

Permethrin pesticide induces NURR1 up-regulation in dopaminergic cell line: is the pro-oxidant effect involved in toxicant-neuronal damage?

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Running title: Redox regulation of Nurr1 by permethrin

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Abstract

The mechanisms associated to the development of neurodegeneration due to pesticide exposure are not clear yet. In this study we evaluated how permethrin pesticide (PERM) can influence the *Nurr1* gene and protein expression, and if a pro-oxidant activity of the pesticide contributes to up-regulation of *Nurr1* in a dopaminergic cell line. Incubation of PC12 cells with 1 μ M PERM for 72 hours, leads to over expression of *Nurr1* gene. This effect occurs with both corn oil and extra virgin olive oil (EVO) used to solubilize the toxicant. In order to investigate if the *Nurr1* up-regulation induced by PERM, was associated to the pro-oxidant activity of the pesticide, anti-oxidants as glutathione (GSH), tocotrienols (TOC) and Electrolyzed Reduced Water (ERW) were tested. RT-PCR of *Nurr1* showed that its up-regulation was significantly reduced in the presence of antioxidants, especially by addition of ERW. Western-blot analysis reveals that ERW was able to counterbalance the up-regulation of *Nurr1* protein induced by permethrin exposure.

Key words: PC12; permethrin; *Nurr1*; antioxidants; redox state

Introduction

Meta-analysis of idiopathic Parkinson's disease (PD) and pesticide exposure reveal a correlation between pesticide toxicity and the onset of dopaminergic neurodegeneration (Ahmed et al., 2017; Priyadarshi et al., 2000; Van Maele-Fabry et al., 2012). However, the mechanisms related to the neuronal damage are complex and not completely identified, suggesting that more factors are responsible for the development of neurodegeneration. Considering the high prevalence of idiopathic PD (representing the 85-90% of the disease) and that PD also occurs in subjects that have not a family history for the disease, it makes to think that the role of environmental factors in the modulation of genetic and epigenetic responses has to be considered (Ahmed et al., 2017).

Exposure to toxicant chemicals contained as residues in food as well as in drinking water and/or environmental metals like Mg, Cu and Fe, contribute to PD development: people living in rural area are more exposed to environmental stress and finally to neuronal damage (Allen and Levy, 2013; Lopert and Patel, 2016). Furthermore, post-mortem brain tissues from PD patients show inhibition of mitochondrial complex I activity, increased oxidative stress at the level of lipids, proteins and DNA and decrease in antioxidant capacity and glutathione (GSH) levels, considered typical biomarkers of disease (Allen and Levy, 2013; Lopert and Patel, 2016).

68 Studies on animal model have been carried out to investigate the mechanisms associated to
69 pesticide-induced neurodegeneration (Fedeli et al., 2017; Nasuti et al., 2017; Vincenzetti et al.,
70 2016). On this basis, early life exposure to permethrin pesticide (PERM) during brain development
71 revealed that PERM at low dosage is able to induce a progressive Parkinson-like disease in animals
72 (Fedeli et al., 2017; Nasuti et al., 2017; Vincenzetti et al., 2016). Changes in oxidative biomarkers
73 at protein, lipid and DNA level have been observed together with an unbalance of endogenous
74 antioxidant enzymes and low GSH content in treated animals (Gabbianelli et al.2013; Carloni et al.,
75 2013; Gabbianelli et al., 2009; Falcioni et al., 2010). After all, increased dopamine turnover leading
76 to low level of dopamine in midbrain, contributes to reactive oxygen species (ROS) production
77 (Nasuti et al., 2007; Nasuti et al., 2013; Carloni et al., 2012). Furthermore, neonatal toxicant
78 exposure promotes also early up-regulation of *Nurr1* together with compensative α -synuclein over
79 expression. A direct effect of toxicant on *Nurr1* gene regulation could be hypothesized since PERM
80 can cross the blood-brain barrier remaining longer in the brain, and molecular docking identified
81 nine sites of interaction of PERM on *Nurr1* protein (Fedeli et al., 2017; Decressac et al., 2012). In
82 addition, early life exposure to PERM has been associated to intergenerational effects: PERM-
83 exposed parents generate a frequency of 40% of male and 50% of female offspring with the same
84 *Nurr1* overexpression at adolescent age of their exposed parents as well as the same global DNA
85 methylation identified in their early-life treated mothers (Bordoni et al., 2015).

