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ARTICLE

# The systemic administration of oleoylethanolamide exerts neuroprotection of the nigrostriatal system in experimental Parkinsonism

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**Abstract**

Oleoylethanolamide (OEA) is an agonist of the peroxisome proliferator-activated receptor $\alpha$ (PPAR$\alpha$) and has been described to exhibit neuroprotective properties when administered locally in animal models of several neurological disorder models, including stroke and Parkinson's disease. However, there is little information regarding the effectiveness of systemic administration of OEA on Parkinson's disease. In the present study, OEA-mediated neuroprotection has been tested on *in vivo* and *in vitro* models of 6-hydroxydopamine (6-OH-DA)-induced degeneration. The *in vivo* model was based on the intrastriatal infusion of the neurotoxin 6-OH-DA, which generates Parkinsonian symptoms. Rats were treated 2 h before and after the 6-OH-DA treatment with systemic OEA (0.5, 1, and 5 mg/kg). The Parkinsonian symptoms were evaluated at 1 and 4 wk after the development of lesions. The functional status of the nigrostriatal system was studied through tyrosine-hydroxylase (TH) and hemeoxygenase-1 (HO-1, oxidation marker) immunostaining as well as by monitoring the synaptophysin content. *In vitro* cell cultures were also treated with OEA and 6-OH-DA. As expected, our results revealed 6-OH-DA induced neurotoxicity and behavioural deficits; however, these alterations were less severe in the animals treated with the highest dose of OEA (5 mg/kg). 6-OH-DA administration significantly reduced the striatal TH-immunoreactivity (ir) density, synaptophysin expression, and the number of nigral TH-ir neurons. Moreover, 6-OH-DA enhanced striatal HO-1 content, which was blocked by OEA (5 mg/kg). *In vitro*, 0.5 and 1 $\mu$M of OEA exerted significant neuroprotection on cultured nigral neurons. These effects were abolished after blocking PPAR$\alpha$ with the selective antagonist GW6471. In conclusion, systemic OEA protects the nigrostriatal circuit from 6-OH-DA-induced neurotoxicity through a PPAR$\alpha$-dependent mechanism.

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**Key words:** Dopaminergic neurons, 6-hydroxydopamine, neuroprotection, Parkinson's disease, PPAR$\alpha$.

## Introduction

Fatty acid acylethanolamides are endogenous lipid modulators generated by the cleavage of the specific membrane phospholipid, *N*-acylphosphatidyl ethanolamide (Piomelli, 2003). These lipids include, among others, oleoylethanolamide (OEA) and palmitylethanolamide (PEA) (Rodriguez de Fonseca et al., 2001; Fu et al., 2003; Lo Verme et al., 2005). Both OEA and PEA are potent endogenous ligands for peroxisome proliferator-activated receptor $\alpha$ (PPAR$\alpha$), a nuclear receptor involved in the transcriptional regulation of lipid metabolism, neuroplasticity and inflammation (Rodriguez de Fonseca et al., 2001; Fu et al., 2003; Lo Verme et al., 2005). Although OEA might act through alternative receptors, including the transient receptor potential vanilloid subtype 1 (TRPV1) (Wang et al., 2005; Thabuis et al., 2008) or the G protein-coupled receptor 119 (GPR119) (Overton et al., 2006), its main actions are mediated through the activation of

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PPARα receptors. Several reports have indicated the physiological relevance of OEA as a modulator of feeding, pain, inflammation, glucose homeostasis and the regulation of metabolism (Rodriguez de Fonseca et al., 2001; Fu et al., 2003; Bordet et al., 2006; Suardiaz et al., 2007; Thabuis et al., 2008; Pavon et al., 2010).

PPARα plays a prominent role in inflammatory and oxidant responses (Peters et al., 1997; Aoyama et al., 1998; Cuzzocrea et al., 2006), and its activation negatively regulates the transcription of inflammatory response genes (Schmidt et al., 1999; Delerive et al., 2001; Lleo et al., 2007; Kono et al., 2009). PPARα ligands, such as synthetic fenofibrates, have been shown to protect against cerebral ischemia by anti-inflammatory and antioxidant mechanisms (Deplanque et al., 2003; Bordet et al., 2006), and these effects are completely absent in PPARα-deficient mice (Deplanque et al., 2003). The synthesis of the endogenous PPARα ligand, OEA, is induced on demand after the local accumulation of stimuli in the brain following an injury, such as cellular stress (Berdyshev et al., 2000; Schabitz et al., 2002; Walter et al., 2002) and tissue damage following ischemia (Schabitz et al., 2002). In fact, the neuroprotective effects of the locally administered agonist OEA and related compounds have been described in animal models of neurological disorders (Lombardi et al., 2007; Sun et al., 2007; Bisogno et al., 2008; Galan-Rodriguez et al., 2009). The therapeutic properties of OEA and its actions on PPARα receptors point to a potential role for this signaling system in neurodegenerative disorders where inflammation and oxidative stress take place (Beltowski et al., 2002; Lo Verme et al., 2005; Suardiaz et al., 2007), such as autoimmune encephalomyelitis (Diab et al., 2004), multiple sclerosis (Racke et al., 2006) and Parkinson's disease (Galan-Rodriguez et al., 2009).

Concerning Parkinson's disease, the *in vivo* retrograde model of nigrostriatal dopaminergic cell degeneration is based on the intrastriatal administration of the neurotoxin 6-hydroxydopamine (6-OH-DA), which leads to an extended and progressive cell death in the substantia nigra (Sauer and Oertel, 1994). Moreover, the insult induces a substantial destruction of nigrostriatal dopaminergic neurons, which is associated with a vigorous inflammatory response (Cicchetti et al., 2002; Na et al., 2010). Interestingly, oxidative stress and neuroinflammation are early events linked to the pathogenesis of Parkinson's disease, a degenerative neurological disorder where the nigrostriatal dopaminergic system is severely damaged ( Jenner and Olanow, 1998; Fernandez-Espejo, 2004). In this sense, PPARα is known to be expressed by dopaminergic neurons of the substantia nigra and the spiny neurons of the dorsal striatum (Kainu et al., 1994; Cullingford et al., 1998; Moreno et al., 2004; Galan-Rodriguez et al., 2009). Because PPARα receptors are involved in oxidative stress and inflammation, which are metabolic situations common to toxic insults induced by neurotoxins (Beltowski et al., 2002; Kreisler et al., 2007), OEA could exert neuroprotective effects against dopamine depletion in the nigrostriatal circuit. OEA might modulate 6-OH-DA-induced neurotoxicity damage of dopaminergic neurons (Ungerstedt, 1968; Schwarting and Huston, 1996) because this neurotoxin acts through the generation of reactive oxygen species (ROS) (Cohen et al., 1976; Cadet et al., 1989; Ben-Shachar et al., 1991). Recently, we have demonstrated that local administration of OEA exerts dose-dependent neuroprotective effects on nigral dopaminergic neurons after intra-striatal injection of 6-OH-DA (Galan-Rodriguez et al., 2009). However, the potentially neuroprotective effects of systemic OEA on the nigrostriatal circuit after 6-OH-DA lesions are still not known. These effects deserve to be studied because systemic treatment is the most widely used route of administration for pharmacological therapies. There is only one report published recently on the neuroprotective actions of OEA administered systemically in a mouse model of brain ischemia (Zhou et al., 2012).

