ARTICLE
Journal of Cellular Biochemistry 116:844–855 (2015)

Journal of Cellular Biochemistry

PPARβ/δ and γ in a Rat Model of Parkinson's Disease: Possible Involvement in PD Symptoms

Roberta Falcone,[1†] Tiziana Marilena Florio,[1†] Erica Di Giacomo,[1] Elisabetta Benedetti,[1] Loredana Cristiano,[1] Andrea Antonosante,[1] Alessia Fidoamore,[1] Mara Massimi,[1] Marcello Alecci,[1,2] Rodolfo Ippoliti,[1] Antonio Giordano,[3,4*] and Annamaria Cimini[1,2,4**]

[1]*Department of Life, Health and Environmental Sciences, University of L'Aquila, Italy*
[2]*Italian National Institute for Nuclear Physics (INFN), L'Aquila, Italy*
[3]*Department of Medicine, Surgery and Neurosciences, University of Siena, Italy*
[4]*Sbarro Institute for Cancer Research and Molecular Medicine and Center for Biotechnology, Temple University, Philadelphia, Pennsylvania*

ABSTRACT

Parkinson's disease is one of the most common neurologic disorder, affecting about 1–4% of persons older than 60 years. Among the proposed mechanisms of PD generation, free radical damage is believed to play a pivotal role in the development and/or progression of the disease. Recently, PPARs, a class of transcription factors involved in several pathways both in physiological and pathological conditions, have been linked by us and others to neurodegeneration. Particularly, PPARγ and its ligands have been indicated as potential therapeutic targets for the treatment of several pathological conditions associated with neuroinflammation within the CNS. The anti-inflammatory function of PPARγ has attracted attention since agonists exert a broad spectrum of protective effects in several animal models of neurological diseases, including psychiatric diseases. On the other hand a detrimental role for PPARβ/δ has been proposed in Alzheimer, being closely related to the decrease of BDNF and Trkfl. On these bases, in this work we used a 6-OHDA hemi-lesioned rat model, inducing loss of dopaminergic neurons, to study the effects of the lesion at three time points from the lesion (1, 2, and 3 weeks), in relevant areas of PD motor symptoms, such as *substantia nigra* and *globus pallidus* and in the area of reward and mood control, the *nucleus accumbens*. In particular, it was studied: (i) the expression of BDNF and its downstream signals; (ii) the modulation of PPARs levels. The results obtained indicate the possible use of a dual PPARβ/δ antagonist/PPARγ agonist to counteract primary and secondary signs of PD neurodegeneration. J. Cell. Biochem. 116: 844–855, 2015.

KEY WORDS: PPARs; NEUROTROPHINS; LIPID PEROXIDATION; MOOD DISORDERS

Parkinson's disease (PD) is one of the most common neurologic disorder, affecting about 1–4% of persons older than 60 years. Motor dysfunctions are assigned as primary symptoms of PD, being all related to events starting on one side of the body. About 60–80% of the dopaminergic neurons are reported to be lost [Chen, 2010]. Dopamine acts as signal between two brain areas, the *substantia nigra* and the *corpus striatum*, to produce smooth and controlled movements. The death of dopaminergic neurons in the *substantia nigra* is the first cause of dopamine decrease. When the levels of dopamine are decreased, the communication between the *substantia nigra* and *corpus striatum* becomes inefficient and the movement start to be impaired [Braak et al., 2004]. Among the proposed mechanisms of PD generation, free radical damage, resulting from dopamine oxidative metabolism, is believed to play a pivotal role in the development and/or progression of the disease [Jenner, 2003]. The oxidative metabolism of dopamine by monoamine oxidase (MAO) leads to the formation of hydrogen peroxide. Hydrogen peroxide is quickly removed by glutathione

[†]These Authors equally contributed to this work.
Grant sponsor: RIA.
*Correspondence to: Antonio Giordano, Department of Medicine, Surgery and Neurosciences, University of Siena, Italy. E-mail: giordano2unisi.it
**Correspondence to: Annamaria Cimini, Department of Life, Health and Environmental Sciences, University of L'Aquila, via Vetoio n. 10, L'Aquila 67100, Italy. E-mail: annamaria.cimini@univaq.it
Manuscript Received: 14 October 2014; Manuscript Accepted: 15 December 2014
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 19 December 2014
DOI 10.1002/jcb.25041 • © 2014 Wiley Periodicals, Inc.

and catalase but, if not adequately cleared, it may result in the formation of highly reactive hydroxyl radicals reacting with cell components such as membrane lipids, resulting in lipid peroxidation and cell damage. Recently, peroxisomes, organelles containing catalase, and peroxisome-related proteins have been linked by us and others to neurodegeneration [Cimini et al., 2009; Kou et al., 2011; Fanelli et al., 2013]. The pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD) and PD has also been described as reduced neurotrophic support. During normal aging and particularly in AD, a decreased ratio between mature Brain-Derived Neurotrophic Factor (BDNF) and its immature form (pro-BDHF) is observed. Pro-BDNF can bind preferentially to pan neurotrophin receptor p75NTR, triggering together with sortilin a death pathway leading to neuronal death (Teng et al., 2005), with a parallel increase of the truncated form of TrkB and decrease of the full length TrkB (TrkBfl) (Tapia-Arancibia et al., 2008). We have previously reported that the peroxisomal proliferator activated receptor β/δ (PPARβ/δ) is involved in the decrease of the TrkBfl, particularly in neurodegeneration [D'Angelo et al., 2009,2011; Cimini et al., 2013a,b]. PPARs are a class of transcription factors involved in the control of several pathways both in physiological and in pathological conditions. In fact, they have been involved in differentiation, energetic metabolism but also in diabetes, carcinogenesis, atherosclerosis, inflammation and, recently, also in neuro-degeneration [Benedetti et al., 2014]. PPARγ is a master regulator of cerebral physiology and potential therapeutic target for the treatment of several pathological conditions associated with neuro-inflammation within CNS. Inflammation within the CNS contributes to many acute and chronic degenerative disorders such as PD and AD [Gonzalez-Scarano and Baltuch, 1999]. Inflammation is also under study for a role in the onset of some psychiatric diseases (i.e., depression, post-traumatic stress disorder [PTSD], schizophrenia) [Hanson & Gottesman, 2005; Dantzer et al., 2008]. The anti-inflammatory function of PPARγ has attracted many attentions since its agonists exert a broad spectrum of protective effects in several animal models of neurological diseases (AD, multiple sclerosis) [Feinstein, 2003]. Similar effects have been also described in animal models of psychiatric diseases: studies have shown that stress enhances the production of 15d-PGJ2 and increases the expression of PPARγ in cerebral cortex as a counterbalancing anti-inflammatory/antioxidant mechanism [García-Bueno et al., 2005a, b].

On the basis of the above considerations in this work we used a hemi-lesioned rat model of PD, where the depletion of dopaminergic neurons was determinated by the unilateral injection of 6-OHDA directly in the *substantia nigra* in the left brain hemisphere, leaving un-lesioned the *substantia nigra* in right brain hemisphere, as internal control. The time-course of the effects of the lesion was followed at three time points from the 6-OHDA injection, (7, 14, and 21 days). In particular, we studied: (i) the expression of BDNF and its downstream signals; and (ii) the modulation of PPARs levels.

The results obtained point towards the possible use of a dual PPARβ/δ antagonist/PPARγ agonist to counteract primary and possibly secondary PD symptoms.

MATERIALS AND METHODS

MATERIALS

Triton X-100, dimethylsulfoxide (DMSO), sodium dodecylsulfate (SDS), Tween20, bovine serum albumine (BSA), l-glutamine, Nonidet P40, sodium deoxycolate, ethylen diamine tetraacetate (EDTA), phenylmethanesulphonylfluoride (PMSF), sodium fluoride, sodium pyrophosphate, orthovanadate, leupeptin, aprotinin, pepstatin, NaCl, polyvinylidene difluoride (PVDF) sheets, rabbit anti P75 NTR, TrkB antibodies, were all purchased from Sigma Chemical Co (St. Louis, CO). rabbit anti-BDNF, rabbit anti-TrkB, rabbit anti-tyrosine hydroxylase antibodies were from St. Cruz Biotechnology (Santa Cruz, CA); horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies were from Vector Laboratories (Burlingame, CA). Micro BCA protein detection kit was from Pierce (Rockford, IL). Vectashield was purchased from Vector Laboratories (Burlingame, CA). All other chemicals were of the highest analytical grade.