86 In this complex scenario, our study aims to evaluate if the overexpression of *Nurr1*, a transcription
87 factor involved in the regulation of the development and the survival of dopaminergic neurons, is
88 associated with redox system alteration. Understanding the mechanism behind the regulation of
89 *Nurr1* gene due to pesticide could open a way to test new compounds able to counterbalance the
90 damage from early stage of the disease in the animal model of progressive PD (Fedeli et al., 2017;
91 Nasuti et al., 2017; Vincenzetti et al., 2016; Gabbianelli et al.2013; Carloni et al., 2013; Gabbianelli
92 et al., 2009; Falcioni et al., 2010; Nasuti et al., 2007; Nasuti et al., 2013; Carloni et al., 2012; Fedeli
93 et al., 2012). To reach this goal *Nurr1* gene expression was evaluated *in vitro* using a rat
94 dopaminergic cell line, PC12 pheochromocytoma cells. These cells are able to express *Nurr1*, that
95 is preferentially induced by depolarization, but not by nerve growth factor or epidermal growth
96 factor (Liu et al., 2003). Considering that PERM neurotoxicity depends on its capacity to bind
97 specially sodium channels leading to membrane depolarization (Soderlund, 2012), and that also
98 hydroperoxides and increase of oxidized glutathione (GSSG) mediate membrane depolarization
99 (Scott et al., 1987; Tretter and Adam-Vizi, 1996), PC12 cells were used to investigate the
100 mechanisms associated to *Nurr1* overexpression, with a particular focus on the role of redox system
101 alteration in this pathway. To this aim, PC12 cells were treated with PERM, in presence or not of

102 compounds with antioxidant capacity as GSH, tocotrienols (TOC) and Electrolyzed Reduced Water
103 (ERW). Nurr1 up-regulation and free radical production due to PERM toxicity in dopaminergic cell
104 line were analysed in order to investigate dopaminergic pathway and oxidative markers.

105

106 **Materials and Methods**

107 Technical grade (75:25, *trans:cis* 92.4% purity) 3-phenoxybenzyl-(1R,S)-cis,trans-3-(2,2-
108 dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate, PERM, (NRDC 143) was generously donated
109 by Dr. A.Stefanini of ACTIVIA, Milan, Italy. Corn oil, glutathione (GSH), 2,2'-Azino-bis(3-
110 ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxydopamine, deoxycolic
111 acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Sodium Dodecyl Sulphate
112 (SDS), diphenyl-1-pyrenylphosphine (DPPP), NP-40 were purchased from Sigma-Aldrich. L-
113 glutamine was obtained by PAA Laboratories GmbH/Austria. Dulbecco's Modified Essential
114 Medium (DMEM), Fetal Bovine serum (FBS), Penicillin/Streptomycin (P/S) were purchased from
115 Corning.

116 Extra Virgin Olive oil (EVO) from the "Raggia" variety, was produced in 2015 in Montegranaro,
117 (Marche region, Central Italy), and the physicochemical characteristics were the following: acidity
118 0.21% oleic acid, peroxide index 6.2 meq/kg oil, oleic acid:linoleic acid ratio 14.6, α -tocopherol
119 284,3 mg/kg, polar polyphenols 632.7 mg/kg.

120

121 *Cell culture and treatment*

122 PC12 cells, *Rattus norvegicus* dopaminergic neuronal phenotype derived from an adrenal
123 tumour, were used as a paradigm for neurobiological and neurochemical studies. These cells
124 possess all enzymes involved in dopamine synthesis and they represent a simply model of neuronal
125 degeneration treated with toxicants (Grau and Greene, 2012). PC12 cells were seeded at a density of
126 1.5×10^6 in 25cm³ flask, cultured in a humid atmosphere (5% CO₂, 37°C) in DMEM supplemented
127 with 10% Fetal Bovine serum, (FBS), 1% Penicillin/Streptomycin (P/S) and 1% glutamine. PC12
128 cells were incubated 72h with permethrin dissolved in corn oil or extra virgin olive oil (EVO) at the
129 concentration of 1 μ M in order to reach a percentage of oil in the cell medium of 0,1%, and treated
130 with test substances (32nM GSH and 1 μ M tocotrienol extracted from *Elaeisis guineensis* oil
131 Venturani et al., 2004) in sterile distilled water, ERW 100 μ l/ml, and 70 μ M 6-hydroxydopamine (6-
132 OHDA) in the presence or absence of 1 μ M permethrin. Various concentrations and time of
133 incubation (6h, 24h,48h and 72h) were studied in the presence of various concentrations of
134 permethrin (from 0.01 μ M to 10 μ M) to identify the lower amount of toxicant able to provoke an

135 effect of Nurr1 gene express. 1 μ M was chosen as the lower able to modulate Nurr1 gene
136 expression.

137

138 *Generation of electrolyzed reduced water (ERW).*

139 ERW was produced by the continuously electrolyzing apparatus (Chanson revolution 9 plates,
140 Taiwan), wherein tap water was the main water source. ERW was prepared by physical filtering
141 followed by electrolysis and collected in a cell equipped with a cathode platinum-coated titanium
142 electrode. ERW was adjusted to pH 7.4 and oxidative-reduction potential (ORP) of -300 mV
143 corresponding to 400 ppb molecular hydrogen.