Taking these data into consideration, the main objectives of the present study were the following: (1) to evaluate the neuroprotective effects of systemic OEA treatment in 6-OH-DA-induced lesions with a rat retrograde model of Parkinsonism and (2) to assess the neuroprotective effects of OEA treatment in 6-OH-DA-induced damage with *in vitro* cell culture and the relationship between the neuroprotective effects and PPARα receptors.

## Methods

### *Animals*

The experiments were performed on male Wistar rats (Charles Rivers, Spain) weighing 275–325 g and 12–15 wk old. The animals were housed in a humidity- and temperature-controlled (22 ± 1 °C) vivarium on a 12 h light/dark cycle. Water and standard laboratory food were available *ad libitum*. All experiments were performed in compliance with the European Animal Research Laws (European Communities Council Directives 2010/63/UE, 90/219/CEE, Regulation (EC) no 1946/2003) and Spanish National and Regional Guidelines for Animal Experimentation and use of genetically modified organisms (RD 53/2013, 178/2004, 320/2010, and Ley 32/2007, 9/2003).

### *Drugs*

For *in vivo* studies, OEA was synthesized in our laboratory, as previously described, (Giuffrida et al., 2000) and administered by i.p. injection in a vehicle solution consisting of 10% dimethylsulfoxide (DMSO) in distilled water. OEA was administered at doses of 20 mg/kg for microdialysis and 0.5, 1 and 5 mg/kg for behavioural tests in a volume of 1 ml/kg body weight. It was administered 2 h before and after the administration of 6-OH-DA. To induce striatal lesions, 6-OH-DA (Sigma-Aldrich, USA) was dissolved in 0.2% ascorbic acid in saline. 6-OH-DA

was centrally injected at 5 μg/μl in a volume of 2 μl ($n$ = 25–30 rats per experimental group).

Regarding the *in vitro* studies, OEA was employed in cultures at doses of 30 and 300 nM, and 1.5, 3 and 6 μM dissolved in a vehicle consisting of 10% ethanol in Neurobasal® medium (Invitrogen, USA). 6-OH-DA was used at 40 and 60 μM dissolved in 0.15% ascorbic acid in saline, and the 6-OH-DA concentrations used were 8.22 and 12.34 μg/ml (volume per well = 100 μl). The selective PPARα antagonist GW6471 [(2S)-2-[[(1Z)-1-Methyl-3-oxo-3-[4-(trifluoromethyl)phenyl]-1-propenyl]amino]-3-[4-[2-(5-methyl-2-phenyl-4-oxazolyl)ethoxyphenylpropyl-carbamic acid ethyl ester] (Tocris-Bioscience, UK) was used at 1 μM dissolved in 10% DMSO.

### *In vivo study*

#### *Microdialysis of extracellular OEA*

Seven male Wistar rats were anaesthetized with an isoflurane/oxygen vapour mixture (1.5–2.0%) and placed in a stereotaxic apparatus. A guide cannula (SciPro, USA) was aimed at the dorsal striatum for each animal (Bregma: anterior–posterior, +1.0 mm; medial-lateral, ±2.6 mm; dorsal-ventral, −3.1 mm) (Paxinos and Watson, 2007). Afterwards, the animals received a minimum of 5 d of post-operative recovery before microdialysis.

During the microdialysis experiment, each animal was lightly anaesthetized (1–2% isoflurane), and a microdialysis probe (2 mm polyethyl sulfone membrane, 15 kDa MW cut off; SciPro) was inserted and secured to the guide cannula. The probes were perfused with artificial cerebrospinal fluid (aCSF) delivered at a 0.6 μl/min flow-rate. Approximately 6 h after the probe implantation, the dialysate samples were collected at 20 min intervals over a 60 min baseline period and during a subsequent post-treatment (OEA, 20 mg/kg) period.

#### *Liquid chromatography/mass spectrometry analysis*

The dialysate level of OEA was determined using liquid chromatography coupled with electrospray ionization mass spectrometry (Agilent 1100 LC-MS Single Quadrupole Mass Spectrometer, Agilent, USA). Previously, external calibration curves were constructed from a minimum of three standard chromatographic concentrations prepared in duplicate, daily. For the dialysate samples, 5 μl of the microdialysate aliquots were spiked with 5 μl of 25 nM of S-2 methanandamide as an internal standard. The standards and samples were loaded onto a microbore precolumn using a 10% methanol (v/v) mobile phase delivered at 55 μl/min by a pump. After a washing period, the mobile phase flow through the precolumn was reversed via a switching valve, and the compounds were delivered to a microbore analytical column using another isocratic mobile phase delivered at 9 μl/min. The following mass/charge (m/z) ratios were used: anandamide (AEA), 370.3 (molecular ion (M)+1 Na); 2-arachidonoylglycerol (2-AG) and 1-arachidonoyl-glycerol (1-AG), 401.3 (M+1 Na); palmitoylethanolamide (PEA), 322.3 (M+1 Na); oleoylethanolamide (OEA), 348.3 (M1+Na); methanandamide (S-2), 384.3 (M+1 Na). Under these proposed analytical conditions, the limits of quantitation were approximately 0.1 nM for each analyte. During the analysis of the data, the peak areas of interest were calculated considering the internal control S-2 methanandamide and the obtained ratios. The ratios were related to the concentrations of the standard curve with known concentrations.

#### In vivo *6-OH-DA-induced striatal lesions*

To assess the neuroprotective effects of OEA, we performed the retrograde classic model of experimental Parkinsonism induced by a 6-OH-DA lesion (Ungerstedt, 1968). Thirty minutes before the 6-OH-DA-induced lesions, the rats were injected i.p. with desipramine (15 mg/kg) and citalopram (1 mg/kg; Sigma-Aldrich) to protect the noradrenergic and serotonergic terminals from 6-OH-DA toxicity, respectively. The rats were anaesthetized (ketamine, 50 mg/kg and xylazine, 10 mg/kg, IM, Sigma-Aldrich) and placed in a Kopf stereotaxic apparatus. A solution containing 6-OH-DA (5 μg/μl) was injected (each μl was infused during 150 s) with a blunted 30-gauge cannula into the dorsal striatum. The sterotaxic coordinates of the dorsal striatum relative to Bregma were: (1) (4 μl): anterior–posterior = +1.6, medial–lateral = −2.6, dorsal–ventral = −5.2 mm; (2) (4 μl): anterior–posterior = +0.5, medial–lateral = −3.0, dorsal–ventral = −5.5 mm; (3) (2 μl): anterior–posterior = −1.3, medial–lateral = −4.0, dorsal–ventral = −5.0 mm (Paxinos and Watson, 2007). The cannula was left in place for 1 min to allow diffusion of the solution after each injection.

#### *Behavioural study*

Motor deficits were evaluated 1 and 4 wk after the unilateral striatal lesion or sham operation, according to methodology previously described (Ungerstedt and Arbuthnott, 1970; Schwarting and Huston, 1996; Fernandez-Espejo et al., 1998; El-Banoua et al., 2004; Galan-Rodriguez et al., 2009). The motor tasks included locomotor directional bias, altered spontaneous locomotion (akinesia and bradykinesia), and forelimb asymmetry. Locomotor directional bias was evaluated by quantifying the rotations induced by acute amphetamine (5 mg/kg, i.p.) and apomorphine (0.5 mg/kg, i.p.). The number of ipsiversive turns was quantified over 60 min beginning 30 min after the amphetamine injection, and only those animals who were observed to make more than 420 turns/h were selected. The apomorphine test was carried out the following day, and the number of contralateral turns was quantified over 60 min beginning 5 min after the injection. Spontaneous locomotion (akinesia and bradykinesia) was evaluated in the locomotion cages (45 × 45 cm) for 10 min. Akinesia was measured by

the distance travelled, and bradykinesia was evaluated by the mean speed of movements. The behaviour was videotaped and analysed with a Smart video-tracking system (Panlab, Spain). Forelimb asymmetry was evaluated by the cylinder test (Kirik et al., 2000), where the animal is allowed to move freely in a transparent cylinder (50 × 30 cm) for 10 min, and rearing postures were observed. The number of left and right forepaw contacts was counted, and the data are presented as the percent right forepaw contacts (right forepaw use ratio). Hemi-Parkinsonian rats with a lesion in the left dorsal striatum present a significant impairment in the contralateral (right) forepaw use, which is indicative of forepaw use asymmetry.

