Neuroprotective effect of sulforaphane in 6-hydroxydopamine-lesioned mouse model of Parkinson's disease

Fabiana Morroni [a,*], Andrea Tarozzi [b], Giulia Sita [a], Cecilia Bolondi [a], Juan Manuel Zolezzi Moraga [a], Giorgio Cantelli-Forti [b], Patrizia Hrelia [a]

[a] Department of Pharmacy and Biotechnology, Alma Mater Studiorum – University of Bologna, Bologna, Italy
[b] Department for Life Quality Studies, Alma Mater Studiorum – University of Bologna, Rimini, Italy

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ABSTRACT

Parkinson's disease (PD) is characterized by the selective loss of dopaminergic nigrostriatal neurons, which leads to disabling motor disturbances. Sulforaphane (SFN), found in cruciferous vegetables, is a potent indirect antioxidant and recent advances have shown its neuroprotective activity in various experimental models of neurodegeneration. This study was undertaken to examine the effects of SFN on behavioral changes and dopaminergic neurotoxicity in mice exposed to 6-hydroxydopamine (6-OHDA). For this purpose, mice were treated with SFN (5 mg/kg twice a week) for four weeks after the unilateral intrastriatal injection of 6-OHDA. The increase in 6-OHDA-induced rotations and deficits in motor coordination were ameliorated significantly by SFN treatment. In addition, SFN protected 6-OHDA-induced apoptosis via blocking DNA fragmentation and caspase-3 activation. These results were further supported by immunohistochemical findings in the substantia nigra that showed that SFN protected neurons from neurotoxic effects of 6-OHDA. The neuroprotective effect of SFN may be attributed to its ability to enhance glutathione levels and its dependent enzymes (glutathione-S-transferase and glutathione reductase) and to modulate neuronal survival pathways, such as ERK1/2, in the brain of mice. These results suggest that SFN may potentially be effective in slowing down the progression of idiopathic PD by the modulation of oxidative stress and apoptotic machinery.

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1. Introduction

Parkinson's disease (PD) is characterized by selective neuronal death affecting the locus coeruleus, dorsal motor nucleus of vagus, nucleus basalis of Meynert and, most importantly, the dopaminergic neurons of substantia nigra pars compacta (SNpc) (Lang and Lonzano, 1998). Breakdown of dopaminergic signaling within the nigrostriatal pathway is believed to result in the cardinal motor symptoms of tremor, rigidity, bradykinesia and postural instability. Current PD therapies, based on dopamine replacement, do not affect the disease progression. Beyond symptomatic relief, treatments that have neuroprotective or disease-modifying properties remain a critical unmet clinical need. Slowing the rate of nigral dopaminergic neuron loss would simplify the management of motor features, and probably improve the quality of life over the duration of the illness. Hence, preclinical research is now focusing on potentially disease-modifying compounds that could prevent (or at least slow down) the degeneration of nigrostriatal dopaminergic neurons – the hallmark of PD (Xia and Mao, 2012). The cause of this neurodegeneration has not been fully elucidated; however, increased oxidative stress, inflammation, excitotoxicity, mitochondrial dysfunction and apoptosis have all been implicated as mechanisms that can mediate cell death in PD (Olanow, 2007; Yacoubian and Standaert, 2009).

6-Hydroxydopamine (6-OHDA) is possibly one of the first toxins used to degenerate the dopaminergic neurons in the substantia nigra (SN) and fibers in the striatum (STR). In the last decade, 6-OHDA has been widely used as model of dopamine neuronal degeneration (Meredith et al., 2008). In the mid-1990s, a variant of the original intranigral administration procedure was proposed, in which 6-OHDA is injected into the striatum. This induces prompt damage of striatal terminals, followed by delayed, progressive cell loss of SNpc neurons, which are secondarily affected through a 'dying back' mechanism. This alternative modality thereby provides a progressive model of nigrostriatal degeneration, which is more similar to the gradual evolution of the neurodegenerative process of human PD (Sauer and Oertel, 1994).

Molecular targets for 6-OHDA toxicity are not well understood and the exact mechanisms through which 6-OHDA induces cell death remain a matter of discussion. 6-OHDA is a redox active neurotoxin that rapidly undergoes non-enzymatic oxidation producing hydrogen peroxide (H_2O_2), superoxide and hydroxyl radicals. The glutathione (GSH) system, responsible for removing free radicals and maintaining protein thiols in their appropriate redox state, is an important protective mechanism for minimizing oxidative stress (Bharath et al., 2002; Mytilineou et al., 2002). Moreover, antioxidant enzymes related to GSH, such as glutathione reductase (GR) and glutathione-S-transferase (GST), are also important mediators in the reduction of oxidative stress.

It has been demonstrated that reactive oxygen species (ROS) can act as a modulator of signal transduction pathways (Suzuki et al., 1997). Many lines of evidence indicate that the activation of the extracellular signal-regulated protein kinase (ERK) branch of the mitogen-activated protein (MAP) kinase superfamily may play a pivotal role in neurons exposed to oxidative stress and in neuronal death induced by 6-OHDA (Stanciu et al., 2000; Kulich and Chu, 2001). Moreover, cytoplasmatic aggregates of activated ERK 1/2 have been observed in the SN of PD patients with Lewy body (Zhu et al., 2002).