Animal experiments were performed in compliance with the European Community Council Directive (86/609/EEC), with the national law 116/95, and under the supervision of the University veterinary service.

Young male Sprague-Dawley rats, weighing 284–381 gr at the starting of the experiment, were used. The animals were housed two per cage and had free access to food and water. The animal room was kept at 21–23°C, under a 12 h/12 h light/dark cycle. Experimental hemi-PD was induced by 6-hydroxydopamine (6-OHDA) injection into the *substantia nigra* on the left hemisphere, while the right hemisphere was left intact and used as inside control. The animals were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and placed in a stereotaxic frame. After exposing the skull, animals received unilateral injection of 6-OHDA (8 μg/4 μL of sterile saline solution containing 0.1% ascorbic acid) via a Hamilton microsyringe, into the *substantia nigra* (SN). Coordinates used for the SN location were AP: 3.7 mm anterior to the interaural line; V: 2.2 mm dorsal to the interaural line; and L: 2.2 mm from the midline according to the Atlas of Paxinos and Watson (1998). 6-OHDA was injected over 5 min, leaving the microsyringe in situ for a further 5 min. Seven days after the injection of 6-OHDA, rats underwent a rotational behavior test induced by the subcutaneous injection of apomorphine (0.5 mg/kg,). Rats were placed in a Plexiglass transparent cilindric box (50 cm diameter), and rotations were counted for all animals. Ipsi-lateral and contro-lateral rotations to the 6-OHDA injected side were counted over a period of 45 min post-injection of apomorphine. Animals, responding to the apomorphine test, were randomly divided into three groups and then, respectively, sacrificed after the first, second and third week from 6-OHDA injection.

TISSUE PREPARATION

After completion of the experiments, respectively one, two, and three weeks after 6-OHDA injection, the animals, under deep chloral hydrate anesthesia (400 mg/kg, i.p.), were sacrificed either for biochemical and morphological studies. For the biochemical processing unfixed brains were rapidly extracted from the skull and rinsed with phosphate saline buffer (PBS) to remove any excess blood. The *substantia nigra*, the *striatum*, the *globus pallidus*, and the

nucleus accumbens areas from each cerebral hemisphere were dissected out under a stereomicroscope. The tissue samples were immediately frozen and stored at −80°C, until use. For the morphological studies, animals were transcardiacally perfused with 50 mL of cold saline containing 0.2 mL of heparin (5000 IU/mL), followed by 250 mL of 4% paraformaldehyde in PBS (pH 7.4). The brains were removed from the skull and post-fixed for 24 h in the same perfusion solution overnight at 4°C. Some brains were taken for Magnetic Resonance Imaging (MRI), as described below. After incubation in a crioprotective 30% sucrose solution, the slabs containing the striatum and the substantia nigra, obtained by a rodent RBM 400 C brain matrix (ASI Instruments, Warren, MI), were cut (30 μm thin coronal sections) using a freezing microtome (Cryomat 1700, Leitz, Wetzlar, Germany).

PROTEINS MEASUREMENT

Proteins were assayed by the micro-BCA kit (Pierce Rockford, IL). Briefly, this assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. The method combines the reduction o Cu^{2+} to Cu^{1+} by protein in alkaline medium (the biuret reaction) with the high sensitive and selective colorimetric detection of the cuprous cation, using a reagent containing BCA. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This complex exhibits a strong absorbance at 562 nm.

WESTERN BLOT ANALYSIS

For Western blotting, tissue were homogenized in ice-cold RIPA buffer (phosphate buffer saline pH 7.4 containing 0.5% sodium deoxycolate, 1% Nonidet P-40, 0.1% SDS, 5 mM EDTA, 100 mM sodium fluoride, 2 mM sodium pyrophosphate, 1 mM PMSF, 2 mM ortovanadate, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL pepstatin), and centrifuged at 13,500*g*. 20–30 μg of proteins were electrophoresed through a 7.5–15% SDS poly-acrilamide gel under reducing condition. Proteins were transferred onto PVDF membrane sheets and nonspecific binding sites were blocked for 1 h at room temperature (RT) in 20 mM TRIS-HCl buffer, 55 mM NaCl and 0.1% Tween 20 pH 7.4 (TBST) containing 5% non-fat dry milk (blocking solution). Membranes were then incubated overnight at 4°C with the primary antibodies: anti-β actin 1:1000 (SIGMA, St. Louis, MO), anti-tyrosine hydroxylase, 1:1000, anti-BDNF 1:200 (Santa Cruz Biotechnology, S.Cruz, CA), anti-Trkb (Santa Cruz Biotechnology, S.Cruz, CA) 1:2000, anti-P75 (SIGMA, St. Louis, MO) 1:200, anti-JNK (Santa Cruz Biotechnology, S.Cruz, CA) 1:200, anti-PPARβ (Novus Biologicals) 1:200, anti-PPARγ (MILLIPORE, CA) 1:200, anti-4HNE (Abcam, Cambridge, UK) 1:1000. Immunostaining was obtained after incubation with secondary anti-rabbit horseradish peroxidase-conjugated IgG (Burlingame, CA) or a secondary anti-mouse horseradish peroxidase-conjugated IgG (Burlingame, CA) 1:1000. Immunoreactive bands visualized by a chemiluminescence detection kit (ECL PLUS Bio-rad). Densitometric analysis on scanned blots was performed using the NIH ImageJ program (NIH, Bethesda, MD) and the relative densities were normalized with respect to β-actin.

IMMUNOCYTOCHEMICAL PROCEDURES FOR TYROSINE HYDROXYLASE

Thin sections (30 μm) were washed three times with 1% PBS and endogenous peroxidase activity was inactivated by incubation for thirty minutes in methanol containing 0.3% H_2O_2. Sections were rinsed, again, with PBS and incubated with normal blocking serum followed by an overnight incubation at 4°C with the primary antibodies anti-TH 1:1000 (Santa Cruz Biotechnology, S.Cruz, CA). TH immunoreactivity was revealed by the biotin-avidin technique (ABC kit, Vector, Vector Laboratories, Burlingame, CA) using 3.3%-diaminobenzidine as the chromogen (peroxidase substrate KIT-DAB, Vector, Vector Laboratories, Burlingame, CA). After DAB staining, sections were rinsed in distillated water for 5 min, dehydrated and mounted with Permount for light microscope examination.

TUNEL ANALYSIS

The tunel reaction mixture (In Situ Cell Death Detection Kit, Fluorescein, Roche) is usually used to detect apoptotic cell death at single cell level via fluorescence microscopy. The reaction is based on labeling of DNA strand breaks. For these experiments, frozen sections have been washed in PBS for thirty minutes; slides were then incubated in permeabilization solution (0.1% sodium citrate in 0.1% triton X-100) for 2 min on ice. Tunel reaction mixture has been added and slides have been then incubated with tunel for one hour at 37°C, in a humidified atmosphere in the dark. Negative controls were performed omitting TdT After rinsing three times for five minutes with PBS, slides were mounted with Vectashield Mounting Medium and observed at florescence microscope AXIOPHOT Zeiss microscope equipped with Leica DFC 350 FX camera. Image acquisition was performed with Leica IM500 program.

MRI

The rat brain fixed in formaldehyde was positioned in a x mL tube and inserted in the 2.35 T Magnetic Resonance Imaging (MRI) scanner (Bruker Biospec, Germany) equipped with a transmit/receive volume birdcage radio frequency coil (Doty Scientific Inc., USA; 8 rungs, diameter 65 mm, length 10 cm) tuned at the proton frequency of 100.3 MHz [Florio et al., 2013]. In this study high-resolution axial gradient echo MR images were acquired with the following conditions: TR = 4500 ms; TE = 46 ms; FOV = 27 mm^2; 512 × 512 pixels; 53 μm resolution; slice thickness = 1 mm; slice number = 25; NEX = 18; TACQ = 11 h 30 min. The MRI images were analyzed with Paravision 4.0 to assess the site of 6-OHDA injection in the *substantia nigra* and the anatomical details between the different areas of the brain, including the ipsi- and contra-lateral *striatum*.