144

145 *RNA extraction and RT-PCR*

146 After 72h of incubation, PC12 cells were collected and total RNA was extracted using RNA
147 Isolation kit (NucleoSpin RNA Purification Kit, Macherey Nagel) according to the manufacturer's
148 instructions. RNA quality was checked by spectrophotometric analysis (OD260/280), while its
149 quantity was measured using the OD260 by Nano-Drop spectrophotometer. Reverse Transcription
150 reactions were performed using iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad Inc.,
151 USA) according to the manufacturer's instructions. RT-PCR was employed to evaluate mRNA
152 expression of genes of interest. The following specific primers were designed on the basis of gene
153 and mRNA sequences available online (<http://www.ncbi.nlm.nih.gov/gene>) and purchased from
154 Metabion (Metabion International AG, Germany): b-Actin,
155 TAAAGACCTCTATGCCAACACAGTGC (fw) and AGAGTACTTGCGCTCAGGAGGAG (rv);
156 Nurr-1, GGTTTCTTTAAGCGCACGGTG (fw) and TTCTTTAACCATCCCAACAGCCAG (rv).
157 qPCR analysis was performed in a total volume of 20 μ l containing 50ng of template cDNA, 0.25
158 μ M sense and antisense primers, 10 μ l of iQ SYBR Green Supermix (Bio-Rad Inc., USA) by using
159 a CFX96 Real-time PCR detection system (Biorad Inc.,USA).

160

161 *Protein cell extraction for western blot analysis*

162 After 72h of incubation, cells were collected and lysed using RIPA/SDS buffer (1% NP40, 0.5%
163 Na-deoxycolic acid and 0.1% SDS in phosphate buffer saline) with freshly added protease
164 inhibitors (Fedeli et al., 2012). The lysates were passed several times through a 22-gauge needle and
165 the releasing of the proteins, followed by the ice incubation for 30 minutes, and vortexed every 5
166 minutes. Samples were then centrifuged for 20 minutes at 4°C at 14 000 rpm, and the supernatant
167 containing the proteins was collected. Proteins concentration was determined using the Pierce BCA
168 Protein Assay Kit (Thermo Scientific).

169 *Nurr1 and tyrosine hydroxylase expression by Western Blot analysis*

170 20 µg for tyrosine hydroxylase or 40 µg for Nurr 1 protein samples were loaded on SDS-PAGE
171 10% along with molecular weight markers, with a range between 250 to 4 kDa (Thermo Scientific
172 Spectra Multicolor Broad Range protein Ladder). After the gel electrophoresis, proteins were
173 electro-blotted on nitrocellulose support (PVDF Transfer Membrane 0,45µM, Thermo Scientific).
174 Reactive sites were blocked by incubating the membrane for 1 h at room temperature with 5% non
175 fat dry milk (Santa Cruz Biotechnology Inc, USA) dissolved in PBS. After two washing with PBS-
176 Tween 0.05%, the membrane was incubated overnight at 4°C under agitation with the polyclonal
177 antibody rabbit anti-NURR1 (Santa Cruz Biotechnology Inc, USA) or with the monoclonal
178 antibody mouse anti-tyrosine hydroxylase (TH) (Santa Cruz Biotechnology Inc, USA), diluted in
179 PBS-T + 2,5% not fat dry milk at the concentration of 1:100 or 1:1000 respectively. After
180 incubation, membrane was washed two times with PBS-T and incubated with secondary antibody,
181 anti-rabbit (Bethyl Laboratories Inc, Montgomery TX) or anti-mouse (Santa Cruz Biotechnology
182 Inc, USA) diluted in PBST with 1% not fat dry milk. β-actin (Thermo Fisher) was utilized as a
183 control for equal protein loading. After three wash with PBST and two with PBS, the membranes
184 were developed using the enhanced chemiluminescence (ECL) system. Image capturing and
185 densitometry analysis were performed using the C-Digit Blot Scanner (LI-COR).

186

187 *Phospholipid hydroperoxide analysis*

188 Lipid hydroperoxides were analyzed using the fluorescent probe diphenyl-1-pyrenylphosphine
189 (DPPP) that if oxidized by reacting with hydroperoxides becomes highly fluorescent. One million
190 of PC12 cells treated as previously described, were homogenized with a small potter in PBS and the
191 protein concentration was measured. Samples were normalized to a final concentration of 25µg in
192 100µl of PBS. DPPP (Cayman, Michigan (USA) was solubilized in methanol and then added to cell
193 suspension at a final concentration of 1µM; followed by incubation at 37°C for 5 min in the dark.
194 Fluorescence intensities of the samples were measured on Spectra Max Gemini XPS microplate
195 reader (Molecular Devices, Sunnyvale, CA, USA) using 351 and 380 nm as excitation and emission
196 wavelengths, respectively.

197

198 *Total anti-oxidant capacity assay (TAC)*

199 The TAC test measures the decrease of absorbance of 2,2'-azinobis (3-ethylbenzthiazoline-6-
200 sulphonic acid (ABTS) solution induced by anti-oxidants contained in the samples under
201 investigation (Pellegrini et al., 2001).