A variety of antioxidant compounds derived from natural products have demonstrated neuroprotective activity in either in vitro or in vivo models of neuronal cell death (Albarracin et al., 2012; Tarozzi et al., 2012, 2007). Sulforaphane (SFN), a naturally occurring isothiocyanate contained in cruciferous vegetables such as broccoli, Brussel sprouts, cauliflower, and cabbage, is known to be a potent NF-E2-related factor 2 (Nrf2) activator and exhibits anti-oxidative and anti-carcinogenic effects via the up-regulation of Antioxidant Responsive Element (ARE) driven genes (Gao et al., 2001; Misiewicz et al., 2004; Matton and Cheng, 2006; Fimognari and Hrelia, 2007; Fimognari et al., 2008). Several studies have reported that SFN has protective effects on neurons of the central nervous system, against neurotoxicity caused by several oxidative insults (Zhao et al., 2007a; Danilov et al., 2009; Ping et al., 2010; Mizuno et al., 2011). However, there are few studies on the detailed mechanism of neuroprotective effects of SFN in the central nervous system.

In the present study, we decided to observe the neuroprotective and anti-apoptotic capacity of SFN on dopaminergic neurons in 6-OHDA-lesioned mice. We examined the effects of SFN on motor impairments and on nigral dopaminergic cell death, DNA fragmentation and caspase-3 activation. In addition, we also determined the role of GSH and its related antioxidant enzymes and the modulation of phosphorylation of ERK1/2 in its mechanism of neuroprotection.

2. Materials and methods

2.1. Animals

Male C57Bl/6 (9 weeks old, 25–30 g body weight at the beginning of the experiment; Harlan, Milan, Italy) mice were housed under 12 h light/12 h dark cycle (lights on from 7:00 a.m. to 7:00 p.m.) with free access to food and water in a temperature- and humidity-controlled room. Briefly, all animal experiments were carried out in accordance with the European Communities Council Directive 86/609/EEC, and Italian (Ministry of Health) laws, and were approved by the local ethics committee (Veterinary Service of the University of Bologna). Care was taken to minimize the number of experimental animals and to take measures to limit their suffering. Mice were allowed to acclimatize for at least 1 week before the start of experiments.

2.2. Experimental design

The experimental protocol was based on the unilateral stereotaxic intrastriatal injection of 6-OHDA. Animals were randomly divided into 4 groups ($n = 10$–12 per group). Two groups received a 6-OHDA injection in the left striatum, while the other two received the same volume of saline solution (sham groups). Each mouse served as its own control, since left-sided levels (ipsilateral to the lesion) were always compared to right-sided levels (contralateral to the lesion) of the same animal. One hour after brain lesion, we started intraperitoneal (i.p.) administration of 5 mg/kg SFN (Lkt Laboratories, St. Paul, MN, USA) or vehicle (VH, saline) in both lesioned and sham mice. We injected mice twice a week. Thus, the four groups are: 6-OHDA/VH; 6-OHDA/SFN; Sham/VH; Sham/SFN.

Four weeks after the lesion, we tested motor function on rotarod apparatus (Ugo Basile, Comerio, VA, Italy), and we also assessed the extent of the lesion using rotational behavior test. At the end of behavioral analysis, mice were sacrificed by cervical dislocation to perform immunohistochemical and neurochemical analysis.

2.3. Surgical procedures

6-OHDA (Sigma–Aldrich, St. Louis, MO, USA) was injected into the left striatum under gaseous anesthesia (2% isoflurane in 1 L/min oxygen/nitrous oxide), using a stereotaxic mouse frame (myNeuroLab, Leica-Microsystems Co, St. Louis, MO, USA) and a 10 μL Hamilton syringe. 6-OHDA was dissolved at a concentration of 4 μg/μL saline in 0.02% ascorbic acid and 2 μL was injected at a rate of 0.5 μL/min. The needle was left in place for 3 min after the injection before slow retraction, followed by cleaning and suturing of the wound. Sham mice received the equivalent volume of saline into the left striatum. The injection was performed at the following co-ordinates: AP: +0.5, ML: −2.0, DV: −2.5, with a flat skull position.

2.4. Behavioral analysis

All tests were carried out between 9.30 a.m. and 3.30 p.m. Animals were transferred to the experimental room at least 1 h before the test in order to let them acclimatize to the test environment. All scores were assigned from the same observer who was unaware of the animal treatment.