STATISTICAL ANALYSIS

Statistical analysis for Western blotting experiments was performed by *t*-test using SPSS software. For all statistical analyses, $^{*}P < 0.01$ and $^{**}P < 0.001$ were considered as statistically significant.

RESULTS

In Figure 1a, the Western blot analysis for tyrosine hydroxylase (TH) shows a reduction of the protein in the lesioned *substantia nigra*, that

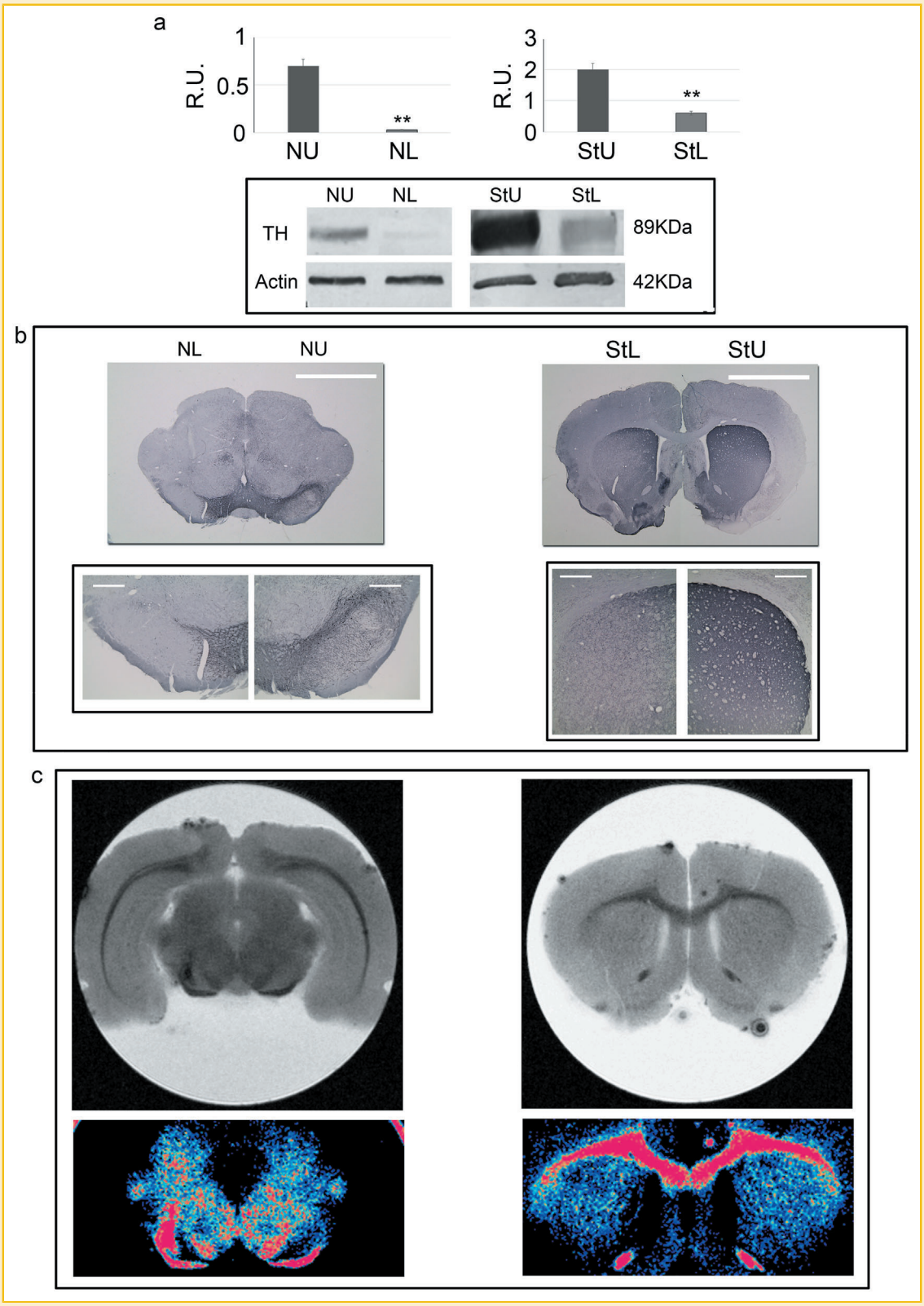

Fig. 1. (a) Western blot analysis for tyrosine hydroxylase (TH) in lesioned (L) and unlesioned (U) *substantia nigra* (N) *and striatum* (St), compared with the normal counterpart. Data are mean ± SEM of three different experiments. $^{**}P < 0.005$, unlesioned versus lesioned. (b) TH immunolocalization in damaged and undamaged *substantia nigra and striatum;* upper images Bar = 3 mm; lower inset images Bar = 500 μm. (c) MRI in lesioned and unlesioned *substantia nigra and striatum,* upper images FOV = 27 mm × 27 mm; lower inset images FOV = 10.4 mm × 4.9 mm.

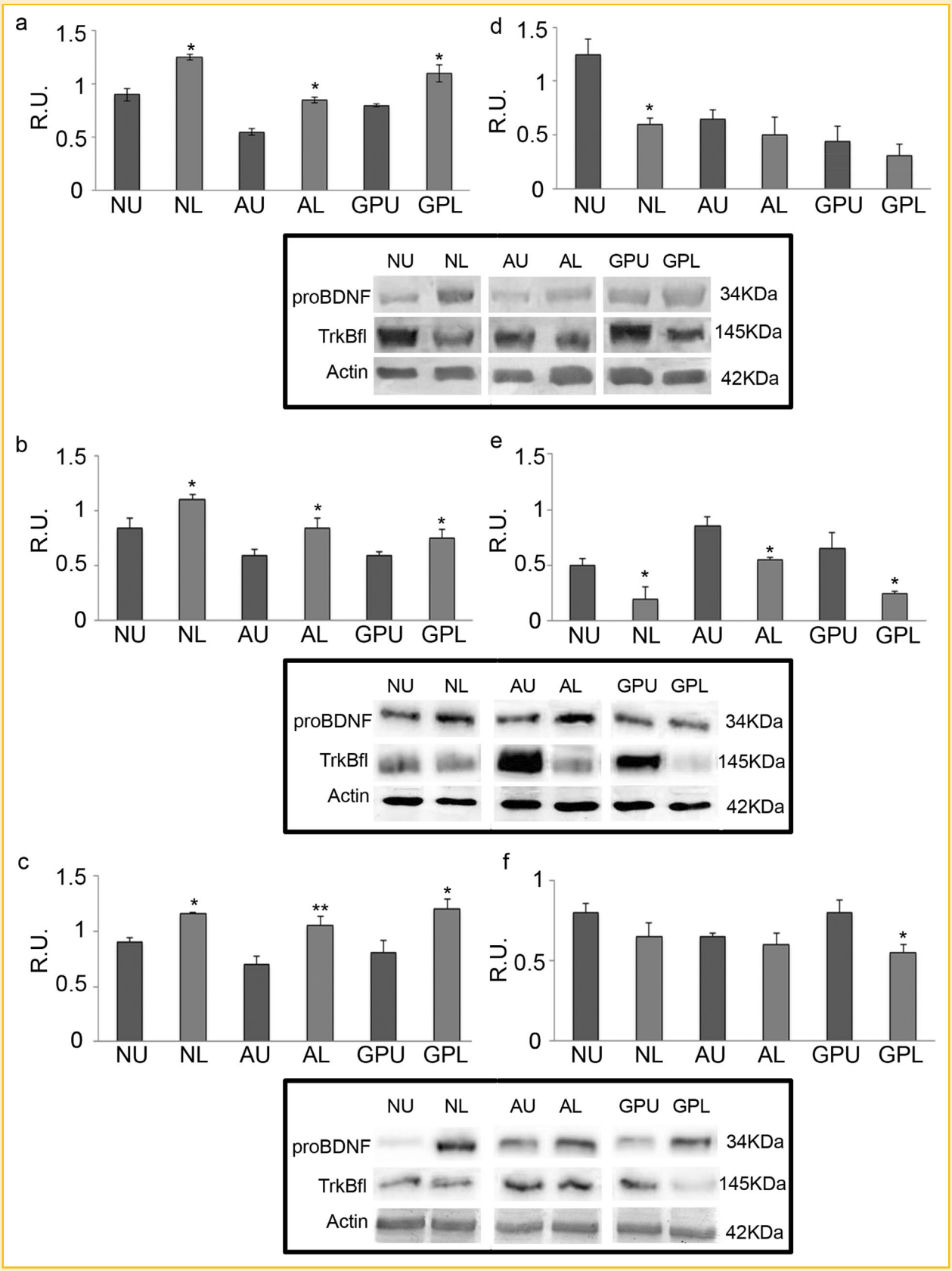

Fig. 2. Western blotting analysis for pro-BDNF (a–c: 1, 2, and 3 weeks, respectively) and its high affinity receptor TrkBfl (d–f: 1, 2, and 3 weeks, respectively) in unlesioned (U) and lesioned (L) areas. N: *substantia nigra*; A: *nucleus accumbens*; GP: *globus pallidus*. Data are mean ± SEM of three different experiments. $^{*}P < 0.05$; $^{**}P < 0.005$.