19.5 mg of ABTS and 3.3 mg potassium persulphate were dissolved in 7 mL of 0,1M phosphate buffer, pH 7.4 and the solution was stored in the dark for 12h for completion of the reaction. For the assay we prepared a solution of ABTS by diluting 3 ml of ABTS solution in 0,1 M phosphate buffer, pH 7,4 and mixed well in order to measure on the spectrophotometer an absorbance value at 734nm of $0,8 \pm 0,2$. Total anti-oxidant capacity (TAC) was used to measure the eventual antioxidant activity of the PC12 cell medium before and after 72h of incubation with the different compounds. The PC12 cell medium after incubation with the different substances was collected, then centrifuged 15 min at 14000 rpm and the supernatant was used to the test. 20µl of supernatant was added to 2 ml of ABTS solution and the absorbance at 734nm was measured in a Varian Cary 219 Spectrophotometer 3 min after the addition. The decrease of absorbance was referred to a standard curve obtained by using known amount of Trolox. Results were expressed as Trolox Equivalent Antioxidant Capacity (µM)

214

215 *Statistical analysis of data*

The results are expressed as mean \pm SEM. Data were analysed using a one-way ANOVA. When appropriate, post-hoc analysis was carried out using the Newman Keul test. Statistical significance was set at $P < 0.05$.

219

220

221 **Results**

Similarly to what observed in the animal model (data under submission), PERM solubilized in corn oil alters the dopaminergic pathway in the PC12 cells.

Figure 1 shows the effect of permethrin and the well-known neurotoxicant 6-hydroxydopamine (6-OHDA) on *Nurr1* gene expression in PC12 cell line incubated for 72 h. As it can be observed both the 6-OHDA and PERM are able to induce a significant up-regulation of *Nurr1* although with a different scope.

In order to screen the impact of the solvent, the lipophilic PERM was solubilized in two different oils: corn oil and extra virgin olive oil. No differential changes in *Nurr1* gene expression were measured comparing the two solvents (Figure 2),

The treatment of PC12 cells with antioxidant compounds as GSH or TOC or ERW is significantly able to control the up-regulation of *Nurr1* gene induced by PERM (Figure 3A) restoring the same levels of expression than controls. The efficacy of the compounds under study was expressed as percent reduction in *Nurr1* gene expression and results were respectively: ERW>TOC>GSH (Figure 3B).

236 Since ERW resulted from RT-PCR analysis the most efficient between the compounds used,
237 western blotting was performed on PC12 cells treated with PERM or with PERM plus ERW (Figure
238 4). Nurr1 protein expression increased by PERM exposure was down-regulated in the presence of
239 ERW (Figure 5), while not significant changes were observed for TH.

240 With the aim to screen the contribution of pro-oxidant effect of PERM in the modulation of Nurr1,
241 lipid hydroperoxide formation was measured in the PC12 cells incubated with PERM alone or plus
242 ERW. As reported in Table 1A, no significant changes were observed between the different
243 conditions; however, when trolox equivalent antioxidant capacity (TEAC) was measured in the
244 medium collected from the same PC12 cells, a significant decrease in terms of antioxidant capacity
245 was measured in samples co-treated with ERW (Table 1B).

246

247 Discussion

248 PERM is a widely used pest control insecticide that can be found in fruits, vegetables, breast milk
249 and also wood where it is used as anti-worm agent (Li et al., 2016) and the exposure to PERM in
250 humans was demonstrated by the detection of its main metabolite, the 3-phenoxybenzoic acid (3-
251 PBA) in people's urine (Li et al., 2016; Yusa et al., 2015; Saillenfait et al., 2015). Its capacity to
252 lead PD has been well demonstrated in an animal model where cognitive, behavioural, biochemical
253 and molecular biomarkers correlate with PD condition have been validated (Fedeli et al., 2017;
254 Nasuti et al., 2017; Vincenzetti et al., 2016; Gabbianelli et al. 2013; Carloni et al., 2013; Gabbianelli
255 et al., 2009; Falcioni et al., 2010; Nasuti et al., 2007; Nasuti et al., 2013; Carloni et al., 2012; Fedeli
256 et al., 2012). What it was not clear in the PD animal model is if PERM could exert its damage on
257 dopaminergic neuron through an oxidative stress mechanism, and in particular, if *Nurr1* gene could
258 be activated by PERM through free radical involvement.

259 Previous studies on Nurr1 demonstrated that its up-regulation follows to a stress condition that, in
260 dopaminergic neurons of PD model, is compensated by overexpression of α -synuclein that
261 translocates into the nucleus to repress Nurr1 transcription (Devine, 2012; Decressac et al., 2012).
262 This effect observed in PERM-early-life exposed rats (Bordoni et al., 2015), comes behind to an
263 age-related progressive down-regulation of Nurr1 expression (Carloni et al., 2013; Carloni et al.,
264 2012), and it combines with a progressive decrease of dopamine in striatum (Nasuti et al., 2007;
265 Nasuti et al., 2013).