2.4.1. Rotarod

Motor coordination and balance were measured 4 weeks after the surgical procedure, using a commercially available mice rotarod apparatus (Ugo Basile). The unit consists of a rotating spindle divided into 5 lanes by gray plastic dividers, a power source for turning the spindle and grids beneath the rotating roller where mice can safely fall, and the time latency to fall (s) is automatically recorded. All mice were pre-trained for 2 days in order for them to reach a stable performance. The apparatus tests five mice at a time, with one mouse in each section of the rod. Each animal was given three independent trials, each lasting 180 s (with a 20 min inter-trial period). Mice were mounted on the rod and the apparatus turned on to a fixed speed of 22 rpm. The latency of fall from the apparatus was recorded. Values were expressed as retention time on the rotating bar over the three test trails.

2.4.2. Rotational behavior

Apomorphine-induced rotations were determined 4 weeks after surgical procedure, according to the method described earlier (Ungerstedt and Arbuthnott, 1970). Mice received a subcutaneous injection of apomorphine (0.05 mg/kg saline), a dopamine D1/D2

receptor agonist. They were acclimatized in plexiglass cylinders for 5 min prior to test. After apomorphine administration, full body ipsilateral and contralateral turns were recorded using an overhead videocamera over a period of 10 min. Subsequently, each 360° rotation of the body axes was manually counted as a rotation. Values were expressed as mean of contralateral turns collected during 10 min.

2.5. Tissue preparation for immunohistochemistry and neurochemical analysis

Once behavioral analysis was completed, mice were deeply anesthetized and sacrificed by cervical dislocation and some of them were perfused with 4% of paraformaldehyde. The brains were removed and were immersed in the fixative solution for 48 h. The non-perfused brains were rapidly removed and placed into dry-ice. The right and left STR and SN were dissected on an ice-cold plastic dish. Samples were then snap frozen in liquid nitrogen, and kept at −80 °C until analysis. Tissues were homogenized in lysis buffer (50 mM Tris, pH 7.5, 0.4% NP-40, 10% glycerol, 150 mM NaCl, 10 μg/mL aprotinin, 20 μg/mL leupeptin, 10 mM EDTA, 1 mM sodium orthovanadate, 100 mM sodium fluoride), and protein concentration was determined by the Bradford method.

2.6. Immunohistochemistry and image analysis

Fixed brains were sliced on a vibratome at 40 μm thickness. Sections were deparaffinized and hydrated through xylene and rinsed in Tris-buffered saline (TBS). After deparaffinization, endogenous peroxidase was quenched with 3% H_2O_2. Non-specific adsorption was minimized by incubating the section in 10% normal goat serum for 20 min. Sections were then incubated overnight, at 4 °C, with a rabbit anti-Tyrosine Hydroxylase antibody (TH; Millipore, Temecula, CA, USA), rinsed in TBS, and re-incubated for 1 h, at room temperature, with a goat biotinylated anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA, USA). Finally, sections were processed with the avidin–biotin technique and reaction products were developed using commercial kits (Vector Laboratories). To verify the binding specificity, some sections were also incubated with only primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations, no positive staining was found in the sections, indicating that the immunoreactions were positive in all experiments carried out.

Image analysis was performed by a blinded investigator, using an Axio Imager M1 microscope (Carl Zeiss, Oberkochen, Germany) and a computerized image analysis system (AxioCam MRc5, Zeiss) equipped with dedicated software (AxioVision Rel 4.8, Zeiss). After defining the boundary of the SN at low magnification (10× objective), the number of TH-positive cells in the SN was counted bilaterally on – at least – four adjacent sections at a higher magnification (40× objective). Neuronal survival in the SN was expressed as the percentage of TH-positive neurons on the lesioned side, with respect to the contralateral, intact side. In the absence of a stereological count, this approach was chosen to avoid methodological biases due to interindividual differences, and has been previously used to assess the extent of 6-OHDA-induced lesion in the SN (Paul et al., 2004; Armentero et al., 2006; Blandini et al., 2007; McCollum et al., 2010).

2.7. Neurochemical analysis

2.7.1. Western blotting

Samples (30 μg proteins) were separated on 10% SDS-polyacrylamyde gels (Bio-Rad, Hercules, CA, USA) and electroblotted onto 0.2 μm nitrocellulose membranes. Membranes were incubated overnight at 4 °C with primary antibodies recognizing either TH (1:1000; Millipore), or phospho-p44/42 MAPK at threonine 202/204 (pERK1/2; 1:1000; Cell Signaling Technology, Danvers, MA, USA). Membranes were washed with TBS-T (TBS + 0.05% Tween20), and then incubated with a horseradish peroxidase (POD) linked anti-rabbit secondary antibody (1:2000; GE Healthcare, Piscataway, NJ, USA). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Pierce, Rockford, IL, USA). The same membranes were stripped and reprobed with a β-actin antibody (1:1000; Sigma–Aldrich) for TH or total ERK1/2 (1:1000; Cell Signaling) for pERK1/2. Data were analyzed by densitometry, using Quantity One software (Bio-Rad). Values were expressed as fold increase versus respective contralateral intact site.