*Immunohistochemistry*

Brain mesencephalic sections (40 $\mu$M) were obtained with a cryostat (Micron, USA) and subjected to TH immunostaining ($n$=6 per group). Briefly, all the sections were incubated overnight with monoclonal mouse anti-TH (1:1000; Sigma-Aldrich) in Phosphate Buffered Saline (PBS) with Triton-X100. On the next day, after PBS washing, sections were incubated for 5 h with a biotinylated anti-mouse antibody (1:200; Sigma-Aldrich). Then, the sections were incubated with the Vectastain ABC kit (1:100; Vector, USA) for 2 h, and the specifically bound antibodies were revealed using 3, 3′-diaminobenzidine (Sigma-Aldrich) as the chromogen with 0.01% hydrogen peroxide. Finally, the tissue sections were washed in PBS, mounted on glass slides and coverslipped.

*Density of the striatal TH+ innervations*

The optical density of striatal TH+ innervation was measured at coronal serial sections (8–12 per rat). The images from the sections including both striata were taken with a digital camera from a microscope (Olympus, Japan). The images were analysed using Scion-Image for Windows (freeware version). The mean density of the entire striatum was calculated. The values are also presented as the mean percentage TH+ density in the lesioned striatum *vs.* the contralateral non-lesioned striatum (considered as 100%).

*Stereology*

The methodology for stereological measurement was based on Aguirre and colleagues (Aguirre et al., 1999). The fractionator and optical dissector methods (Gundersen et al., 1988) were used to estimate the total number of TH+ neurons. Stereological measurements were performed using the CAST-Grid software (Olympus, Germany). This software package generated sampling frames with a known area, and directed the motorized X–Y stage (LANG GmbH &Co. KG, Germany) and a MT-12 microcator (Heidenhain Co., Germany) which monitored the movements in the $Z$-axis with a resolution of 0.5 mm. The substantia nigra pars compacta (SNpc) of each 40 $\mu$M section ($\bar{t}$) was delineated using a 4 × objective. The CAST-grid software produced a set of crosses, the Cavalieri or hitting points, which were counted using the appropriate counting criteria (Lagares and Avendaño, 1999). The area associated with each point ($a(p)$) was 23 767.7 $\mu$M$^2$. The estimated volume ($V$) of the SNpc was calculated as follows (Aguirre et al., 1999; Lagares and Avendaño, 1999):

$$V = \Sigma Pi \times a(p) \times \bar{t} \times n$$

where $\Sigma Pi$=total number of hitting points; $n$=number of sections. The estimated number of neurons was counted using a 20 × objective. The system produced a serial of frames, the first one being placed randomly, and the remainder frames were placed following a random systematic arrangement (Sterio, 1984). The frame size was 15% of the observation area, and the sampling covered 20% of the SNpc. The distance between two adjacent sections was $\bar{t}$=160 $\mu$M. The associated area of each frame ($a$(ret)) was 21 391 $\mu$M$^2$. First, the estimated dissector volume ($V$(dis)) was calculated as follows:

$$V(\text{dis}) = a(\text{ret}) \times \text{number of counting frames, } \bar{t}$$

Second, the estimated total number of neurons ($N$) was calculated as follows (Gundersen et al., 1988; Aguirre et al., 1999), according to the following formula:

$$N = \Sigma Q^{-} \times V(\text{ref})/V(\text{dis})$$

where $\Sigma Q^{-}$=total number of cells counted; $V$(ref)=estimate of the volume; $V$(dis)=estimated dissector volume (Gundersen et al., 1988). The coefficient of error (CE) for each estimation and animal ranged from 0.01 to 0.05. The total CE for each group (CE group value) ranged from 0.02 to 0.04. The mean volume of the TH-immunoreactivity (ir) neurons was estimated by means of the point sampled intercept method based on Cavalieri's principle (Gundersen et al., 1988). The estimations of the mean volume are obviously influenced by shrinkage, which was close to 50%, and the volume results were multiplied by 2.

*Protein immunoblotting*

Other groups of Parkinsonian rats ($n$=4 per group) were used for western blotting analysis. These rats were subjected to the same OEA and 6-OH-DA regimen as explained previously and were sacrificed 48 h after the lesion to study the striatal heme-oxygenase 1 (HO-1) and synaptophysin expression. The brains were removed from decapitated rats and cooled in ice-cold physiological buffer. Both dorsal striata were obtained by careful dissection under microscopic observation. The brain samples were lysed, and the protein levels were quantified using the Bradford method (Bradford, 1976). Samples containing 25 $\mu$g of protein each, were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electrophoretically

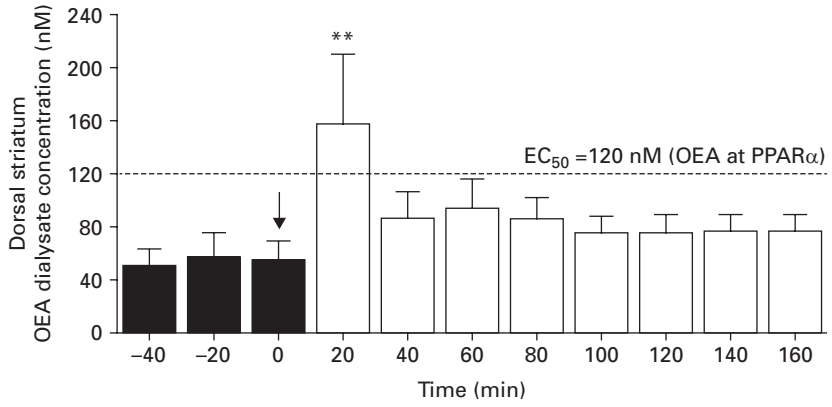


**Fig. 1.** Dorsal striatum OEA dialysate concentration (nM) before and after systemic OEA administration (20 mg/kg, i.p.), as measured by brain microdialysis/HPLC-MS in rats. The bars represent the means±S.E.M. of the OEA dialysate concentrations at 20 min intervals. The black bars are the baseline OEA levels 1 h before the treatment. The white bars are the OEA levels after the OEA treatment. $^{**}p<0.01$ *vs.* baseline OEA concentrations. The black arrow indicates the OEA injection.

transferred to polyvinylidene difluoride (PVDF) membranes. Immunolabeling was conducted with goat polyclonal anti-HO1 (1:500; Santa Cruz, USA), mouse monoclonal anti-synaptohysin (1:1000; GmbH, Germany), and mouse monoclonal anti-$\alpha$-actin antibody (reference protein; Santa Cruz). The primary antibodies were detected with peroxidase-linked secondary antibodies (Santa Cruz) with enhanced chemiluminescence (Amersham-GE, Sweden) and autoradiography. The band densities were quantified using the Scion-Image. The values are given as a percentage band density with respect to the corresponding striatum of rats injected with vehicle (considered as 100%).