266 The focus on the orphan receptor Nurr1 is due to its key role in the midbrain, where it can modulate
267 a set of genes involved in dopamine metabolism and transport, and where it is able to inhibit pro-
268 inflammatory genes, protecting against neurodegeneration (Lammi et al., 2007; Kwang-Soo, 2017).
269 Its activation is regulated by phosphorylation of ERK, AKT, sumoylation by STAT- γ , and by

dimerization with glucocorticoid or retinoid receptors. In addition, its regulatory activity, as transcription factor, depends to its cytoplasmic/nuclear ratio localization (Lammi et al., 2007; Kwang-Soo, 2017; Jie Dong et al., 2016). In neurons, the oxidative stress can modulate post-translational modifications of Nurr1 influencing its trafficking from cytoplasm to nucleus (Patel et al., 2011). Previous evidences support the role of oxidative stress in the nuclear export of Nurr1, underling that alterations in redox system lead to loss of Nurr1 transcription (Garcia-Yague et al., 2013). Furthermore, the cytosolic accumulation of Nurr1 protein, has been reported in dopaminergic SH-SY5Y cells treated with 6-OHDA (No et al., 2010).

In this scenario, the present study shows that Nurr1 mRNA and protein increase following PERM incubation, as well as in the presence of the 6-OHDA toxicant. Tested bioactive compounds involved in the redox system are able to counterbalance this overexpression. Among antioxidants, GSH was chosen because it has a key role in the redox system, and it is significantly decreased in dopaminergic neurons from PD patients and PD animal model exposed to PERM (Falcioni et al., 2010); on the other hand, the TOC, a mix of tocotrienols, with its lipophilicity works properly to contrast the oxidation in the hydrophobic sites and was already effective to contract PERM damage (Nasuti et al., 2008). With regards to ERW, it was tested because it is characterized by a high concentration of hydrogen molecules, negative oxidation-reduction potential (ORP -300 mV) and ROS scavenging activity in dopaminergic cell lines and in several neurodegenerations (Scirahata et al., 2012; Hamasaki et al., 2008) as well as in the PD animal model developed by PERM early-life exposure (Coman et al., 2014; Nasuti et al., 2016).

Despite the positive effect of antioxidants on Nurr1 gene expression, no changes in hydroperoxide formation in cells or TEAC in growth medium were measured in the presence of PERM. However, the involvement of ROS due to PERM cannot be excluded.

In fact the low concentration of PERM used (1 μ M), could be able to perturb homeostasis leading to the membrane depolarization and/or low level of radicals that were neutralized by antioxidants, even without any detection of oxidation at the lipid hydroperoxide level and any change of the TEAC in the medium from treated cells. Furthermore, the significantly reduced TEAC in the presence of ERW measured with and without PERM, suggests that the stress induced by the low amount of PERM is moderated, making the strong antioxidant ERW (ORP -300 mV) to work as a pro-oxidant in absence of any radicals to scavenge. Being ERW a powerful antioxidant with a short life-time, it can be hypothesised that its excessive presence in the medium leads to a pro-oxidant damage of the proteins contained in the medium where cells were incubated. In support of that, previous studies on rats exposed to PERM together with antioxidants demonstrated similar results when too much antioxidant was used (Gabbianelli et al., 2004).

304 Anyway, given the protective effect exerted by GSH, TCO and ERW on *Nurr1* gene expression
305 (Figure 3), it could be hypothesized that PERM toxicity is due, at least in part, to free radical
306 production in accordance with previous works that demonstrated its ability to induce oxidative
307 stress on DNA, protein and lipids (Wang et al., 2016). Furthermore, free radical production has
308 been associated to membrane depolarization and antioxidants can mitigate it by scavenger activity
309 (Lee et al., 2000). Additionally, previous studies report that membrane depolarization due to radical
310 production can be observed also without a marked lipid peroxidation, like if free radical mediates
311 primary nerve dysfunction without a detectable oxidation (Tretter and Adam-Vizi, 1996). On the
312 other hand, to explain this evidence beyond the direct protection against free radical, PERM could
313 interact with Nurr1 protein in one or more of the nine sites of binding previously identified (Fedeli
314 et al., 2017), and the presence of antioxidants could interfere with this link.
315 Further studies aimed to assess redox status impairment using other techniques as well as
316 investigation in other dopaminergic cellular models could be useful to clarify the molecular
317 mechanisms associated to the PERM damage.

318

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323

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487 **Legend to figures**

488

489 **Figure 1.** Representation of the *in vitro* model of dopaminergic pathway impairment after 72h of
490 PERM exposure: Nurr1 increasing in PC12 cell line. Exposure to PERM induce Nurr1 increasing in
491 PC12 cells, similarly to what happens with 6-OHDA.

492

493 **Figure 2.** Effects of PERM solved in different oils in Nurr1 expression. Damage induced by PERM
494 is not reduced if EVO is used as solved instead of corn oil. Despite its well-known antioxidant
495 properties, EVO have the same effect than corn oil in terms of Nurr1 expression in this model.

496

497 **Figure 3.** Protective effect exerted by GSH, tocotrienols and electrolyzed reduced water in an in
498 vitro model on Nurr1 overexpression induced by PERM exposure of dopaminergic neurons. (A)
499 ERW (B) GSH (C) Tocotrienol (TOC). Representation of protective efficacy as percentage
500 reduction of Nurr1 expression in cells treated with PERM in Corn Oil normalized for control (corn
501 oil) of each experiment (D).

502

503 **Fig. 4** Western blot analysis of Nurr1 from PC12 cell lysates obtained after 72h of incubation with
504 1 μ M PERM dissolved in co and in presence and absence of ERW.

