed in parallel. First strand cDNA was amplified by using Phire Hot Start II DNA Polymerase (Finnzymes Oy, Finland) in a total volume of 20 μl containing 100 ng of cDNA, 0.5 μM of sense and antisense gene-specific primers and 200 μM of dNTP Mix (Fermentas, Thermo Fisher Scientific Inc., USA). The temperature profile was as follows: 30 s at 98 °C; 35 cycles of 5 s at 98 °C, 5 s at 63 °C, 15 s at 72 °C and held 1 min at 72 °C . The amplification products were analyzed by electrophoresis on a 1.7% agarose gel in 1 × TAE buffer (40 mM Tris–acetate and 1 mM EDTA) containing 0.5 μg/ml ethidium bromide. GeneRuler 100 bp DNA Ladder (Fermentas, Thermo Fisher Scientific Inc., USA) was used to size DNA fragments. Gel images were captured using the KODAK Image Station 2000r Systems. qPCR analysis was performed in a total volume of 20 μl containing 25 ng of template cDNA, 0.25 μM sense and antisense primers, 10 μl of iQ SYBR Green Supermix (Bio-Rad Inc., USA) by using a Stratagene MX3000P. The same RT-PCR specific sense and antisense primers described above were used. The real-time PCR program was: initial denaturation at 95 °C for 3 min; and 40 cycles at 95 °C for 30 s, 63 °C for 30 s and 72 °C for 1 min. The program was terminated by a final extension at 95 °C for 1 min, 60 °C for 30 s and 95 °C for 30 s. Relative mRNA expression on each tissue sample was quantified according to the ΔΔCt method. A ΔCt value was first calculated by subtracting the Ct value for the housekeeping gene β-actin from the Ct value for each sample. A ΔΔCt value was then calculated by subtracting the ΔCt value of the control from the ΔCt value of treated samples. Fold changes compared to the controls were then determined by $2^{-\Delta\Delta Ct}$. Each PCR experiment was run three times in triplicate.

2.6. Tissues preparation

Leukocytes from rats' blood were isolated using Mono-Poly Resolving Medium (ICN, Biomedicals, Milan, Italy), then they were recovered from gradients and washed using PBS. Pools of striatum,

prefrontal cortex, hippocampus and leukocytes obtained from control and treated rats were lysated using RIPA buffer [1% NP40, 0.5% Na-deoxycolic acid and 0.1% SDS in phosphate buffered saline (PBS)] with freshly added protease inhibitors. The lysates were passed several times through a 22-gauge needle in order to shatter the DNA molecules.

2.7. Western blot

Lysates were quantified using the Lowry method (Lowry et al., 1951) and equal amounts (30 μg) of protein from each cell lysate were separated using SDS-PAGE (10%) and electrophoretically blotted on a nitrocellulose support (Hybond C, Amersham Bioscience, Little Chalfont, UK).

Reactive sites were then blocked with PBS containing 0.05% Tween 20 and bovine serum albumin (10%) for 1 h at room temperature and incubated with polyclonal anti-rabbit Nurr-1 antibody (Santa Cruz Inc, USA) diluted 1:100 and then with secondary anti-rabbit antibody diluted 1:5000, (KPL, USA). β-actin was utilized as a control for equal protein loading: membranes were stripped and reprobed for β-actin with an anti-rabbit monoclonal antibody (Abcam plc, UK) diluted 1:3000. Antibodies were diluted in PBS containing 0.05% Tween 20 and bovine serum albumin (2%). Every gel was loaded with molecular weight markers including proteins with MW from 250 to 4 kDa (Invitrogen,USA). HeLa cell lysate was used as positive control (data not shown). Image capturing and densitometry analysis were performed using the KODAK Image Station 2000r Systems.