### *In vitro study*

#### *Neuron culture*

Primary cultures of substantia nigra neurons were established as previously described by Cardozo (1993), with some modifications (Mena et al., 1997; Burke et al., 1998; Smeyne and Smeyne, 2002). Postnatal pups (PD0) were killed by decapitation, and the brains were removed and placed in cold Hank's balanced salt solution (GIBCO, USA). Under a dissecting microscope, a 0.8–1.0 mm thick coronal section of the mesencephalon was made using a scalpel, and the regions containing the substantia nigra were isolated. The tissue was digested in a solution of papain (20 units/ml; Worthington, USA) with 0.2 mg/ml L-cysteine in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich), 100 mM $CaCl_2$, and 50 mM EDTA for 40 min at 37 °C in an incubation bath. Afterwards, the tissue was mechanically dissociated and centrifuged (1000 r/min) for 5 min. The pellet was collected and layered on top of the inactivation medium. The pellet was resuspended with feeding medium consisting of Neurobasal®-A supplemented with 2% B-27® and 1% Glutamax (Life Technologies, USA), and cell counts were made. The cell suspension was adjusted to 30000 cells per well in a standard 96-well plate.

#### *6-OH-DA/OEA cell culture treatment and lactate dehydrogenase (LDH) assay*

The exposure to 6-OH-DA was initiated after 4–5 d *in vitro*. The medium was removed and changed to Neurobasal® without B-27® for 2 h (Molina-Holgado et al., 2005). OEA (0.5, 1, and 5 $\mu$M in 1% DMSO in Neurobasal®) was added to the neurons before and after the treatment with 6-OH-DA for 2 h. The time and dose are based on Su and colleagues (Su et al., 2006). Freshly prepared, 6-OH-DA (40 and 60 $\mu$M) with 0.15% ascorbic acid in saline was added for 15 min to induce specific death of the dopaminergic neurons (Ding et al., 2004). The medium was removed immediately after 6-OH-DA or 2 h after 6-OH-DA for OEA treatment. Next, the cultures were gently washed, twice, with Neurobasal® and then further incubated for 24 h to perform the lactate dehydrogenase (LDH) assay. If GW6471 was used, it was added to the medium for 15 min just before 6-OH-DA (1 $\mu$M), and OEA was given after the insult. Cytotoxicity was evaluated by the release of the cytosolic enzyme, LDH, into the culture medium by dead and dying cells (Cytotoxicity Detection kit; Roche, Germany). The total LDH release was calculated by incubating untreated cells with 0.5% Triton X-100 for 1 h to induce maximal cell lysis. The basal death was calculated from untreated wells without B-27®. The treatment values were expressed as a percent of the maximal LDH release.

#### *Statistical analyses*

Two-way analysis of variance (ANOVA) was used for analysing behaviour, striatal density, stereological results and cell culture data, followed by the *post-hoc* Newman–Keuls test for comparisons among groups, or Student's *t*-test to compare two groups. With respect to western

blotting, the density of the bands was compared with the non-parametric Kruskal–Wallis test followed by the *post-hoc* Wilcoxon test. In all cases, when the variance was not homogeneous, the data were logarithmically (log($x$)) transformed prior to analysis (Gravetter and Wallnau, 2007).

## Results

### *OEA crosses the blood–brain barrier as revealed by monitoring its interstitial concentration in dorsal striatum after systemic administration*

The first experiment was designed to confirm that OEA injected peripherally can cross the blood–brain barrier. Figure 1 shows the extracellular levels of OEA in the dorsal striatum after a single i.p. injection of OEA (20 mg/kg). Baseline levels of OEA were established for 1 h at 20 min sampling intervals and the concentrations were as follow: 49.69±13.07 nM (60–40 min pre-injection), 57.12±18.34 nM (40–20 min pre-injection) and 54.50±15.20 nM (20–0 min pre-injection) ($N$=7). The peripheral administration of OEA induced an increase in the interstitial levels of OEA obtained by microdialysis. In fact, the maximum peak was reached after the OEA injection: 157.28± 53.66 nM at 20 min post-injection ($p$<0.01). This OEA concentration reached at 20 min post-injection (157.28± 53.66 nM) is within the range reported to produce stimulation of PPAR$\alpha$ receptor-dependent transcription (120 nM) (Fu et al., 2003). These results show that OEA rapidly crosses the blood–brain barrier increasing its extracellular concentrations, although a fast clearance occurs thereafter. Such increase was followed by a rapid clearance (87.08±19.56 nM, at 40 min post-injection). However, the dialysate levels after 60 min remained slightly above the baseline (93.85±21.84 nM at 60 min; 86.09±15.40 nM at 80 min; 76.29±12.05 nM at 100 min; 75.35±14.46 nM at 120 min; 77.30±10.70 nM at 140 min; 76.86±12.01 nM at 160 min post-injection), although these values were not different when compared to the baseline (Bonferroni *post hoc* test). Additionally, we have now provided statistical data by one-way ANOVA showing the stability of the baseline of OEA levels that were evaluated at 60–40, 40–20 and 20–0 min before OEA injection to show no differences in baseline OEA dyalisates ($F_{2,18}$=0.05, $p$=0.9). This kinetics is similar to the observed in plasma after peripheral administration of OEA (Rodriguez de Fonseca et al., 2001).

### *OEA given systemically significantly reduces behavioural Parkinsonian symptoms*

In order to evaluate the potential neuroprotective actions of OEA, we measured the onset of Parkinsonian symptoms in animals treated with either OEA, 6-OH-DA or its combination. Regarding amphetamine-induced rotations, two-way ANOVA revealed a significant interaction effect ($F_{3,75}$=6.5, $p$<0.05). *Post-hoc* analysis revealed that number of rotations were significantly lower in animals treated with 5 mg/kg OEA relative to the other groups, at both time point (1 and 4 wk, $p$<0.05) (Fig. 2(*a*)). Two-way ANOVA also revealed a significant dose effect ($F_{3,75}$= 5.5, $p$<0.05) in apomorphine-induced rotations. But in this case, *post-hoc* analysis revealed that number of rotations were only significantly lower in animals treated with 5 mg/kg OEA relative to the other groups, at 4 wk post-lesion ($p$<0.05) (Fig. 2(*b*)).

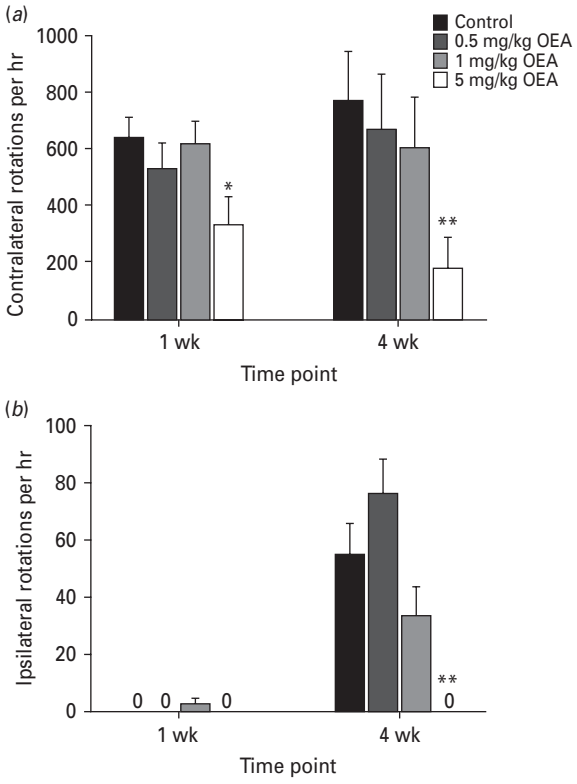


**Fig. 2.** Amphetamine- and apomorphine-induced rotations in rats treated with systemic OEA before or after 6-OH-DA intrastriatal toxic insult. (*a*) Ipsiversive amphetamine-induced rotations were reduced in animals receiving OEA 5 mg/kg, at both time points (1 wk, $p$<0.05; 4 wk, $p$<0.01). (*b*) Contraversive apomorphine-induced rotations were enhanced in each group over time, except for in the animals treated with OEA 5 mg/kg at 4 wk post-lesion ($p$<0.01). Mean ±S.E.M., *$p$<0.05; **$p$<0.01 *vs.* the other animals at the same time point (Newman–Keuls). The data are expressed as the number of rotations/hr.