505 # $p < 0.05$ vs naif * $p < 0.05$ vs co+PERM

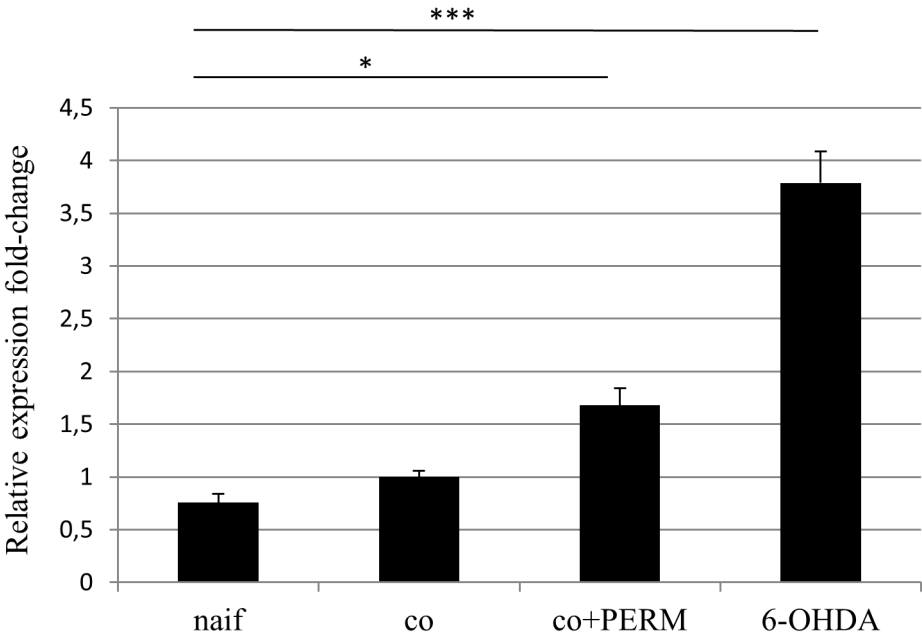
506

507 **Fig. 5.** Western blot analysis of tyrosine hydroxylase from PC12 cell lysates obtained after 72h of
508 incubation with 1 μ M permethrin dissolved in co and in presence and absence of ERW.