2.7.2. Determination of DNA fragmentation

The determination of cytoplasmatic histone-associated DNA fragments was performed using the Cell Death Detection ELISA[PLUS] kit (Roche Diagnostics, Mannheim, Germany) according to the protocol from the company. The assay is based on a quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones, respectively. This allows the specific determination of mono- and oligonucleosomes (histone-associated DNA fragments) in the fraction of tissue lysates. Duplicates with lysates corresponding to 80 μg of proteins were used at each reaction. The amount of nucleosomes demonstrating DNA degradation was quantified by POD retained in the immunocomplex. POD was determined photometrically at 405 nm with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) as a substrate by a microplate reader (GENios, TECAN®, Männedorf, Switzerland) after 15 min of substrate reaction time. Values were expressed as mean of optical density (OD) of each experimental group.

2.7.3. Determination of caspase-3 activity

Caspase-3 enzyme activity was determined using a protocol adapted by Movsesyan et al. (2002). The assay is based on the hydrolysis of the p-nitroaniline (pNA) moiety by caspase-3. Briefly, tissue lysates were incubated with assay buffer (50 mmol/L Hepes, pH 7.4; 0.2% CHAPS; 20% sucrose; 2 mmol/L EDTA; and 10 mmol/L dithiothreitol) and a 50 μmol/L concentration of chromogenic pNA specific substrates (Z-Asp-Glu-Val-Asp-pNA; AlexisBiochemicals, San Diego, CA, USA) for caspase-3. In a final volume of 100 μL (containing 120 μg of protein), each test sample was incubated for 3 h at 37 °C. The amount of chromogenic pNA released was measured with a microplate reader (GENios, TECAN®) at 405 nm. Values were expressed as the mean of optical density (OD) of each experimental group.

2.7.4. Determination of GSH content

GSH content was estimated using the protocol described earlier (Jollow et al., 1974) with slight modifications. Aliquots of 25 μL of samples were precipitated with 25 μL of sulfosalicylic acid (4%). The samples were kept at 4 °C for at least 1 h and then subjected to centrifugation at 3000 rpm for 10 min at 4 °C. The assay mixture contained 25 μL of aliquot and 50 μL of 5-5′-dithio-bis (2-nitrobenzoic acid) (4 mg/mL in phosphate buffer, 0.1 M, pH 7.4) in a total volume of 500 μL. The yellow color that developed was read immediately at 412 nm (GENios, TECAN®) and results were calculated using a standard calibration curve. Values were expressed as mmol GSH/mg of total lysate proteins per assay.

2.7.5. Determination of GST activity

GST activity was assessed by the ability to conjugate GSH to CDNB, as previously shown (Sharma et al., 1997). Tissue lysates were added to 0.1 mol/L potassium phosphate-1 mmol/L EDTA with 20 mmol/L GSH and 20 mmol/L CDNB. The rate of appearance of the GSH-CDNB conjugate was measured at 340 nm (GENios,

TECAN®). GST activity was expressed as GST units/mg of total lysate proteins per assay.

2.7.6. Determination of GR activity

GR activity was determined using the Glutathione Reductase Assay Kit according to the manufacturer's instructions (Sigma–Aldrich). Briefly, the assay mixture consisted of phosphate buffer (pH 7.6), EDTA (1 mM), oxidized GSH (2 mmol/L) and NADPH (2 mmol/L). GR was determined by measuring the disappearance of NADPH at 340 nm (GENios, TECAN®). The specific activity of GR was expressed as GR units/mg of total cellular protein per assay.

2.8. Statistical analysis

Data are reported as mean ± SEM. Statistical analysis was performed using one-way ANOVA with Bonferroni post hoc test. Differences were considered significant at $p < 0.05$. Analyses were performed using PRISM 5 software (GraphPad Software, La Jolla, CA, USA).

3. Results

All animals well tolerated surgical operations and there was no mortality due to treatments. There was also no significant change in weight of animals in each group.

3.1. Behavioral analysis

Four weeks after the lesion induced by 6-OHDA, we measured motor coordination in the rotarod test. As shown in Fig. 1, the latency time to fall off from the rod was reduced in 6-OHDA-injected mice (62% of sham mice), while there was no difference in motor performance between Sham/VH and Sham/SFN. However, the latency time to fall off increased to 50% in mice treated with 5 mg/kg of SFN after 6-OHDA lesion with respect to mice treated with saline. The results indicate that SFN treatment showed a significant improvement in rotarod performance for 6-OHDA-induced motor deficits.

We subsequently examined rotational behavior. Apomorphine-induced rotation was neither increased nor different in either direction in Sham-injected animals. In contrast, mice exhibited contralateral rotational behavior following apomorphine challenge 4 weeks after the unilateral administration of 6-OHDA. A significant increase in the number of apomorphine-induced rotations was observed in lesioned mice compared with sham mice. More interestingly, the 6-OHDA/SFN group exhibited a significant attenuation of the asymmetric motor behavior compared to the 6-OHDA/VH group (Fig. 2).