Altered spontaneous locomotion was evaluated in locomotion cages. Two-way ANOVA revealed significant interaction effects for speed of movement or bradykinesia index ($F_{3,62}$=5.7, $p$<0.05) and distance traveled (akinesia; $F_{3,62}$=5.5, $p$<0.01). *Post-hoc* analysis showed that bradykinesia and akinesia were lower in those animals treated with 5 mg/kg OEA at both 1 and 4 wk after lesion (Fig. 3 (*a*, *b*)). Forelimb asymmetry was measured with the cylinder test. Two-way ANOVA indicated a significant interaction effect ($F_{3,62}$=2.9, $p$<0.05). *Post-hoc* analysis revealed that right forepaw use ratio was significantly enhanced in animals treated with 5 mg/kg OEA relative to the

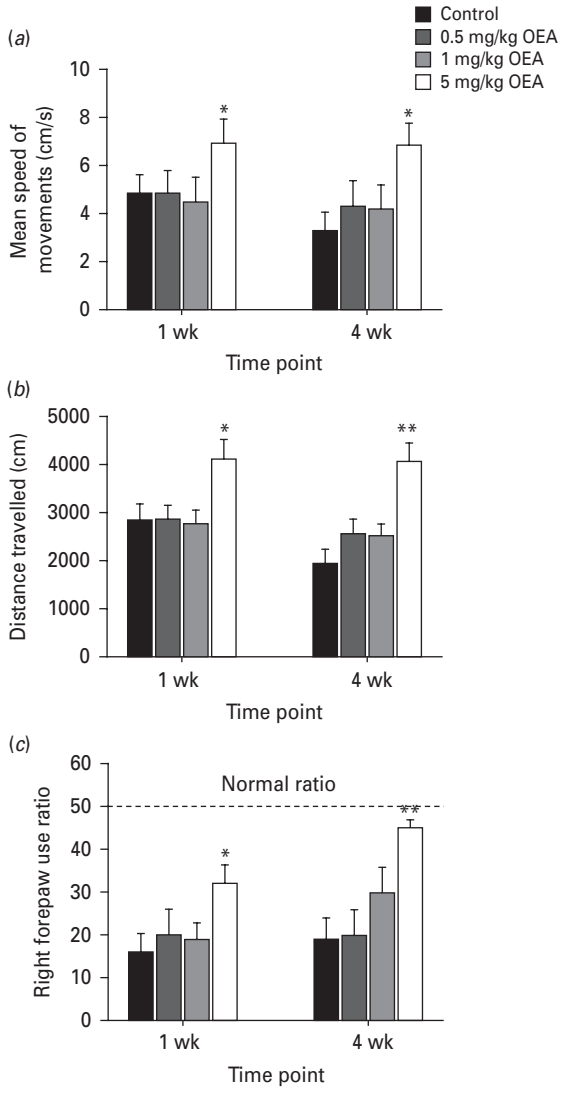


**Fig. 3.** Spontaneous motor tests in rats treated with systemic OEA before or after 6-OH-DA intrastriatal toxic insult. Both, (*a*) Bradykinesia (mean speed of movements (cm/s)) and (*b*) akinesia (distance traveled (cm)) measured in an open field, were significantly attenuated in animals receiving OEA at 5 mg/kg at both time points (1 wk, $p<0.05$; 4 wk, $p<0.05$ and $p<0.01$). (*c*) Forepaw asymmetry (right forepaw use ratio), as measured by the cylinder test, was significantly lower in animals treated with OEA at 5 mg/kg at 1 wk ($p<0.05$) and 4 wk post-lesion ($p<0.01$). Mean±S.E.M., *$p<0.05$; **$p<0.01$ *vs.* the other animals at the same time point (Newman–Keuls).

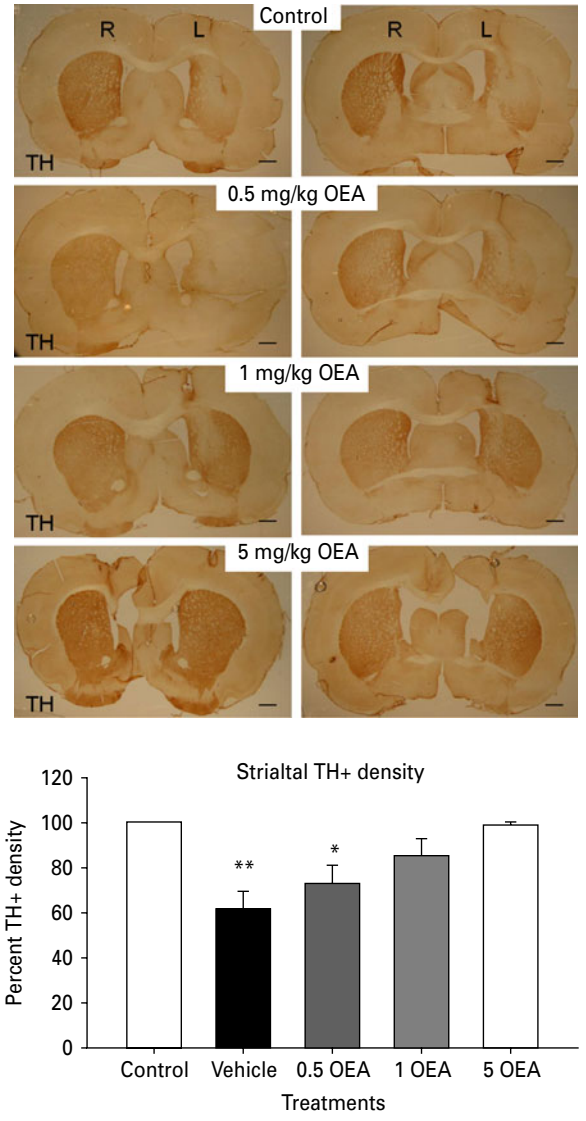


**Fig. 4.** Striatal TH immunostaining density (TH+ density). *Top*: Representative photomicrographs of the striatum at different levels with TH immunostaining. The left striatum (L) was injected with 6-hydroxydopamine, and the animals were treated with systemic vehicle (control) or OEA (0.5, 1 and 5 mg/kg doses). 4 wk later, a strong reduction of TH-positive signal was observed in the vehicle-treated hemi-Parkinsonian animals as well as the animals treated with 0.5 mg/kg OEA, however, this signal was reliably improved after 1 and 5 mg/kg OEA treatment. *Bottom*: Percent TH+ density values of the left striatal tissue relative to the contralateral striatum of the vehicle-treated group (considered as 100%, control). The percent TH density of the whole striatum was reduced in the vehicle-treated and 0.5 mg/kg OEA-treated-Parkinsonian rats at 4 wk after 6-OH-DA infusions relative to the normal contralateral striatum (−45±8%, vehicle-treated, $p<0.01$; −27±8%, 0.5 mg/kg OEA-treated, $p<0.05$), but the percent striatal TH density was not reliably reduced in animals that were treated with 1 mg/kg (−15±8%) or 5 mg/kg OEA (−1±0.2% *vs.* contralateral striatum). Mean±S.E.M., *$p<0.05$, **$p<0.01$ *vs.* contralateral striatum (Wilcoxon test). Bar: 1 mm. Abbreviations: L, left: R, right.

other groups, at 1 ($t=2.9$, $p<0.01$) and 4 wk post-lesion ($t=2.1$, $p<0.05$) (Fig. 3(*c*)).