509

510

511 **Figure 1**



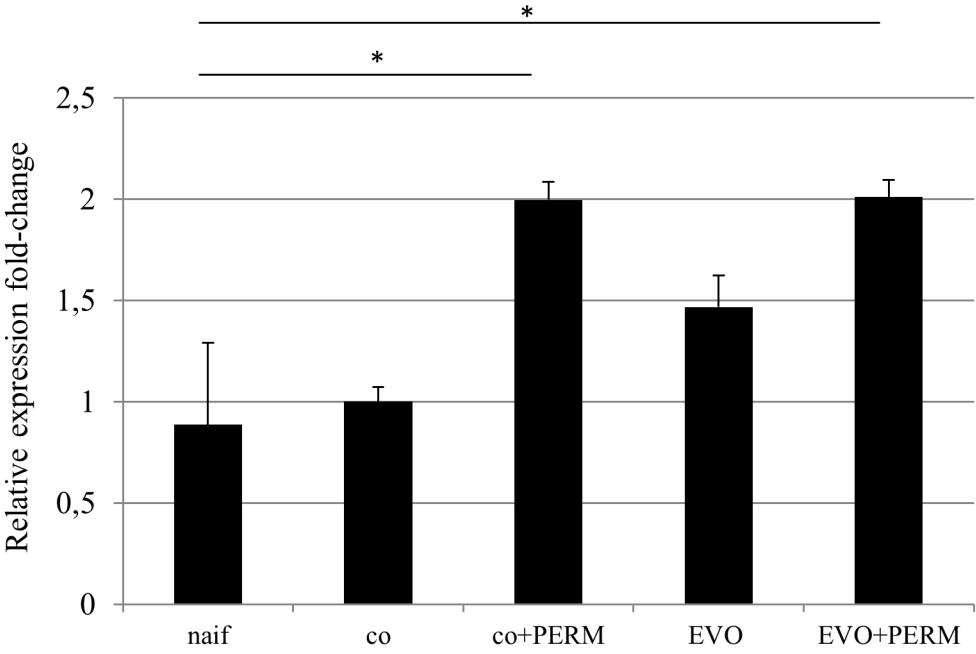
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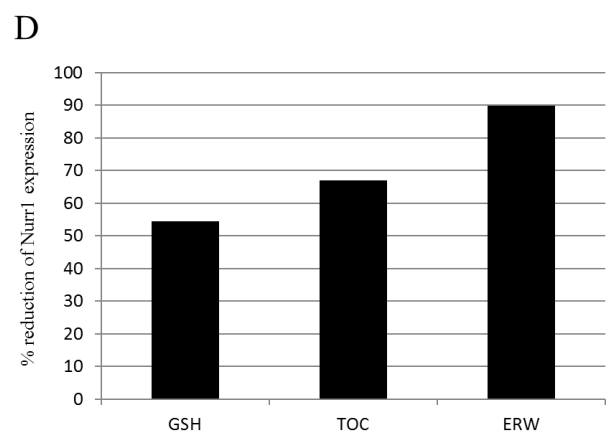
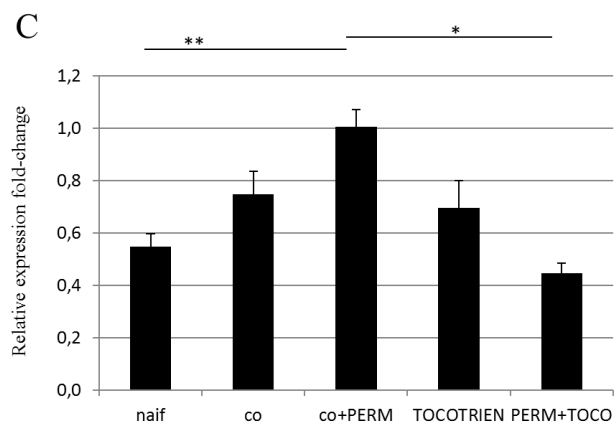
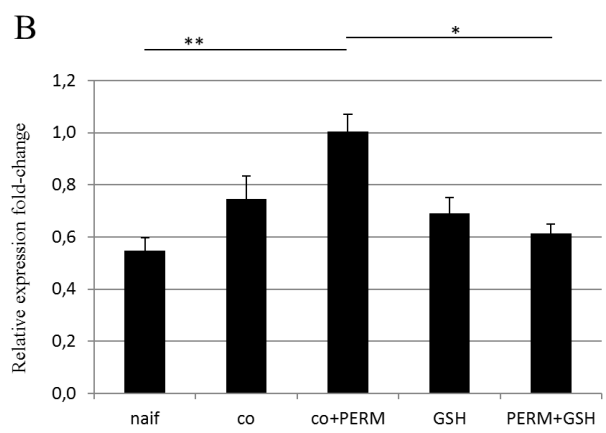
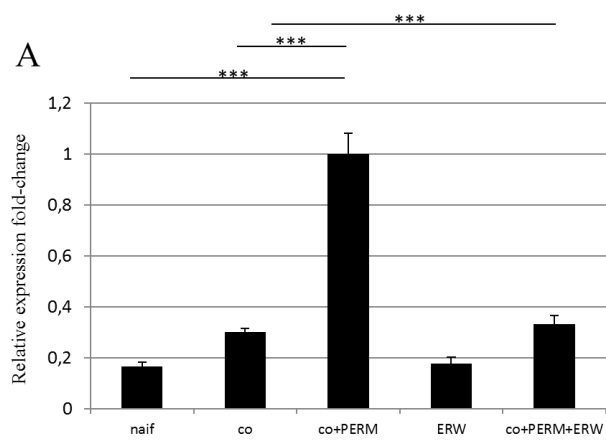
514 **Figure 2**