2.8. Glutamate levels

Glutamate quantification on pools of striatum, prefrontal cortex and hippocampus tissue homogenate obtained from control and treated rats was assessed using a commercially available enzymatic assay kit (BioVision Inc., USA) according to the manufacturer's instructions. Glutamate levels are expressed in nmol glutamate/mg protein.

2.9. Calcium levels

Ca^{++} level quantification on plasma and pools of striatum, prefrontal cortex and hippocampus tissue homogenate obtained from control and treated rats was assessed using Calcium Green-1AM (Invitrogen, USA) as fluorescent indicator. Calcium Green-1AM was reconstituted in high-quality anhydrous DMSO as stock solution at 5 mM concentration. Calcium levels were measured in a reaction mixture containing 5 μM Calcium Green-1AM, and equal amounts (0.3 mg/ml) of protein from each brain area tissue homogenate or 50 μl of plasma obtained from control and treated rats. The fluorescence was monitored using a Hitachi 4500 spectrofluorometer, emission wavelength — 531 nm, excitation wavelength — 506 nm, excitation slit — 5, emission slit — 5. Measurements were recorded from 0″ to 10″. A standard curve was obtained using $CaCl_2$ in PBS at different concentrations in the presence of Calcium Green-1AM (5 μM final concentration) to report the results as nmol Ca^{++}/mg protein or nmol/ml plasma.

2.10. NO levels

NO quantification on plasma and pools of striatum, prefrontal cortex and hippocampus tissue homogenate obtained from control and treated rats was assessed using a commercially available non enzymatic assay kit (Neogen Corporation, USA) according to the manufacturer's instructions. NO levels are expressed in nmol NO/mg protein for each brain area tissue homogenate and in nmol NO/ml for plasma level measurements.

2.11. Superoxide dismutase levels

Superoxide dismutase (SOD) was determined according to the method reported in Misra and Fridovich (1972). SOD level was monitored in a reaction mixture containing 0.05 M carbonate buffer, 0.1 M EDTA, pH 10.2, 0.05 M adrenaline and 50 μl of plasma. The change in absorbance was recorded spectrophotometrically at 480 nm at 37 °C. A standard curve was used to report the results as μg SOD/ml plasma.

2.12. Phospholipid hydroperoxide detection

Intracellular lipid hydroperoxides were detected using a membrane-localized fluorescent probe DPPP that reacts specifically with hydroperoxides and becomes highly fluorescent when oxidized (Takahashi et al., 2001). Control and PERM treated rat brain cells at the protein concentration of 0.4 mg/ml, were incubated with 1 μM DPPP in PBS at 37° for 5 min in the dark. After incubation cells were washed three times with PBS and resuspended in the same buffer and the fluorescence intensities of the samples were measured with a Hitachi 4500 spectrofluorometer using 351 and 380 nm as excitation and emission wavelengths, respectively.

2.13. Statistical analysis

Data are expressed as mean values ± SEM. Each experiment was performed in triplicate and repeated three times on pooled samples of treated and control animals. Statistical analysis was carried out using a one-way analysis of variance or an analysis of variance for repeated measurements followed by post hoc Newman–Keuls test (Statsoft Statistica Software, 9.0). A P value <0.05 was considered statistically significant.

3. Results

3.1. Gene expression

Extracted total RNA from pool of striatum tissue homogenate obtained from control and treated rats were processed for RT-PCR and qPCR analyses. Fig. 1 indicates that the RT-PCR produced the expected size genes (141 bp for Nurr-1 and 145 bp for β-actin). qPCR was performed to determine changes in mRNA levels of Nurr-1 due to PERM early life treatment. The results show a 0.77-fold decrease ($P<0.05$) in Nurr-1 mRNA in treated rats' striatum when compared to the control rats (Fig. 1).