### *OEA-induced neuroprotection: striatal TH density is strongly reduced after 6-OH-DA infusion, but this reduction is weaker if animals are treated with OEA*

In order to confirm if OEA helps to protect dopaminergic neurons after 6-OH-DA insult, we measured the striatal density of the TH enzyme. ANOVA indicated an interaction treatment × striatum effect on TH density

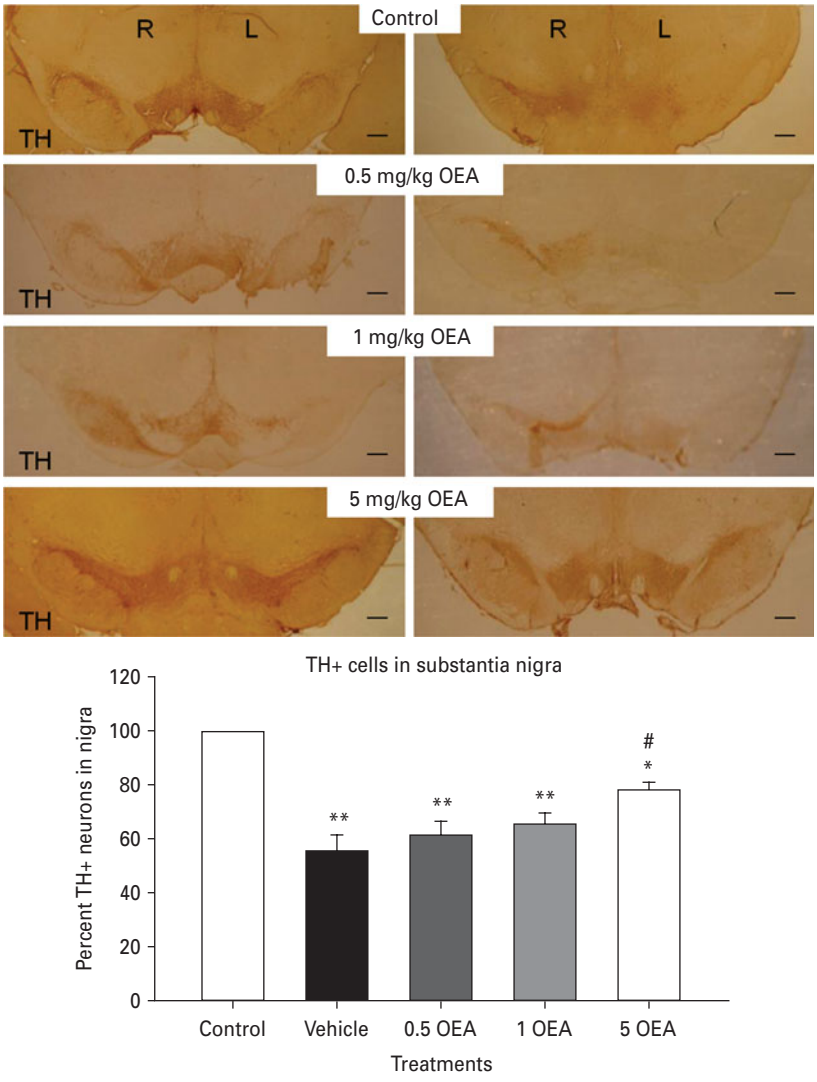


**Fig. 5.** Nigral TH immunostaining density (TH+ density). *Top*: Representative photomicrographs of the substantia nigra at different levels following TH immunostaining. The left striatum was injected with 6-hydroxydopamine, and the animals were treated with systemic vehicle (control) or OEA (0.5, 1 and 5 mg/kg doses). 4 wk later, a strong reduction of TH+-expressing cells is observed in the left substantia nigra of vehicle-treated hemi-Parkinsonian animals as well as the animals treated with 0.5 and 1 mg/kg OEA; however, the number of TH+ neurons was significantly less reduced after administration of 5 mg/kg OEA. *Bottom*: Percent reduction in the number of TH+ cells in the substantia nigra relative to the contralateral substantia nigra of the vehicle group (considered as 100%, control). The percentage reduction of 5 mg/kg OEA-treated rats (−17.2±2%) was significantly lower ($p<0.05$) than that of the remainder of the hemi-Parkinsonian groups (vehicle, −52.1±6%; 0.5 mg/kg OEA, −56.1±6%; 1 mg/kg OEA, −35.5±7%; $p<0.01$). Mean±S.E.M., *$p<0.05$, **$p<0.01$ *vs.* contralateral substantia nigra of controls; #$p<0.05$ *vs.* the other groups treated with OEA or vehicle (Wilcoxon test). Bar: 1 mm. Abbreviations: L, left: R, right.

($F_{4,49}=8.9$, $p<0.01$), as well as a treatment effect ($F_{3,49}=12.4$, $p<0.02$) suggesting a neuroprotective effect. Percent TH density of the whole striatum was found to be reduced in vehicle-treated and 0.5 mg/kg OEA-treated-Parkinsonian rats at 1 and 4 wk after 6-OH-DA infusions relative to normal contralateral striatum (−55±8%, vehicle-treated; −31±8%, 0.5 mg/kg OEA-treated; $p<0.01$), but percent striatal TH density was not reliably reduced if animals were treated with 1 mg/kg (−4±1%) or 5 mg/kg OEA (−1±0.2% *vs.* contralateral striatum), as shown in Fig. 4.

### *OEA-induced neuroprotection: the number of TH+ neurons in the substantia nigra is significantly reduced after intrastriatal 6-OH-DA infusion, and it was less severe following treatment with OEA*

The neuroprotection induced by OEA were also observed when the number of substantia nigra neurons was monitored after 6-OH-DA insult. Two-way ANOVA revealed a significant interaction effect on number of TH+ neurons in the substantia nigra of Parkinsonian groups ($F_{1,19}=78.9$, $p<0.001$). The number of TH+ cells was lower in