appears more pronounced than in the damaged *striatum*, compared with the intact counterparts. TH enzyme has been used as control to verify the toxic action of 6-OHDA to dopaminergic neurons. *Striatum* was investigated in order to evidentiate a damage also to the nigro-striatal pathway, which may, in turn, result in an alteration of the *striatum* efferent pathways to the *globus pallidus*. The occurrence of the lesion, in both areas, was further confirmed by immunochemical staining for TH (Fig. 1b) and by MRI examination (Fig. 1c). It is possible to observe a reduced immunoreactivity in the ipsi-lateral *striatum* as well as in the area of lesioned *substantia nigra*, thus demonstrating the loss of DA neurons. The high resolution (53 μm) MRI of the ipsi-lateral *striatum* shows an evident loss of structure, probably due to fiber alteration (Fig. 1c).

In Figure 2 the Western blotting analyses for pro-BDNF and TrkBfl at the different time-points from the lesion are shown. One week after 6-OHDA injection (Fig. 2a), it is possible to observe a significant increase of pro-BDNF in all damaged brain areas considered. This event is followed by a marked decrease of TrkBfl in the *substantia nigra* (Fig. 2d). A similar situation appears in the second week after the lesion (Fig. 2b), with pro-BDNF always significantly up-regulated in the damaged side and TRkBfl always decreased (Fig. 2e). Three weeks after the lesion, (Fig. 2c) pro-BDNF is still significantly up-regulated, while TrkBfl is almost restored to the healthy levels, with the only exception of *globus pallidus* (Fig. 2f).

In Figure 3 the death pathway has been studied by Western blotting analyses for p75 and JNK, at different weeks from the injection of 6-OHDA. One week after the lesion, (Fig. 3a) p75 is increased in the damaged *substantia nigra* and *nucleus accumbens,* although significantly only in the former area, while unaffected in the *globus pallidus*. JNK at the same time-point appears up-regulated only in the *substantia nigra* (Fig. 3d). Two weeks after the lesion (Fig. 3b), p75 is increased in all damaged areas. JNK appears always increased, although significant only in *substantia nigra* (Fig. 3e). Three weeks after the lesion, p75 is almost restored to control value in *globus pallidus*, still up-regulated in the other areas (Fig. 3c). JNK is up-regulated in all damaged brain areas, but significantly only in the *globus pallidus* (Fig. 3f).

In Figure 4 the TUNEL analysis shows the apoptotic cells in the damaged and intact brain areas, at different weeks from the lesion. In Figure 4.1 TUNEL analysis for intact (a–c) and lesioned (d–f) *substantia nigra* is shown. After the lesion, it is possible to observe apoptotic nuclei with chromatin margination in the damaged side (d–f), although at different extent, particularly evident in the second week, in agreement with the increase of p75/JNK, observed at this time point. In Figure 4.2 TUNEL analysis for intact (a–c) and damaged (d–f) *nucleus accumbens* is shown. Two weeks after lesion, a marked apoptosis in damaged side (e), is observed, still present in the third week, although at lower extent (f). In Figure 4.3 TUNEL analysis for intact (a–c) and damaged (d–f) *globus pallidus* is reported. The presence of apoptosis is apparent at any time-points, particularly at three weeks after lesion (f), where a strong apoptosis is maintained in damaged side, in agreement with the persistent increase of JNK, observed at this time-point.

Since the strong involvement of PPARs, particularly PPARγ, in neuro-inflammation and, mainly PPARβ/δ, in the control of oxidative stress, the expression of PPARs was also assessed (Figs. 5 and 6). Our findings show that PPARα does not exhibit significant variations during the time-course considered, consequently only PPARβ/δ and PPARγ are presented, the former together with a marker of lipid peroxidation, 4-hydroxynonenal (4-HNE), a known PPARβ/δ ligand [Coleman et al., 2007]. The product of lipid peroxidation, 4-HNE, is considered one of the main signaling molecules in the pathogenesis of neurodegenerative diseases and it is also known as an intracellular agonist of PPARβ/δ. Since during oxidative challenge, 4-HNE protein adducts were reported to be increased in neurodegeneration [Benedetti et al., 2014], the levels of 4-HNE protein adducts were also assayed by Western blotting in the different brain areas and at the three different time-points.

One week after the lesion (Fig. 5a), PPARβ/δ shows a trend to up-regulation in lesioned *substantia nigra* and in *globus pallidus*, while it is not affected in the *nucleus accumbens*; 4-HNE protein adducts are not affected at this time-point (Fig. 5d). Two weeks after the lesion, PPARβ/δ is significantly increased (Fig 5b), only in the damaged *substantia nigra*, while 4-HNE adducts are significantly up-regulated in damaged *substantia nigra* and *nucleus accumbens* (Fig. 5e). Three weeks after the lesion (Fig. 5c), PPARβ/δ results still increased, significantly only in the damaged *substantia nigra,* while 4-HNE is now significantly increased only in the damaged *globus pallidus* (Fig. 5f). At any time considered (Fig. 6a–c), PPARγ appears strongly down-regulated in all damaged brain areas.

DISCUSSION

PD is a neurodegenerative syndrome characterized by the death of dopaminergic neurons in the *substantia nigra pars compacta* [Samii et al., 2004]. Neurodegenerative diseases [Jenner, 2003; Reale et al., 2012], have oxidative stress as one of the main causes, thus availability of antioxidants may help to antagonize oxidative damage to neurons. Oxidative stress and inflammation are also reported to be causative of PD [Allan and Rothwell, 2003]. The PD animal models, obtained by treatment with 6-OHDA, causing selective destruction of dopaminergic neurons by a mechanism generating reactive oxygen species, [Duty and Jenner, 2011] is a well-recognized model of the disease, evaluated by the characteristic rotational or circling behavior of affected rodents in response to the administration of dopamine-mimetics [Bové and Perier, 2012]. Besides pro-oxidative effects of 6-OHDA, it has also reported that this molecule can induce, during the neuronal development period, architectural changes in the SN due to BDNF deficiency [Li et al., 2013]. Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, supports the survival of existing neurons and maintains the growth and differentiation of new neurons and synapses [Huang and Reichardt, 2001]. Animals born with defects in the production of BDNF will suffer developmental defects in the brain, indicating that BDNF plays a pivotal role in normal neural progression [Hyman et al., 1991]. BDNF is implicated in the survival, proliferation, and differentiation of neural cells [Zhou et al., 2005] and it is known that BDNF-therapy reduces neural degeneration and promotes neuronal repair [Kaplan et al., 2010].