3.2. Western blot

Fig. 2 shows the Western blot analysis using anti-Nurr-1 antibody in striatum, prefrontal cortex, hippocampus tissue homogenate and leukocytes obtained from control and treated rats. Anti-Nurr1 antibody detected a prominent band at an approximate molecular mass of 67 kDa. β-actin was utilized as a control for equal protein loading. The densitometry analysis of the blot shows that in striatum, the intensity of the treated band was lower (93.60 ± 0.27%) ($P<0.05$) with respect to the control (100%), while no differences were observed in other brain regions. For this reason, RNA from prefrontal cortex and hippocampus were not processed for RT-PCR and qPCR analyses. Nurr1 was also checked in leukocytes to determine if it could be used as a biomarker of neurodegeneration. No changes in Nurr1 in leukocytes were observed.

3.3. Glutamate levels

Fig. 3 shows the glutamate levels in striatum, prefrontal cortex and hippocampus tissue homogenates obtained from control and treated rats. A significant decrease in glutamate level (45.30 ± 0.14 nmol/mg

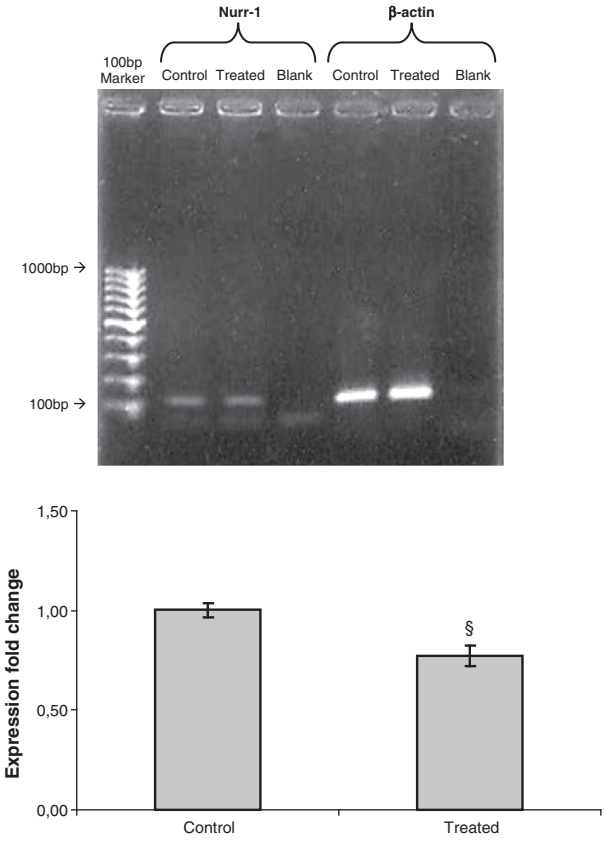

Fig. 1. RT-PCR assay and qPCR for quantifying changes in gene expression of Nurr-1 in control and treated rats. All expression values were normalized to the value of β-actin gene used as an internal control. $P<0.05$ § vs control. RT-PCR picture is representative of 3 experiments.

protein) was observed in hippocampus of treated rats with respect to the control (68.04 ± 1.34 nmol/mg protein) ($P<0.05$), while no significant changes were observed in the other brain regions.

3.4. Calcium levels

Fig. 4 shows the Ca^{++} levels in striatum, prefrontal cortex, hippocampus and leukocyte tissue homogenates obtained from control and treated rats. A significant Ca^{++} level decrease (120.65 ± 3.74 nmol/mg protein) was observed in striatum of treated rats with respect to the control (304.40 ± 2.38 nmol/mg protein) ($P<0.05$). The same behavior was observed in hippocampus (124.44 ± 3.55 nmol/mg protein for control and 103.35 ± 7.17 nmol/mg protein for treated, $P<0.05$) and leukocytes (85.88 ± 2.83 nmol/mg protein for control and 43.71 ± 2.82 nmol/mg protein for treated, $P<0.05$), while no change was observed in prefrontal cortex. Fig. 5 shows Ca^{++} plasma levels, where a significant decrease is observed in treated rats ($1.32 \times 10^3 \pm 0.11$ nmol/ml plasma) with respect to the control ($2.22 \times 10^3 \pm 0.17$ nmol/ml plasma) ($P<0.05$).