3.2. Immunohistochemistry and neurochemical analysis

We next examined whether SFN could affect the survival of dopaminergic neurons. Lesioned animals showed a consistent reduction of TH-positive cells in the left SN, compared to the intact side, with a neuronal loss of 71% (Fig. 3a and b). SFN treatment induced a significant decrease of dopaminergic cell loss in the SN (37.5% compared to 6-OHDA/VH). Sham-operated animals showed symmetrical expression of TH-positive cells in the SN, which was not modified by chronic treatment with SFN. Moreover, western blot analysis of TH protein confirmed a large decrease in SN and STR of TH expression (87.5% and 79% of control, respectively, Fig. 4a and b) after 6-OHDA treatment. Lesioned animals treated with SFN showed a slight but significant increase in the expression of TH protein in SN compared with the 6-OHDA/VH group (Fig. 4a), whereas in the STR only a trend of increase of TH protein was found in the 6-OHDA/SFN group (Fig. 4b).

Chromosomal breakdown of DNA into 200-bp nucleosomal fragments and DNA condensation are hallmarks of cellular apoptosis (Cohen, 1997). We recently showed that SFN reduced apoptotic cell death induced by 6-OHDA in SH-SY5Y cells (Tarozzi et al., 2009). To quantify apoptotic cell death in our in vivo model, we used an ELISA DNA fragmentation assay which measures low molecular weight histone-associated DNA. In our experimental model, 6-OHDA injection caused an increase of DNA fragmentation in SN samples by 98% compared to Sham animals. SFN significantly blocked 6-OHDA-induced DNA fragmentation as shown in Fig. 5.

Previous experiments with SH-SY5Y cells have shown that 6-OHDA induces caspase-dependent cell death via the intrinsic apoptotic pathway (Tarozzi et al., 2009). The ability of SFN to inhibit the appearance of apoptotic traits that accompany caspase-dependent apoptosis was therefore examined in our in vivo model. Intrastriatal injection of 6-OHDA increased the levels of caspase-3 enzymatic activity in SN samples (Fig. 6); as expected SFN treatment completely inhibited the activation of caspase-3.
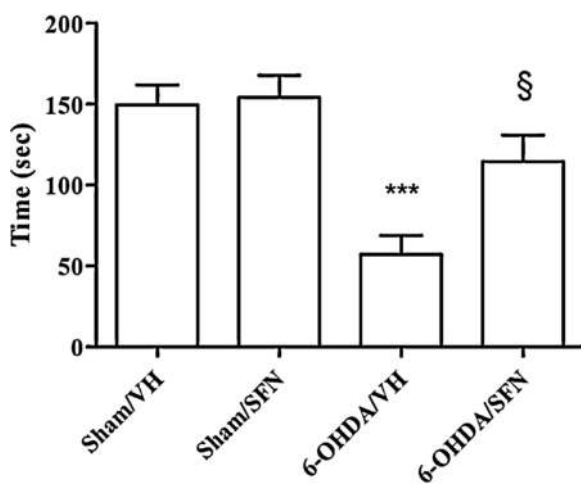

Fig. 1. Effects of SFN on rotarod performance in 6-OHDA lesioned mice. Four weeks after surgery, SFN significantly increased the latency of fall from the rotarod apparatus. Values are expressed as mean of retention time (s) on the rotating bar ± SEM ($n = 10$) (***$p < 0.001$, 6-OHDA/VH vs. Sham/VH, §$p < 0.05$, 6-OHDA/VH vs. 6-OHDA/SFN; ANOVA, post hoc test Bonferroni).
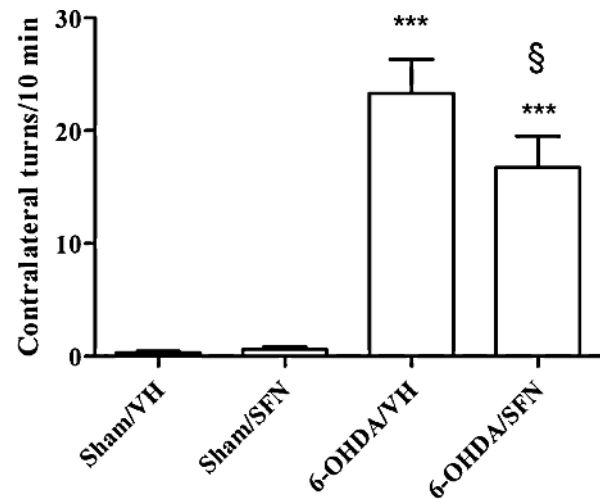