It has been demonstrated that anti-oxidants increase DA production in brain, which in turn up-regulates BDNF synthesis

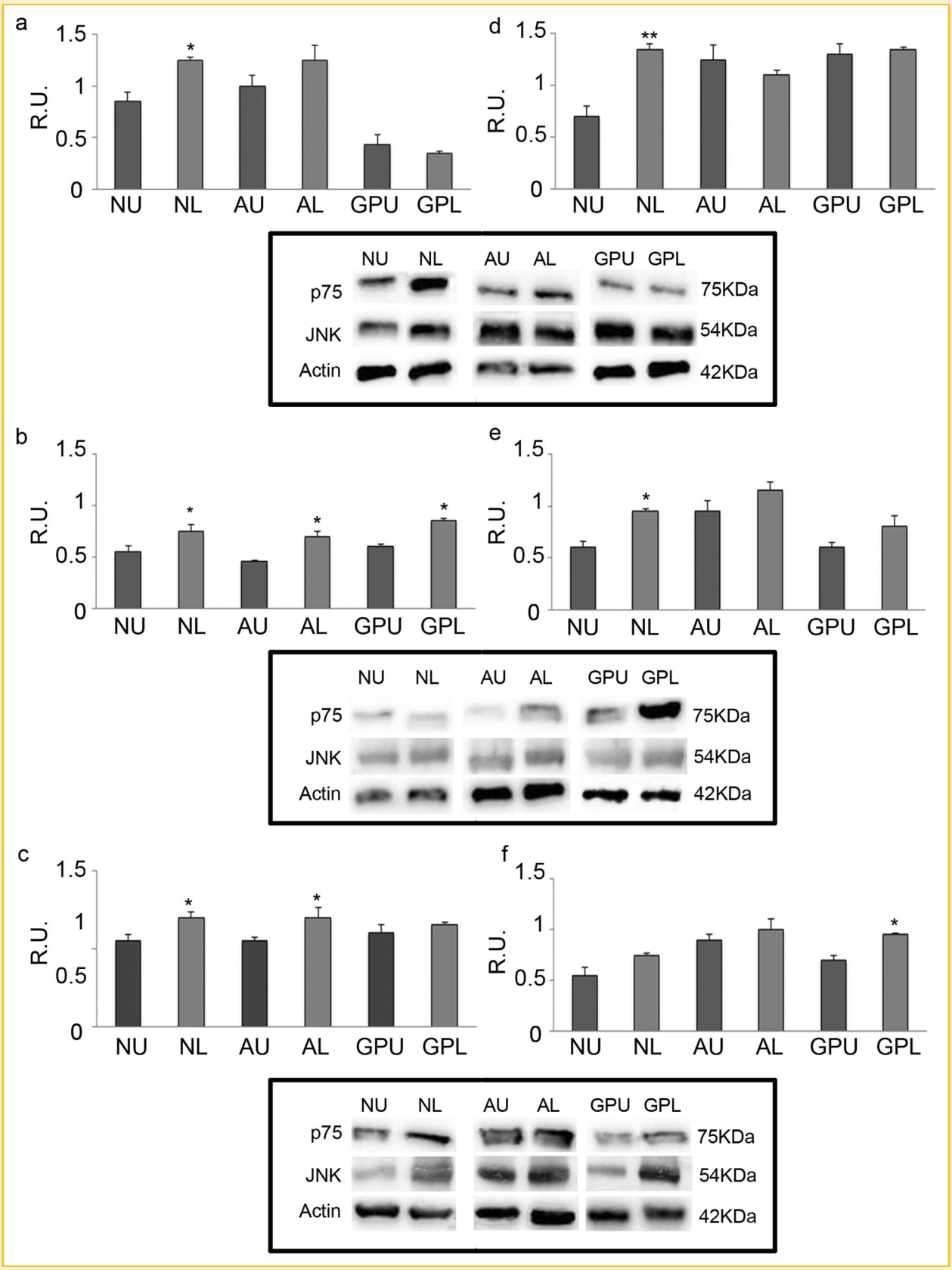

Fig. 3. Western blotting analysis for p75 (a–c: 1, 2, and 3 weeks, respectively) and JNK (d–f: 1, 2, and 3 weeks, respectively) in unlesioned (U) and lesioned (L) areas. N: *substantia nigra*; A: *nucleus accumbens*; GP: *globus pallidus*. Data are mean ± SEM of three different experiments. $^{*}P < 0.05$; $^{**}P < 0.005$.

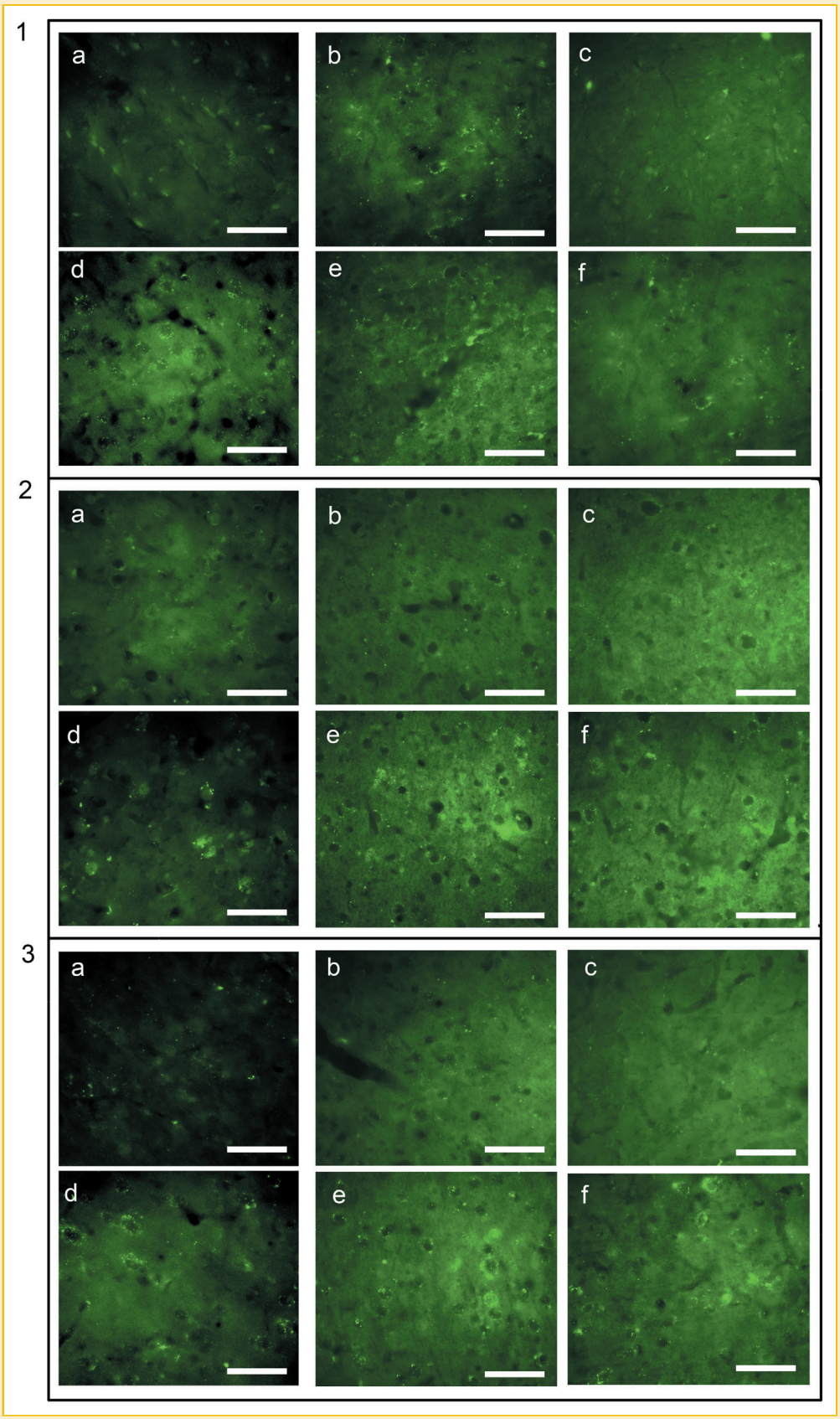

Fig. 4. Tunel assay in intact and lesioned *substantia nigra* (1a–c, unlesioned; d–f, lesioned), *nucleus accumbens* (2a–c, unlesioned; d–f, lesioned) and *globus pallidus* (3a–c, unlesioned; d–f, lesioned) at three different time-points from the 6-OHDA injection (1, 2, and 3 weeks, a–d; b–e and c–f, respectively). Bar = 50 μm.