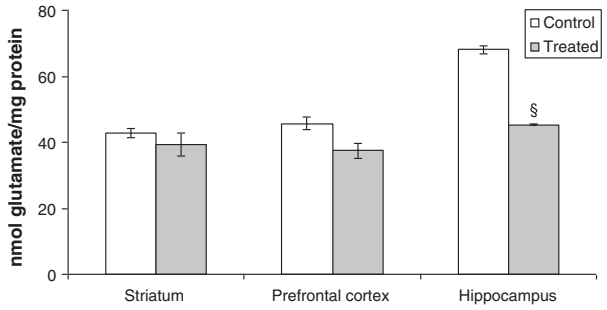

Fig. 3. Glutamate levels on striatum, prefrontal cortex and hippocampus obtained from control and treated rat groups. $P<0.05$ § vs control.

3.5. NO levels

Fig. 6 shows the NO levels in striatum, prefrontal cortex, hippocampus and leukocyte tissue homogenates obtained from control and treated rats. A significant NO level decrease (2.29 ± 0.12 nmol/mg protein) was observed in striatum of treated rats with respect to the control (6.83 ± 0.30 nmol/mg protein) ($P<0.05$). The same behavior was observed in hippocampus (3.13 ± 0.36 for control and 1.44 ± 0.12 nmol/mg protein for treated, $P<0.05$), while no changes were observed in leukocytes and prefrontal cortex. Fig. 5 shows NO plasma levels, where a significant increase is reported in treated rats (3.99 ± 0.05 nmol/ml plasma) with respect to the control (3.45 ± 0.05 nmol/ml plasma) ($P<0.05$).

3.6. Superoxide dismutase level

Fig. 5 shows the effect of early life PERM treatment on SOD plasma content. The data show a significant decrease in treated rats

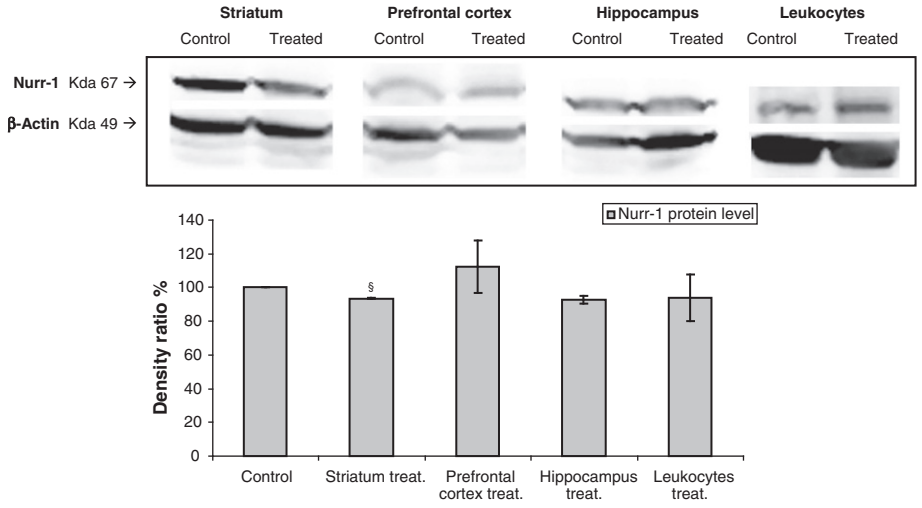

Fig. 2. Western blotting detection and densitometry analysis of Nurr-1 protein expression on striatum, prefrontal cortex, hippocampus and leukocytes. Density ratio percentage of each treated area sample was referred to its respective control fixed as 100%. $P<0.05$ § vs control. The immunoblot exhibited in the figure is representative of 3 experiments.