Fig. 2. Effects of SFN on apomorphine-induced rotation in 6-OHDA lesioned mice. Four weeks after surgery, SFN significantly decreased contralateral rotations induced by apomorphine. Values are expressed as mean of contralateral turns collected during 10 min ± SEM ($n = 10$) (***$p < 0.001$, 6-OHDA groups vs. Sham groups, §$p < 0.05$, 6-OHDA/VH vs. 6-OHDA/SFN; ANOVA, post hoc test Bonferroni).
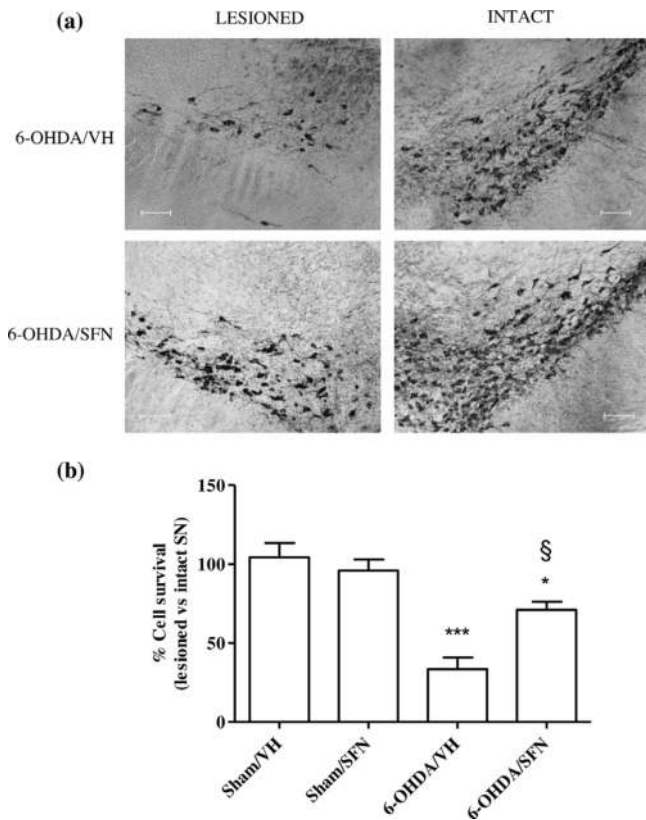
Fig. 3. Effect of SFN on degeneration of dopaminergic neurons in 6-OHDA lesioned mice. (a) TH immunohistochemistry. Representative photomicrographs of brain coronal sections containing SN in the intact and lesioned hemispheres from 6-OHDA/VH and 6-OHDA/SFN groups. Scale bar 100 μm. (b) Histogram representing dopaminergic cell survival in the SN. SFN induced a significant increase on dopaminergic neuron survival. Values are expressed as mean ± SEM ($n = 10$) of the percentage of surviving TH-positive cells of the left, lesioned hemisphere, compared to the right, intact hemisphere (*$p < 0.05$, Sham/VH vs. 6-OHDA/SFN, ***$p < 0.001$, Sham/VH vs. 6-OHDA/VH, $^{\S}p < 0.05$, 6-OHDA/VH vs. 6-OHDA/SFN; ANOVA, post hoc test Bonferroni).

Fig. 5. Effects of SFN on DNA fragmentation in 6-OHDA lesioned mice. DNA fragmentation into oligosomes was detected using DNA-based ELISA. SFN significantly decreased DNA fragmentation in SN samples. Values are expressed as mean of optical density (OD) of each experimental group ± SEM ($n = 10$) ($^{\S\S\S}p < 0.001$, 6-OHDA/VH vs. 6-OHDA/SFN, ***$p < 0.001$ Sham/VH vs. 6-OHDA/VH; ANOVA, post hoc test Bonferroni).

GSH depletion, the earliest biochemical event occurring in PD, is responsible for oxidative stress, mitochondrial dysfunction, and ultimately cell death (Chinta and Andersen, 2006; Zeevalk et al., 2008). Therefore, we determined whether the protective effect of SFN on 6-OHDA-toxicity was related to the inhibitory effect on GSH depletion. In our experimental model, 6-OHDA dramatically decreased GSH levels (Fig. 7). As shown in Fig. 7, the level of GSH was not altered significantly in the Sham/SFN group, whereas SFN showed a significant increase of GSH content in 6-OHDA lesioned mice compared to 6-OHDA/VH mice. We then determined the ability of SFN to modulate the activity of GSH-related enzymes, GST and GR. In the 6-OHDA/VH group we observed alterations of brain antioxidant status compared to the sham group as evidenced by a decrease of GST (Fig. 8a) and GR (Fig. 8b) activities. By contrast,
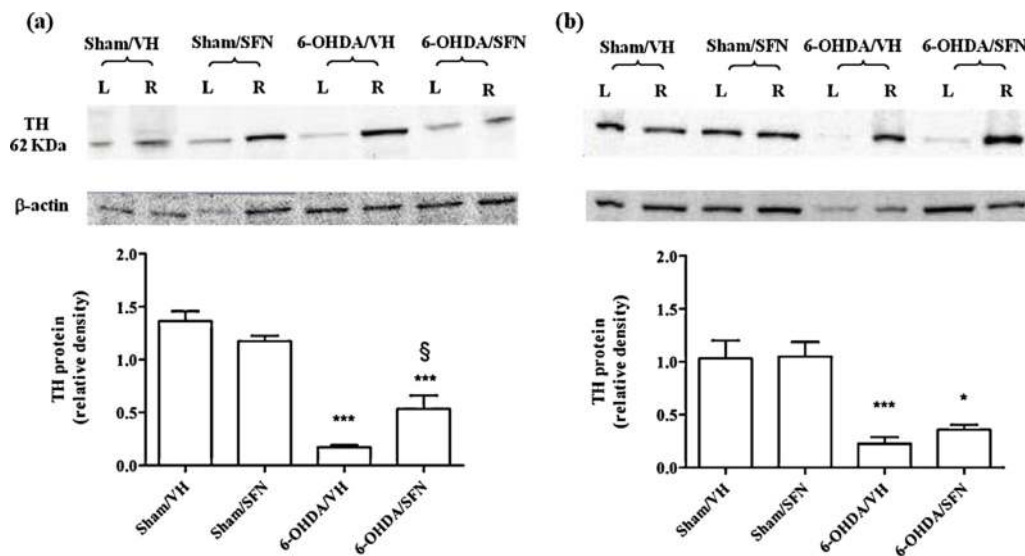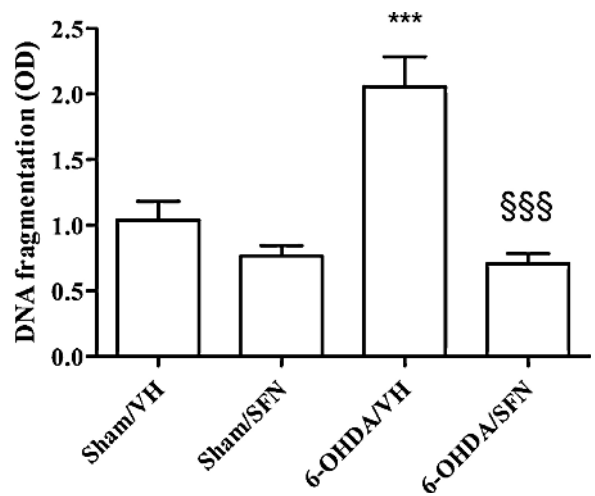