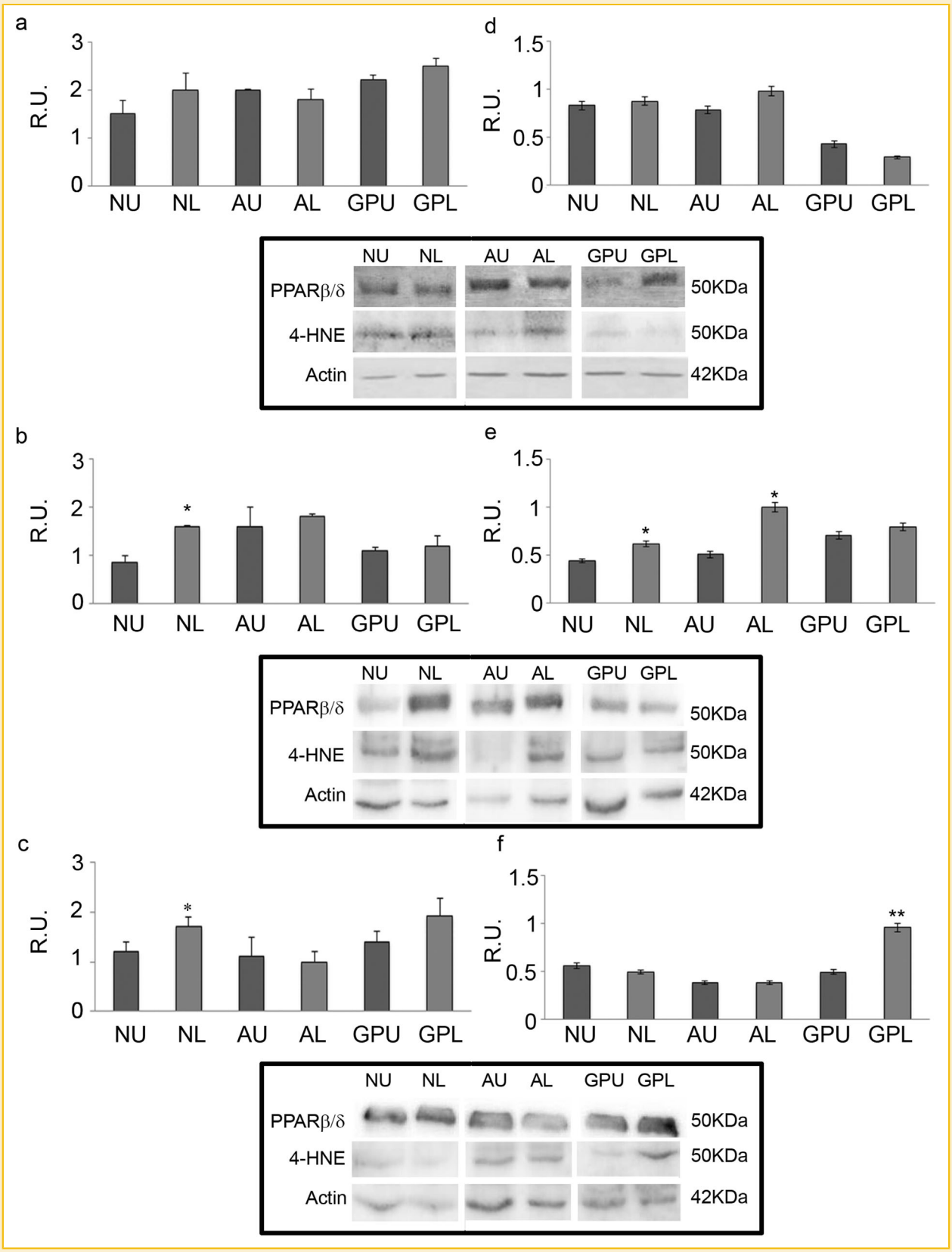

Fig. 5. Western blotting analysis for PPAR**β/δ** (a–c: 1, 2, and 3 weeks, respectively) and 4-HNE (d–f: 1, 2, and 3 weeks, respectively) in unlesioned (U) and lesioned (L) areas. N: *substantia nigra*; A: *nucleus accumbens*; GP: *globus pallidus*. Data are mean ± SEM of three different experiments. $^{*}P < 0.05$; $^{**}P < 0.005$.

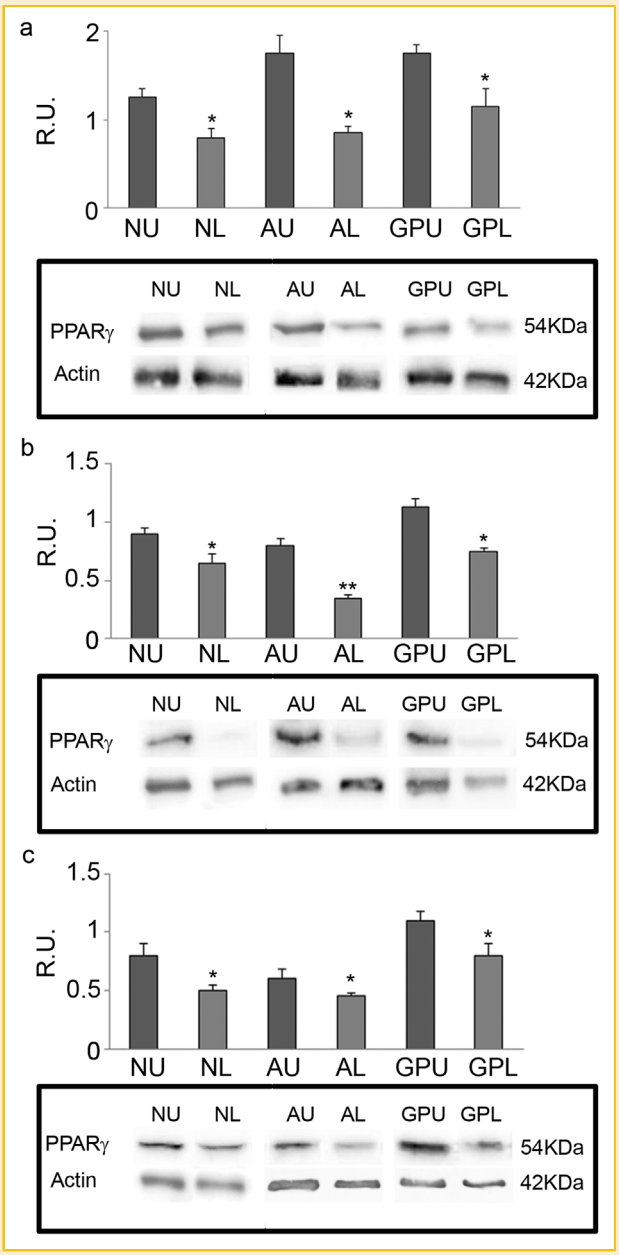

Fig. 6. Western blotting analysis for PPARγ (a–c: 1, 2, and 3 weeks, respectively) in unlesioned (U) and lesioned (L) areas. N: *substantia nigra*; A: *nucleus accumbens*; GP: *globus pallidus*. Data are mean ± SEM of three different experiments. $^{*}P < 0.05$; $^{**}P < 0.005$.

The Ken & Ann Charitable Foundation Trust

and release, thereby promoting the growth of neurons via the TrkB pathways [Massa et al., 2010; Yoshii and Constantine, 2010]. Moreover, the endogenous production of BDNF is responsible for blocking neuronal degeneration in *substantia nigra* tissue.

Our results show that, along the time course of 6-OHDA lesion, an increase of oxidative stress is observed, as highlighted by 4-HNE levels during the second week from the lesion. This event may contribute to the activation of PPARβ/δ, known oxidative stress sensor, leading to TrkBfl decrease and consequently to cell death. PPARβ/δ belongs to PPARs subfamily (PPAR) included in the superfamily of nuclear hormone receptors [Houseknecht et al., 2002; Zolezzi and Inestrosa, 2013; Skerrett et al., 2014]. They act as ligand-dependent transcription factors, binding to DNA in specific regions (PPAR response elements, PPREs) and regulating the expression of genes related to lipid and glucose metabolism, inflammatory processes and cellular differentiation [Kapadia et al., 2008]. Interestingly, PPARs are expressed in the great majority of brain areas [Moreno et al., 2004; Heneka and Landreth, 2007].

Our group has already demonstrated a relationship between pro-BDNF signaling and PPARβ/δ, particularly related to neurogeneration [D′Angelo et al., 2009; Cimini et al., 2013a,b; Benedetti et al., 2014]. In our experimental conditions, it seems that, all brain areas ipsi-lateral to the 6-OHDA injection, show an increase of pro-BDNF paralleled by a decrease of TrkBfl and an increase of p75. A similar finding was as already demonstrated by us in AD models [Benedetti et al., 2014]. These events are accompanied by a general increase of JNK, thus suggesting the activation of a death pathway through pro-BDNF-p75-JNK, as previously described by Teng et al. (2005) and also supported by our TUNEL analysis. The decrease of TrkBfl is concomitant with a general increase of PPARβ/δ, mainly in *substantia nigra*, in agreement with our previous in vitro observations [D′Angelo et al., 2009; Cimini et al., 2013a,b] and with a recent work in the AD mouse model [Benedetti et al., 2014]. It is worth noting that the increase of the nuclear receptor is not a necessary condition for its activation. In fact, the transcription factor may be active even if not increased. In this view, we hypothesize that its activation depends on the availability of its ligand, that is, HNE, that in our experimental conditions, appears significantly up-regulated in all damaged brain areas, although with different timing.