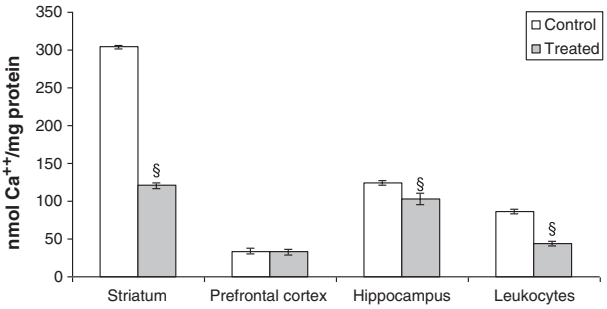

Fig. 4. Ca^{++} levels on striatum, prefrontal cortex, hippocampus and leukocytes obtained from control and treated rat groups. $P<0.05$ § vs control.

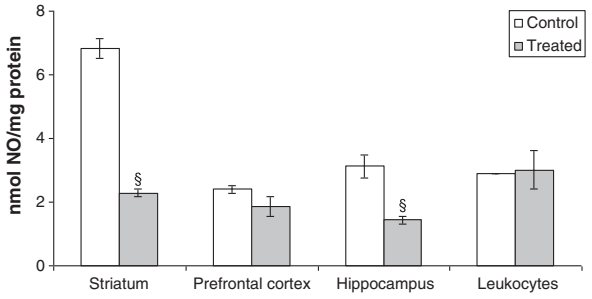

Fig. 6. NO levels on striatum, prefrontal cortex, hippocampus and leukocytes obtained from control and treated rat groups. $P<0.05$ § vs control.

(6.39 ± 1.18 μg/ml plasma) with respect to the control (13.34 ± 0.24 μg/ml plasma) ($P<0.05$).

3.7. Lipid peroxidation

DPPP fluorescence was used for detection of membrane lipid peroxidation in striatum, prefrontal cortex and hippocampus tissue homogenates obtained from control and treated rats. An increase in DPPP fluorescence was obtained in striatum (5275 ± 7.68 for control and 6292 ± 2.98 fluorescence arbitrary units for treated, $P<0.05$), while no changes were observed in prefrontal cortex and hippocampus (Fig. 7).

4. Discussion

The development of age-related disorders is associated with chronic low grade inflammatory activity, neurodegeneration and cardiovascular diseases (Guarente and Franklin, 2011). We previously reported that early life PERM treated rats showed an increase in pro-inflammatory cytokines (i.e. L-1β, IL-2, IFN-γ, rat-Rantes) and changes in heart cells in adult age (Vadhana et al., 2011). In this paper, adult rats from the same treatment were studied to investigate the effect of early life permethrin intake on Nurr1 expressed in different brain areas and in leukocytes. Nurr1 is a transcription factor able to regulate tyrosine hydroxylase gene expression, whose function is related to dopamine synthesis in midbrain dopaminergic neurons (Bensinger and Tontonoz, 2009; Galleguillos et al., 2010). It is a member of NR4A subfamily of orphan nuclear receptors (Nur77/NR4A1, Nurr1/NR4A2, and NOR1/NR4A3), that are expressed in various metabolically demanding and energy dependent tissues such as skeletal muscle, adipose, heart, kidney, T-cells, liver and brain (Maxwell and Muscat, 2005). Furthermore, the NR4A subgroup can be induced by physiological signals such as fatty acids, stress, prostaglandins, growth factors, calcium and inflammatory cytokines (Maxwell and Muscat, 2005). Low Nurr1 gene expression and/or mutations were observed in midbrain and lymphocytes from Parkinson's disease patients (Sleiman et al., 2009; Le et al., 2008; Fan et al., 2009). Nurr1 has neuroprotective effects because it inhibits the production of inflammatory mediators in microglia by interaction with CoREST repressor and promotes the bound formation with NF-kB subunit p65 (Bensinger and Tontonoz, 2009; Fan et al., 2009). On the other hand, peripheral Nurr1 levels are increased during inflammation; in fact some authors reported that following lipopolisaccharide and cytokine (i.e. IFNγ) stimulation, Nurr1 is highly expressed in macrophages (Pei et al., 2006; Murphy et al., 2001). Our results show a decrease in Nurr1 mRNA and protein in striatum from adult rats treated in early life with permethrin, while no changes in protein level were observed in hippocampus and prefrontal cortex. The decrease in striatum Nurr1 gene and protein expression was previously found in patients with Parkinson's disease (Kaoru et al., 2009). This outcome together with the increase in lipid hydroperoxide content measured in striatum, confirms a neurodegenerative process that has occurred in this area in 300 day-old rats. Since these findings characterize Parkinson's disease, we could hypothesize that early life permethrin treatment could induce a Parkinson-like neurodegeneration. This hypothesis is supported by previous experimental evidence, such as increased dopamine turnover and protein oxidation, DNA damage, decrease in GSH content and inhibition of mitochondria complex I measured in striatum (Nasuti et al., 2007; Falcioni et al., 2010). Since Nurr1 plays a relevant role in the development of dopaminergic neurons during early life, corresponding to pesticide treatment in our model, the hypothesis that permethrin induces striatum damage through epigenetic mechanisms may be advanced. Decreased Nurr1 in striatum can reflect an impairment of the dopaminergic system which is correlated with lower calcium and NO levels observed in the same area of treated rats. NO is formed following activation of glutamate receptors, and after this activation, calcium increases in the cytosol and forms a complex with calmodulin that activates neuronal nitric oxide synthase (NOS). Since calcium levels are low in treated rats, NOS is probably less activated with respect to the control