515

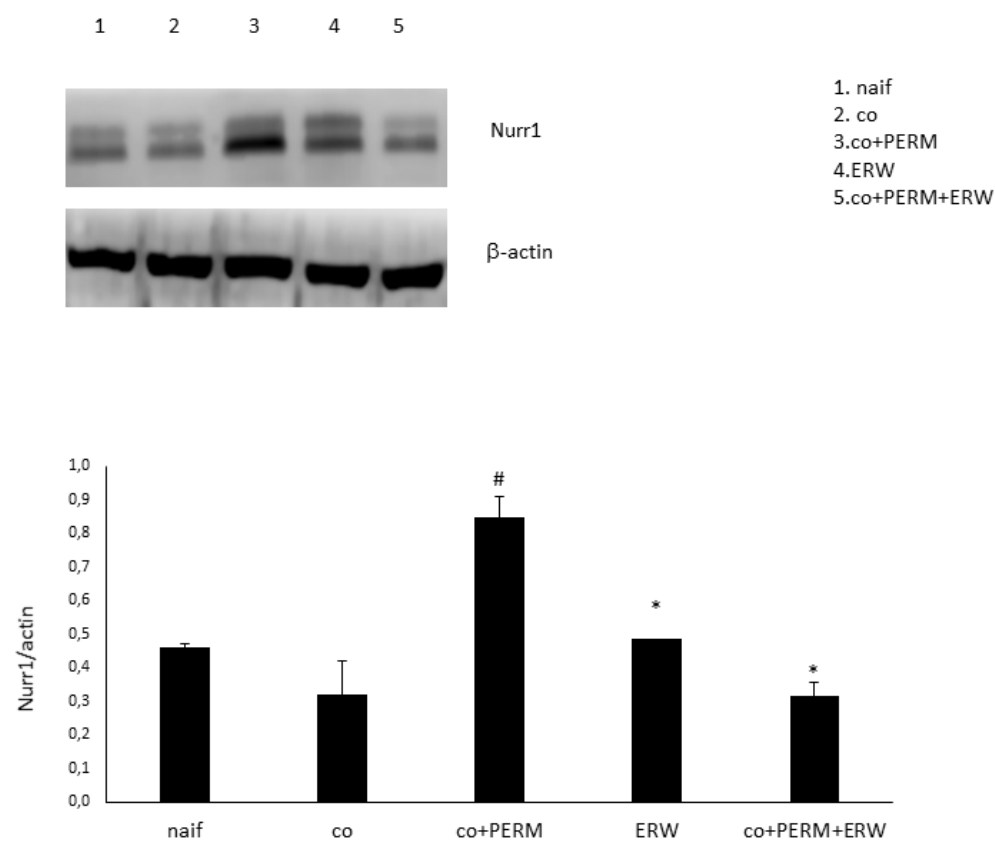
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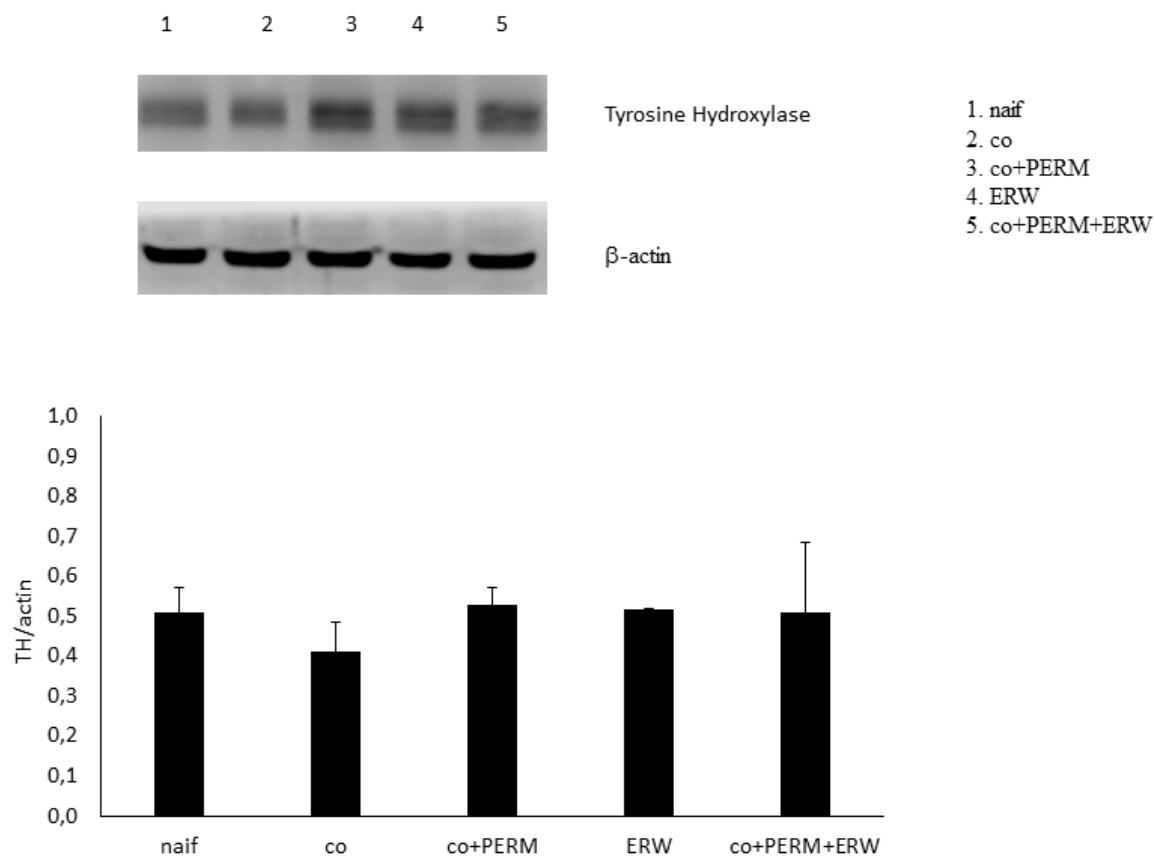


519 **Figure 4**



520
521

522 **Figure 5**



523
524

525 **Table 1**

526 A) Lipid hydroperoxides formation in PC12 cells incubated 72h in presence of ERW or co and 1µM
527 PERM. Data are represented as means ± ES of replicated experiments

528

Sample	Fluorescence Intensity (arbitrary unit) (±ES)	529
naif	2683 ± 68,5	530
co	2557 ± 29,7	531
co+PERM	2680 ± 55,1	
ERW	2290 ± 423	532
co +PERM + ERW	2579 ± 381	533

534

535

536 B) Trolox Equivalent Antioxidant Capacity (TEAC) of PC12 culture medium at 0 h and after 72h
537 incubation in the presence of ERW or co and 1µM Permethrin. Data are represented as means ± ES
538 of at least three replicated experiments. *P<0.05 vs co+PERM, naif, co

539

	TEAC µM Trolox (±ES)	
Sample	0h	72h
naif	10,09±0,92	11,15±0,37
co	10,57±0,80	12,20±0,31
co+PERM	11,19±0,69	13,37±0,18
ERW	11,26±0,121	6,06±0,08*
co +PERM + ERW	12,31±0,45	7,30±0,07*

540