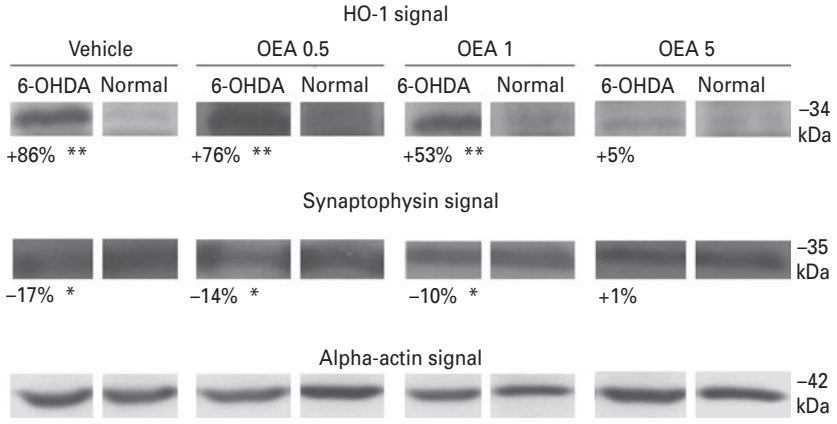


**Fig. 6.** Representative immunoblots showing the expression signal of HO-1, synaptophysin, and control $\alpha$-actin in both striata (lesioned with 6-OH-DA and normal), after the OEA treatments (vehicle and 0.5, 1 and 5 mg/kg OEA). Below each panel, the percent density values relative to the corresponding contralateral striatum (considered as 100%) are shown. Mean±S.E.M., *$p<0.05$, **$p<0.01$ *vs.* vehicle-treated lesioned striatum of hemi-Parkinsonian rats.

all Parkinsonian rats comparing TH+ neurons/mm$^3$ contralateral *vs.* lesioned side: vehicle-treated (6676±1432 *vs.* 3237±1476), 0.5 mg/kg OEA (7643±1083 *vs.* 2968±782), 1 mg/kg OEA (7841±900 *vs.* 4362±1293), and 5 mg/kg OEA (7484±551 *vs.* 5600±644). However, considering corresponding contralateral substantia nigra as control (100%), statistical study revealed that percentage reduction of 5 mg/kg OEA-treated rats was significantly lower (−23±4%, $p<0.05$) than vehicle-, 0.5 and 1 mg/kg OEA-treated groups (vehicle, −44±5%; 0.5 mg/kg OEA, −39±6%; 1 mg/kg OEA, −31±6%) (Fig. 5).

### *OEA improves additional biomarkers of lesion: analysis of HO-1 and synaptophysin*

#### *HO-1 expression is enhanced in the 6-OH-DA-lesioned striatum except following treatment with OEA*

The Kruskal–Wallis test revealed a significant interaction effect on HO-1 expression (H=7.8, $p<0.05$). *Post-hoc* analysis indicated that HO-1 expression was significantly enhanced in all striata of Parkinsonian rats (vehicle, +86±4%; 0.5 mg/kg OEA, +76±9%; 1 mg/kg OEA, +53±13%; $p<0.01$ *vs.* corresponding normal contralateral striatum), and this reduction was significantly lower in 5 mg/kg OEA-treated rats (+5±17% *vs.* contralateral one). Increased HO-1 expression in 5 mg/kg OEA-treated rats was significantly lower than that of the remaining Parkinsonian groups ($p<0.05$), as shown in Fig. 6.

#### *Synaptophysin content is reduced in the 6-OH-DA-lesioned striatum except following treatment with OEA*

Two-way ANOVA revealed a significant interaction effect on synaptophysin expression ($F_{3,14}=6.3$, $p<0.01$). *Post-hoc* analysis indicated that synaptophysin expression was significantly reduced in striata of Parkinsonian rats (vehicle, −17±4%; 0.5 mg/kg OEA, −14±3%; 1 mg/kg OEA, −10±2%; $p<0.05$ *vs.* corresponding contralateral striatum), and this reduction was not observed in 5 mg/kg OEA-treated rats (1±2% *vs.* contralateral one), as shown in Fig. 6.

### *Mechanisms of action: 6-OH-DA-induced death of cultured substantia nigra dopaminergic neurons is attenuated by* in vitro *0.5 and 1 μM OEA and reversed by the PPARα agonist GW6471*

OEA in culture produced neuroprotection against 6-OH-DA-induced insult. Two-way ANOVA revealed a significant interaction OEA×6-OH-DA effect ($F_{6,71}=4.9$, $p<0.05$), as well as OEA ($F_{3,71}=78$, $p<10^{-6}$) and 6-OH-DA effects ($F_{6,71}=99$, $p<10^{-6}$). *Post-hoc* analyses indicated that cell death, as measured through the LDH signal, was significantly lower in three groups: 6-OH-DA 40 μM +OEA 0.5 μM ($t=2.3$, $p<0.05$); 6-OH-DA 40 μM+OEA 1 μM ($t=2.4$, $p<0.05$), and 6-OH-DA 60 μM+OEA 1 μM ($t=3.5$, $p<0.05$), all relative to the corresponding 6-OH-DA alone group (Fig. 7(*a*)). OEA-induced effects followed U-shaped curves, with the highest OEA dose (5 μM) inducing significant cell death in its own right (Fig. 7(*a*)).

In order to test whether OEA actions depend on PPAR$\alpha$ receptor, cell death was tested in the presence of the selective antagonist GW6471. Basal cell death was not affected in culture neurons after pretreatment with GW6471 (Fig. 7(*c*)). Regarding OEA-induced effects, two-way ANOVA did not indicate a significant interaction effect, but showed a dose effect ($F_{3,71}=55$; $p<5\times10^{-5}$). On the other hand, OEA-induced neuroprotection of 6-OH-DA-treated cells was abolished, and 5 μM OEA-induced cytoxicity was not eradicated in these cells as measured through the LDH test.

## Discussion

In the present study, the potentially neuroprotective efficacy of systemically administered OEA in a model