During the preparation of this manuscript, an interesting paper dealing with the possible neuro-protective effect of PPARβ/δ in a similar PD animal model was reported [Das et al., 2014], in contrast with our findings. A possible explanation of this discrepancy may be due to the fact that in the present work we studied the situation of the transcription factors in the PD model without any treatments, while the Authors presented evidences of a neuro-protective effects of the synthetic PPARβ/δ ligand, GW0742, but did not demonstrated the activation of the receptor or if the amount of the ligand in brain may be sufficient to activate the receptor or to compete with natural ligands, such as 4-HNE. We agree with a role for this transcription factor in neuronal maturation, but its behavior may change in relation to cellular environment modification, such as increased 4-HNE levels. Moreover, PPAR-independent effects for GW agonists have been described, as also shown for thiazolidinediones.

PPARγ and its ligands are master regulators of cerebral physiology so that pathological conditions associated with neuro-inflammation can be potentially treated targeting this receptor within CNS [Bright et al., 2008]. The anti-inflammatory functions of PPARγ have received much attention since its agonists exert a broad spectrum of protective effects in several animal models of neurological diseases [Feinstein, 2003].

Similar effects have been also described in animal models of psychiatric diseases: studies have shown that stress enhances the production of 15d-PGJ2, known PPARγ ligand, and increases the expression of PPARγ in cerebral cortex counteracting inflammation

and oxidative stress [García-Bueno et al., 2005a, b]. It has also demonstrated that both synthetic and natural PPARγ ligands prevent inflammatory and oxidative/nitrosative consequences of stress exposure in the cerebral cortex of rats subjected to restraint stress [García-Bueno et al., 2005a, b] All these observations suggested that the PPARγ pathway may act as a mediator of neuro-protection during inflammation/oxidative stress conditions [Galea et al., 2003].

In our experimental conditions, a strong decrease of PPARγ was observed over the entire time course and in any considered damaged brain areas. These data suggest that in addition to oxidative stress increase and activation of cell death pathway, the onset of the disease may be also related to the decrease of this transcription factor, crucial for neuro-protection [Schintu et al., 2009].

Of particular interest it appears our findings, following PD induction, in the *nucleus accumbens*, known to be involved in the mood control and in the reward system. Apathy is a complex, behavioral disorder associated with reduced spontaneous initiation of actions. Although present in mild forms in some healthy people, it is a pathological state in conditions such as AD and PD, where it can have profoundly devastating effects. Interestingly, it has been reported that reward insensitivity is associated with basal ganglia dysfunction and that it might be an important component of apathy observed in neurodegenerative diseases [Adam et al., 2013].

Our results show that in the *nucleus accumbens* a strong and sustained decrease of PPARγ is paralleled by a significant increase of the death pathway. The neuronal death observed in this area may likely account for the reported mood disorders in PD, also in the light of the recent involvement of PPARγ decrease in neuropsychiatric disease [García-Bueno et al., 2010].

Regarding *globus pallidus*, our results indicate that this area is affected at later time, after the others have been significantly modified. Particularly, we observed a sustained cell death in the third week after the lesion as also highlighted by the JNK levels at this time point. Chronic loss of dopamine from cortico-basal ganglia circuits profoundly alters neuronal activity therein and often leads to the emergence of excessively synchronized oscillations, as documented in patients with PD [Moran et al., 2008] and in parkinsonian animal models [Sharott et al., 2005]. The changes in microcircuit properties that give rise to these changes remain to be elucidated, but it has been suggested that altered interactions in the reciprocally connected network of glutamatergic subthalamic nucleus (STN) neurons and GABAergic *globus pallidus* external segment (GPe) neurons contribute to these oscillations. Moreover, it has been reported that chronic dopamine depletion changes firing rates and led to strong beta-frequency oscillations in the STN-GPe network, accompanied by a pronounced increase in bidirectional interactions between these nuclei. Therefore this increased strength of reciprocal effective coupling may not only contribute to excessive beta synchrony in Parkinsonism but also may impede the information flow and representation within the STN-GPe network [Cruz et al., 2011]. These findings may be probably explained with the biochemical alterations observed by us in the *globus pallidus*, accompanied by neuronal death in this area. It is noteworthy that the alterations observed in this area appear delayed with respect to *substantia nigra*, thus suggesting that the loss of dopaminergic connections gradually impairs *globus pallidus* functionality.

These observations, may lead to consider that the nuclear receptors PPARs have an interesting potential as promising therapeutic targets to consider in the future for this disease. In fact, the data obtained in this work suggest the possible use of a dual PPARβ/δ antagonist/PPARγ agonist approach to counteract PD neurodegenerative primary and possibly secondary symptoms.

ACKNOWLEDGMENTS

This work has been supported by RIA fund (Prof Cimini). The Authors thank to the Human Health Foundation for the support. Many of the experiments have been performed in the Research Center for Molecular Diagnostics and Advanced Therapies granted by the Abruzzo Earthquacke Relief Fund (AERF). The Authors wish to thank The Ken & Ann Charitable Foundation Trust for their support.

REFERENCES

Adam R, Leff A, Sinha N, Turner C, Bays P, Draganski B, Husain M. 2013. Dopamine reverses reward insensitivity in apathy following globus pallidus lesions. Cortex 49:1292–1303.

Allan SM, Rothwell NJ. 2003. Inflammation in central nervous system injury. Philosophical transactions of the royal society. London: Series B: Biological Sciences 358:pp 1669–1677.

Benedetti E, D′Angelo B, Cristiano L, Di Giacomo E, Fanelli F, Moreno S, Cecconi F, Fidoamore A, Antonosante A, Falcone R, Ippoliti R, Giordano A, Cimini A. 2014. Involvement of peroxisome proliferator-activated receptor β/δ (PPAR β/δ) in BDNF signaling during aging and in Alzheimer disease: Possible role of 4-hydroxynonenal (4-HNE). Cell Cycle 13:1335–1344.

Bové J, Perier C. 2012. Neurotoxin-based models of Parkinson's disease. Neuroscience 211:51–76.

Braak H, Ghebremedhin E, Rub U, Bratzke H, Del Tredici K. 2004. Stages in the development of Parkinson's disease-related pathology. Cell Tissue Res 318:121–134.

Bright JJ, Kanakasabai S, Chearwae W, Chakraborty S. 2008. PPAR regulation of inflammatory signaling in CNS diseases. PPAR Res 2008:658520.

Chen JJ. 2010. Parkinson's disease: Health-related quality of life, economic cost, and implications of early treatment. Am J Manag Care 16:S87–S93.

Cimini A, Benedetti E, D′Angelo B, Cristiano L, Falone S, Di Loreto S, Amicarelli F, Cerù MP. 2009. Neuronal response of peroxisomal and peroxisome-related proteins to chronic and acute Abeta injury. Curr Alzheimer Res 6:238–251.

Cimini A, Gentile R, Angelucci F, Benedetti E, Pitari G, Giordano A, Ippoliti R. 2013a. Neuroprotective effects of PrxI over-expression in an in vitro human Alzheimer's disease model. J Cell Biochem 114:708–715.

Cimini A, Gentile R, D′Angelo B, Benedetti E, Cristiano L, Avantaggiati ML, Giordano A, Ferri C, Desideri G. 2013b. Cocoa powder triggers neuro-protective and preventive effects in a human Alzheimer's disease model by modulating BDNF signaling pathway. J Cell Biochem 114:2209–2220.

Coleman JD, Prabhu KS, Thompson JT, Reddy PS, Peters JM, Peterson BR, Reddy CC, Vanden Heuvel. 2007. The oxidative stress mediator 4-hydroxynonenal is an intracellular agonist of the nuclear receptor peroxisome proliferator-activated receptor-beta/delta (PPARbeta/delta). Free Radic Biol Med 42:1155–1164.