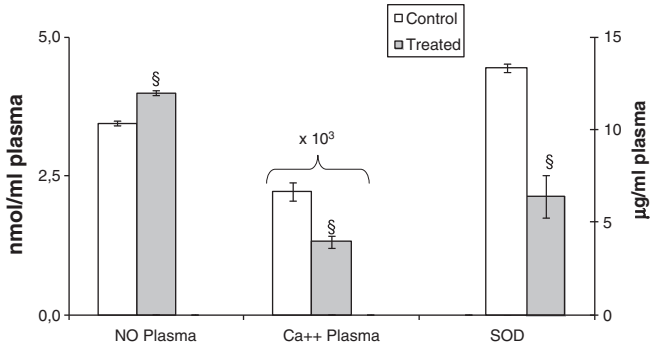

Fig. 5. NO, Ca^{++} and SOD plasma levels from control and treated rat groups. $P<0.05$ § vs control.

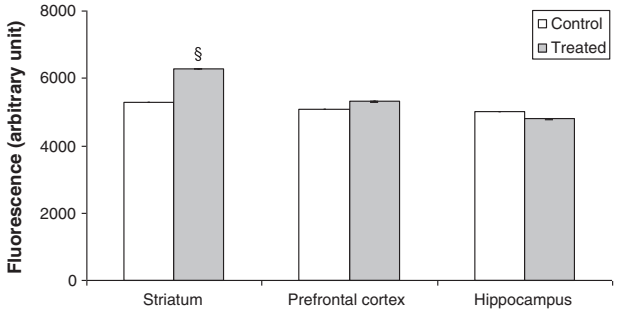

Fig. 7. Lipid hydroperoxide formation on striatum, prefrontal cortex and hippocampus obtained from control and treated rat groups. $P<0.05$ § vs control.