Fig. 4. Effects of SFN on TH levels in 6-OHDA lesioned mice. TH levels were determined in SN (a) and STR (b) samples by Western blotting. Top: representative images of the protein expression. We detected TH at 62 kDa and the loading control β-actin at 42 kDa. Bottom: quantitative analysis of the Western blot results for the TH levels. SFN significantly increased TH levels in SN samples. Values are expressed as mean of fold increase versus respective contralateral intact site ± SEM ($n = 10$) (a: $^{\S}p < 0.05$ 6-OHDA/SFN vs. 6-OHDA/VH, ***$p < 0.001$ Sham/VH vs. 6-OHDA groups; b: *$p < 0.05$, Sham/VH vs. 6-OHDA/SFN, ***$p < 0.001$ Sham/VH vs. 6-OHDA/VH; ANOVA, post hoc test Bonferroni).
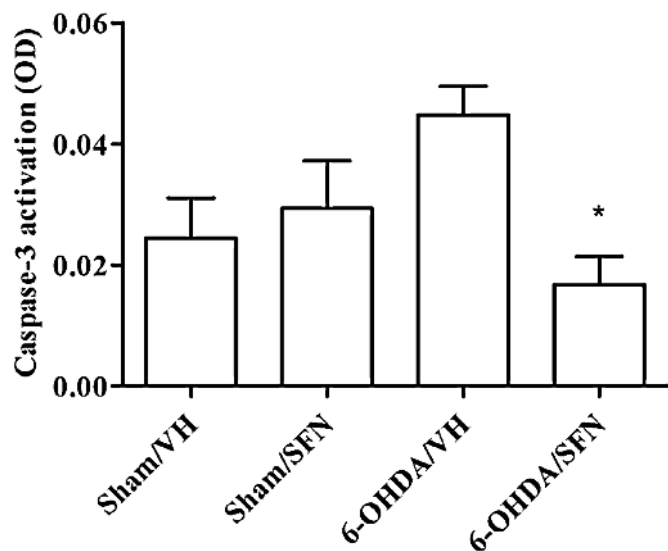
Fig. 6. Effects of SFN on caspase-3 activation in 6-OHDA lesioned mice. Caspase-3 activation was determined using specific chromogenic substrate. SFN significantly decreased caspase-3 activation in SN samples. Values are expressed as mean of optical density (OD) of each experimental group $\pm$ SEM ($n = 10$) ($^*p < 0.05$, 6-OHDA/VH vs. 6-OHDA/SFN; ANOVA, post hoc test Bonferroni).

Fig. 7. Effect of SFN on GSH content in 6-OHDA lesioned mice. GSH content was measured using a colorimetric assay. SFN significantly increased GSH levels in SN samples. Values are calculated using a standard calibration curve and expressed as mmol GSH/mg protein ($^*p < 0.05$, Sham/VH vs. 6-OHDA/VH, $^{§§}p < 0.01$, 6-OHDA/SFN vs. 6-OHDA/VH; ANOVA, post hoc test Bonferroni).

the 6-OHDA/SFN group evidenced a significant increase of GST (41%) and GR (69%) activities compared to the 6-OHDA/VH group.