Cruz AV, Mallet N, Magill PJ, Brown P, Averbeck BB. 2011. Effects of dopamine depletion on information flow between the subthalamic nucleus and external globus pallidus. J Neurophysiol 106:2012–2023.

D′Angelo B, Benedetti E, Di Loreto S, Cristiano L, Laurenti G, Cerù MP, Cimini A. 2011. Signal transduction pathways involved in PPARβ/δ-induced neuronal differentiation. J Cell Physiol 226:2170–2180.

D′Angelo B, Santucci S, Benedetti E, Di Loreto S, Phani RA, Falone S, Amicarelli F, Cerù MP, Cimini A. 2009. Cerium oxide nanoparticles trigger neuronal survival in a human Alzheimer disease model by modulatine BDNF pathway. Curr Nanosci 5:167–176.

Dantzer R, O′Connor JC, Freund GG, Johnson RW, Kelley KW. 2008. From inflammation to sickness and depression: When the immune system subjugates the brain. Nat Rev Neurosci 9:46–56.

Das NR, Gangwal RP, Damre MV, Sangamwar AT. 2014. Sharma SS A PPARβ/δ is neuroprotective and decreases cognitive impairment in a rodent model of Parkinson's disease. Curr Neurovasc Res 11:114–124.

Duty S, Jenner P. 2011. Animal models of Parkinson's disease: A source of novel treatments and clues to the cause of the disease. Br J Pharmacol 164:1357–1391.

Fanelli F, Sepe S, D′Amelio M, Bernardi C, Cristiano L, Cimini A, Cecconi F, Ceru′ MP, Moreno S. 2013. Age-dependent roles of peroxisomes in the hippocampus of a transgenic mouse model of Alzheimer′s disease. Mol Neurodegener. 8:1–19.

Feinstein DL. 2003. Therapeutic potential of peroxisome proliferator-activated receptor agonists for neurological disease. Diabetes Technol Ther 5:67–73.

Florio T, Confalone G, Sciarra A, Sotgiu A, Alecci M. 2013. Switching ability of over trained movements in a Parkinson's disease rat model. Behav Brain Res 250:326–333.

Galea E, Heneka MT, Dello Russo C, Feinstein DL. 2003. Intrinsic regulation of brain inflammatory responses. Cell Mol Neurobiol 23:625–635.

García-Bueno B, Caso JR, Leza JC. 2008. Stress as a neuroinflammatory condition in brain: Damaging and protective mechanisms. Rev Neurosci Biobehav 32:1136–1151.

García-Bueno B, Madrigal JL, Lizasoain I, Moro MA, Lorenzo P, Leza JC. 2005a. The anti-inflammatory prostaglandin 15d-PGJ2 decreases oxidative/nitrosative mediators in brain after acute stress in rats. Psychopharmacology (Berlin) 180:513–522.

García-Bueno B, Madrigal JL, Lizasoain I, Moro MA, Lorenzo P, Leza JC. 2005b. Peroxisome proliferator-activated receptor gamma activation decreases neuroinflammation in brain after stress in rats. Biol Psychiatry 57:885–894.

García-Bueno B, Perez-Nievas BG, Leza JC. 2010. Is there a role for the nuclear receptor PPARγ in neuropsychiatric diseases. Int J Neuropsychopharmacol 13:1411–1429.

Gonzalez-Scarano F, Baltuch G. 1999. Microglia as mediators of inflammatory and degenerative diseases. Annu Rev Neurosci 22:19–240.

Hanson DR, Gottesman II. 2005. Theories of schizophrenia: A genetic-inflammatory-vascular synthesis. BMC Med Genet 11:7.

Heneka MT, Landreth GE. 2007. PPARs in the brain. Biochim Biophys Acta 1771:1031–1045.

Houseknecht KL, Cole BM, Steele PJ. 2002. Peroxisome proliferator-activated receptor gamma (PPARgamma) and its ligands: A review. Domest Anim Endocrinol 22:1–23.

Huang EJ, Reichardt LF. 2001. Neurotrophins: Roles in neuronal development and function. Annu Rev Neurosci 24:677–736.

Hyman C, Hofer M, Barde YA, Juhasz M, Yancopoulos GD, Squinto SP, Lindsay RM. 1991. BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. Nature 350:230–232.

Jenner P. 2003. Oxidative stress in Parkinson's disease. Ann Neurol 53:26–36.

Kapadia R, Yi JH, Vemuganti R. 2008. Mechanisms of anti-inflammatory and neuroprotective actions of PPAR-gamma agonists. Front Biosci 13:1813–1826.

Kaplan GB, Vasterling JJ, Vedak PC. 2010. Brain-derived neurotrophic factor in traumatic brain injury, post-traumatic stress disorder, and their comorbid conditions: Role in pathogenesis and treatment. Behav Pharmacol 21:427–437.

Kou J, Kovacs GG, Höftberger R, Kulik W, Brodde A, Forss-Petter S, Hönigschnabl S, Gleiss A, Brügger B, Wanders R, Just W, Budka H, Jungwirth S, Fischer P, Berger J. 2011. Peroxisomal alterations in Alzheimer's disease. Acta Neuropathol 122:271–283.

Li R, Xu LY, Liang T, Zhang SJ, Li YW, Duan XQ. 2013. Effect of puerarin on the expressions of BDNF, TrkB, caspase-3 in substantia nigra tissue of Parkinson's rats. Chin J Exp Tradit Med Formulae 19:204–207.

Massa SM, Yang T, Xie Y, Shi J, Bilgen M, Joyce JN, Nehama D, Rajadas J, Longo FM. 2010. Small molecule BDNF mimetics activate TrkB signaling and prevent neuronal degeneration in rodents. J Clin Invest 120:1774–1785.

Moran A, Bergman H, Israel Z, Bar-Gad I. 2008. Subthalamic nucleus functional organization revealed by parkinsonian neuronal oscillations and synchrony. Brain 131:3395–3409.

Moreno S, Farioli-Vecchioli S, Cerù MP. 2004. Immunolocalization of peroxisome proliferator-activated receptors and retinoid X receptors in the adult rat CNS. Neuroscience 123:131–145.

Paxinos G, Watson C. 1998. The rat brain in stereotaxic coordinates. 4th ed. San Diego: Academic Press.

Reale M, Pesce M, Priyadarshini M, Kamal MA, Patruno A. 2012. Mitochondria as an easy target to oxidative stress events in Parkinson's disease. CNS Neurol Disord Drug Targets 11(4):430–438.

Samii A, Nutt JG, Ransom BR. 2004. Parkinson's disease. Lancet 363:1783–1793.

Schintu N, Frau L, Ibba M, Caboni P, Garau A, Carboni E, Carta AR. 2009. PPAR-gamma-mediated neuroprotection in a chronic mouse model of Parkinson's disease. Eur J Neurosci 29:954–963.

Sharott A, Magill PJ, Harnack D, Kupsch A, Meissner W, Brown P. 2005. Dopamine depletion increases the power and coherence of beta-oscillations in the cerebral cortex and subthalamic nucleus of the awake rat. Eur J Neurosci 21:1413–1422.

Skerrett R, Malm T, Landreth G. 2014. Nuclear receptors in neurodegenerative diseases. Neurobiol Dis 72PA:104-116.

Tapia-Arancibia L, Aliaga E, Silhol M, Arancibia S. 2008. New insights into brain BDNF function in normal aging and Alzheimer disease. Brain Res Rev 59:201–220.

Teng HK, Teng KK, Lee R, Wright S, Tevar S, Almeida RD, Kermani P, Torkin R, Chen ZY, Lee FS, Kraemer RT, Nykjaer A, Hempstead BL. 2005. ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. J Neurosci 25:5455–5463.

Yoshii A, Constantine PM. 2010. Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity and disease. Dev Neurobiol 70:304–322.

Zhou XF, Li WP, Zhou FH, Zhong JH, Mi JX, Wu LL, Xian CJ. 2005. Differential effects of endogenous brain-derived neurotrophic factor on the survival of axotomized sensory neurons in dorsal root ganglia: A possible role for the p75 neurotrophin receptor. Neuroscience 132:591–603.

Zolezzi JM, Inestrosa NC. 2013. Peroxisome proliferator-activated receptors and Alzheimer's disease: Hitting the blood-brain barrier. Mol Neurobiol 48:438–451.