group hence NO levels result lower. The main NO target is soluble guanylyl cyclase which is activated to form cyclic GMP, thus regulating ion channels, and mitochondrial cytochrome c oxidase, which is inhibited, altering the energy metabolism of brain and gene expression (Moncada and Bolanos, 2006). For this reason, the reduced NO levels observed in striatum from treated rats contribute further to neuronal distress induced by lower Nurr1 gene and protein expression. Since glutamate levels resulted unchanged in treated rats, the low calcium level appears unrelated to the lack of activation of glutamatergic receptors, whereas it could be linked to an increased calcium discharge from the organism in agreement with the lower calcium plasma level observed with respect to the control rats. The increased calcium elimination could be a consequence of calcium channel blockage induced by permethrin (Hildebrand et al., 2004). The maintenance of the calcium level in neurons is important to conserve optimal neuronal activity, while calcium homeostasis alterations will affect the neuronal network as observed in neurodegenerative processes (Gleichmann and Mattson, 2011). The NO and calcium decrease in striatum may support the hypothesis that permethrin induces a Parkinson's-like neurodegeneration because a decrease in NOS has been found in post-mortem Parkinsonian brains (Eve et al., 1998; West and Tseng, 2011; Chan et al., 2009). Since the low calcium level is responsible, via N-type ion channels, for the alteration of Nurr1 gene expression, leading to dopaminergic alterations in striatum, it may be considered as a key factor for the development of various neurobehavioral disturbances such as Huntington, Parkinson's disease and obsessive and compulsive disorders, which share many similarities such as striatum damage (Brosenitsch and Katz, 2001; Kovalovsky et al., 2002; Douaud et al., 2009; van den Heuvel et al., 2009).

When the same markers were studied in hippocampus of treated rats, a decrease in glutamate, NO and calcium was observed with unchanged Nurr1 protein expression. Since the glutamatergic system plays a selective role in hippocampal-dependent memory processes, lower glutamate levels suggest a reduced activity of the glutamatergic system that lowers calcium influx into the neuron. Consequently, NO production by NOS is also reduced and certain physiological signals (e.g. neurotransmission) may result inadequate (Steinert et al., 2010). As reported by some authors, elevated glutamate is lethal whereas too low levels are also detrimental for hippocampal neurons, suggesting the maintenance of a glutamate window optimal for their survival. This interpretation is supported by the finding that inhibition of calcium influx blocks the trophic effects of depolarization activating intracellular signaling pathways (Bambrick et al., 1995).

In plasma of early life PERM treated rats, NO levels significantly increased while SOD levels were decreased in adult rats. A decrease in this antioxidant enzyme activity leads to an increase in free superoxide anion which reacts with the increased NO levels to form peroxynitrite. This in turn induces protein nitrotyrosination causing a loss in plasma protein activity (Peluffo and Radi, 2007). These data are in accordance with previous results (Ónody et al., 2003; Lonneke et al., 2006), where increased NO levels are associated with both hypercholesterolemia and hypoalbuminemia measured in the same animal model (Vadhana et al., 2011). These findings, considered together with striatum oxidative stress and higher levels of plasma pro-inflammatory cytokines (Nasuti et al., 2007; Vadhana et al., 2011), support the hypothesis that early life permethrin treatment plays a key role in the onset of neurodegeneration. Further evidence sustaining our findings, is the decrease in antioxidant status which is associated with neurodegeneration and with reduced neurogenesis in the brain (Goshen et al., 2008; Campbell and MacQueen, 2006; Stockmeier et al., 2004; Koo and Duman, 2008).

In conclusion, we show that early life permethrin treatment, at a dose close to the NOAEL (25 mg/kg), has important consequences because it reduces both NO and calcium levels, and Nurr1 gene and protein expression in striatum nucleus in adulthood. At the peripheral level, the decrease in SOD and the increase in NO levels measured in plasma, contribute to impair the redox status. All these changes could be useful to identify the impact of early life pesticide treatment on the development of neurodegeneration. Since the dose of permethrin used in this study is very close to NOAEL used to calculate the human acceptable dose intake (ADI), we can affirm that the exposure to permethrin can represent a real risk factor for the development of neurodegenerative diseases in humans. In order to define the phenotype and characterize the mechanisms related to neurodegeneration, behavioral tests and epigenetic studies are in progress in our animal model.

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