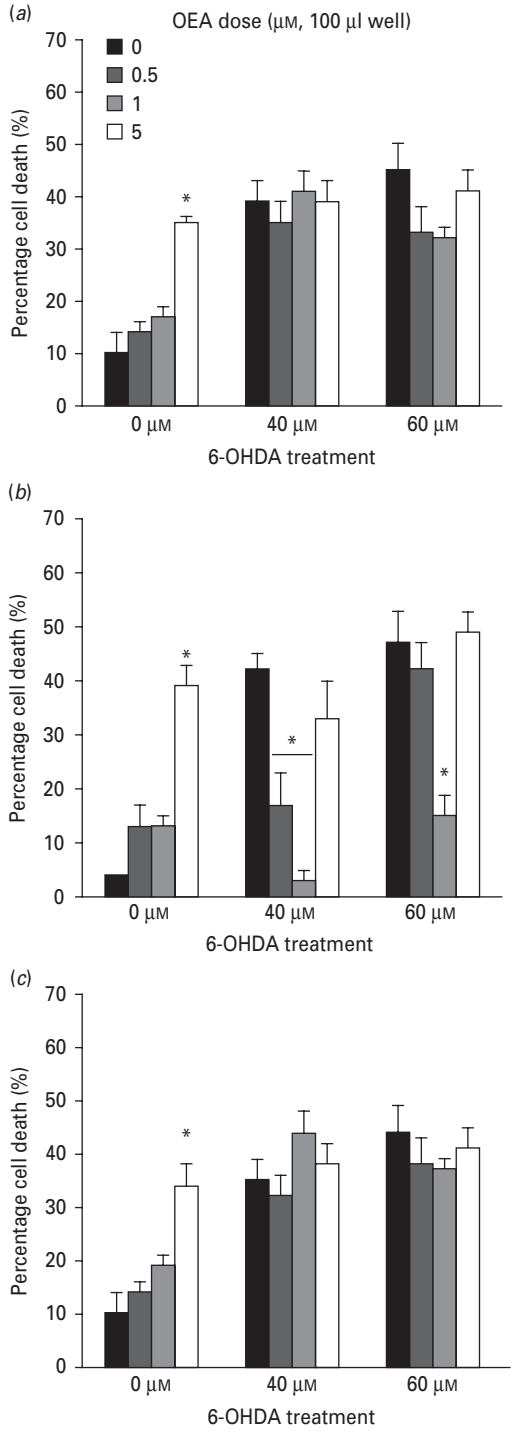


**Fig. 7.** Bars represent the percentage of cell death in cultured neurons of the substantia nigra after different treatments, as evaluated through the LDH test. (*a*) OEA added to cell culture before 6-OH-DA-induced insult at 0.5 and 1 $\mu$M concentrations does not protect cells. (*b*) OEA added to cell culture before 6-OH-DA-induced insult at 0.5 and 1 $\mu$M concentrations protects against 6-OH-DA-induced insult at 0.5 and 1 $\mu$M concentrations on cells treated with 40 or 60 $\mu$M 6-OH-DA ($p<0.05$). The deleterious effect of 5 $\mu$M OEA can also be observed. (*c*) GW6471 treatment (1 $\mu$M) before OEA given after insult was able to block the neuroprotective effects of OEA. Mean±S.E.M., *$p<0.05$ *vs.* groups without OEA (Student's *t*-test).

of neurotoxin-induced Parkinsonism was assessed. OEA administered by i.p. injection can cross the blood brain barrier and reach active concentrations in the basal ganglia (Fig. 1). At this concentration, OEA can target PPAR$\alpha$ receptors and produce anti-inflammatory/antioxidant responses. This finding is relevant because the *in vivo* model selected in the present study was based on the neurotoxin 6-OH-DA, which induces rapid oxidative and inflammatory responses in the striatum (Tatton and Kish, 1997; Mogi et al., 2000; Nagatsu and Sawada, 2005) and progressive cell death in the substantia nigra (Munoz et al., 2005; Rey et al., 2007), leading to overt Parkinsonian symptoms. This model provides a method for better discerning the potentially neuroprotective effects (Sauer and Oertel, 1994; Przedborski et al., 1995).

The present findings revealed that 5 mg/kg OEA administered i.p. was able to reliably reduce behavioural deficits in Parkinsonian rats, including motor asymmetries, forepaw use asymmetry and akinesia/bradykinesia. Doses of 0.5 and 1 $\mu$M (given after 6-OH-DA insult) were able to antagonize the damage in cell culture of substantia nigra neurons. The functional effects are indicative of an effective antagonism to dopamine depletion caused by 6-OH-DA, while the cellular effects seem to be mediated by PPAR$\alpha$. OEA can exert its effects on nigral dopamine neurons because the nuclear receptors, PPAR$\alpha$, are expressed by dopamine neurons of the substantia nigra and the intrinsic neurons of the dorsal striatum (Kainu et al., 1994; Cullingford et al., 1998; Moreno et al., 2004; Galan-Rodriguez et al., 2009).

Systemic 5 mg/kg OEA was able to induce long-term neuroprotection in the nigrostriatal circuit, and the severe reduction of striatal TH observed after 6-OH-DA was reliably attenuated after this dose but not with the other doses. This result confirms that OEA exerts anti-Parkinsonian and neuroprotective effects within a narrow dose range, as observed in our previous work (Galan-Rodriguez et al., 2009), where OEA was locally administered. OEA-induced neuroprotection followed U-shaped dose response curves with beneficial effects at low doses (Galan-Rodriguez et al., 2009) and toxicity due to a high drug concentration at the high dose (Calabrese and Baldwin, 2001). Dopamine cell death in the substantia nigra was clearly reduced because a higher number of dopamine cells survived long-term after the toxic insult following administration of systemic 5 mg/kg OEA. This dose also reduced a short-term oxidative response in the dorsal striatum, as measured through HO-1. HO-1 was employed as a marker for oxidative stress (Munoz et al., 2005), and its expression is strongly induced after oxidant and noxious stimuli (Applegate et al., 1991). Moreover, systemic administration of 5 mg/kg OEA was also effective in antagonizing the 6-OH-DA-induced reduction of the synaptophysin level within the striatum. Synaptophysin is a major integral membrane protein of small presynaptic vesicles and is used as a molecular indicator for synaptic density (Marqueze-Pouey et al.,

1991). 6-OH-DA destroys dopaminergic terminals and reduces synaptic contacts, leading to a significant reduction of synaptophysin. The neuroprotection achieved by 5 mg/kg OEA indicates that the integrity of dopaminergic terminals was maintained after this OEA dose. In this sense, OEA could exert these effects by way of central actions because: (1) OEA crosses the brain blood barrier after peripheral injection (Plaza-Zabala et al., 2010), (2) OEA has been reported to exert neuroprotective actions after intracerebral (striatum) injection (Galan-Rodriguez et al., 2009), (3) PPARα has been extensively reported to be expressed in the brain (Kainu et al., 1994) including mesolimbic circuitries, (4) without discarding other molecular targets of OEA, the activation of PPARα is also known to induce neuroprotective effects (Deplanque et al., 2003) and (5) here, OEA exerts neuroprotection in cultured neurons and this action is prevented with PPARα antagonists.

Regarding the *in vitro* study, OEA protected cultured substantia nigra dopamine neurons from 6-OH-DA-induced toxicity at 0.5 and 1 μM doses. The protective effects also followed U-shaped curves, with the highest OEA dose (5 μM) being ineffective in all cases and even inducing cytotoxicity of cultured neurons. In this context, a U-shaped curve normally suggests a pleiotropic effect that could be due to the existence of two different populations of receptors because this type of curve is well described by two exponential functions (Meuth et al., 2002). In fact, the protective effects disappeared when the cell culture was treated with GW6471, a selective antagonist of PPARα. Hence, the findings can be accounted for by a neuroprotective effect mediated by OEA through PPARα. An alternative explanation is that opposing intracellular pathways are activated by different OEA doses, because biphasic responses to lipid transmitters have been noted in several studies (Okada et al., 1992; Glass and Felder, 1997; Sulcova et al., 1998).

The molecular mechanisms through which OEA exerts dose-dependent neuroprotective effects *in vivo* may well be diverse. OEA actions can mimic the protective and antioxidant actions of PPARα agonists, such as the fibrates (Deplanque et al., 2003). It is known that fenofibrate and bezafibrate exert neuroprotective effects in Parkinsonian mice after MPTP insult (Kreisler et al., 2007), and fenofibrate treatment is known to favourably modulate the oxidant–antioxidant balance (Beltowski et al., 2002). The activation of PPARα induces neuroprotective effects also involving both cerebral and vascular mechanisms (Bordet et al., 2006). Activation of the main family of PPAR receptors (both α and γ isoforms) decreases neuronal death through a vascular protection, and these vascular effects result from a decrease in oxidative stress and prevention of adhesion proteins (Bordet et al., 2006). Apoptosis and inflammation are also regulated by PPARα, whose activation negatively regulates the transcription of inflammatory response genes by antagonizing the AP-1 and nuclear factor-κB signaling pathways (Schmidt et al., 1999; Delerive et al., 2001; Lleo et al., 2007).

In summary, we have demonstrated that systemic administration of OEA, an endogenous PPARα agonist, dose-dependently protects the nigrostriatal circuit from 6-OH-DA-induced toxicity as well as dopamine neurons in culture subjected to oxidative stress. These effects open the possibility of reducing the impact of nigrostriatal neurodegeneration by targeting PPARα receptors with a natural agonist.

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## Statement of interest

None.

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