To further elucidate the neuroprotective mechanism of SFN, the ability to decrease 6-OHDA-dependent ERK1/2 phosphorylation was examined. In line with growing evidence indicating that ERK activation can alter GSH metabolism (Benassi et al., 2006) and promote cell death (Chu et al., 2004), the 6-OHDA/VH group showed a significant increase in ERK1/2 activation. As shown in Fig. 9 of Western blot analysis, SFN treatment could dramatically down-regulate the phosphorylation of ERK1/2 induced by 6-OHDA, reaching levels comparable to those in Sham-operated mice.

4. Discussion

This study demonstrates that SFN counteracts SN cell loss following striatal injection of 6-OHDA in mice and investigates the potential molecular mechanisms by which SFN protects neurons from 6-OHDA-induced neurotoxicity. Current studies have focused attention on SFN protective effects in several experimental models of brain and neuronal injury. In particular, SFN reduces the infarct size in rats induced by ischemia and reperfusion, also protecting
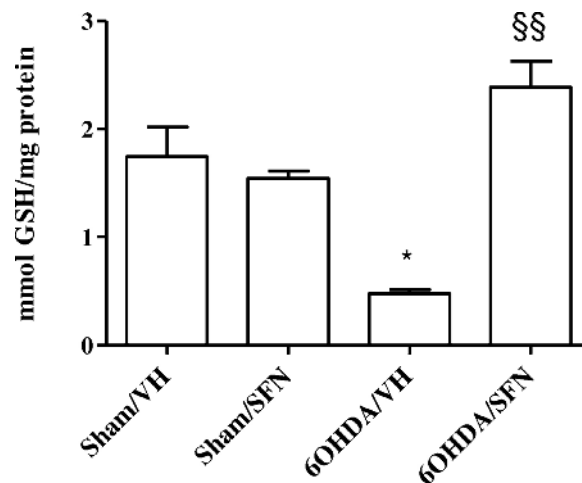
the blood brain barrier after brain injury and attenuating inflammation in mouse hippocampus in a model of lipopolysac-charide-induced inflammation (Zhao et al., 2006, 2007b; Inna-morato et al., 2008). Moreover, Jazwa et al. (2011) have recently demonstrated that SFN induces an Nrf2-dependent phase II response in the basal ganglia, and protects against nigral dopaminergic cell death, astrogliosis, and microgliosis in the MPTP mouse model of PD.

Considering all these previous findings and our in vitro study (Tarozzi et al., 2009), we decided to investigate the neuroprotective effect of SFN after injection of 6-OHDA into the striatum. This model causes progressive retrograde neuron death (Berger et al., 1991; Sauer and Oertel, 1994; Przedborski et al., 1995), and the dying neurons exhibit a varied morphology including some features of apoptosis (Martí et al., 1997). This model creates a therapeutic window, that can be used for the investigation of potentially neuroprotective treatments for the disease.

A major finding of this study is that SFN ameliorated behavioral impairments such as motor coordination and rotational behavior. To the best of our updated knowledge, this study is the first to demonstrate a restorative role for SFN in regulating PD-associated motor impairments and we believe that the behavioral assessment is a powerful endpoint in evaluating neuroprotection. In particular,
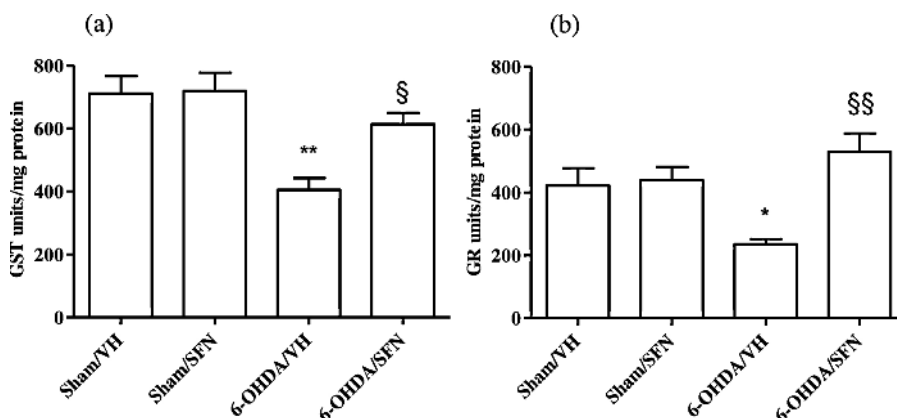
(a)   (b)

Fig. 8. Effect of SFN on GST and GR activity in 6-OHDA lesioned mice. GST and GR activities were measured using a colorimetric assay. SFN significantly increased GST (a) and GR (b) activities in SN samples. Values are expressed as enzyme units/mg of proteins. (a: $^{**}p < 0.01$, Sham/VH vs. 6-OHDA/VH, $^{§}p < 0.05$, 6-OHDA/SFN vs. 6-OHDA/VH; b: $^*p < 0.05$ Sham/VH vs. 6-OHDA/VH, $^{§§}p < 0.01$ 6-OHDA/SFN vs. 6-OHDA/VH; ANOVA, post hoc test Bonferroni).
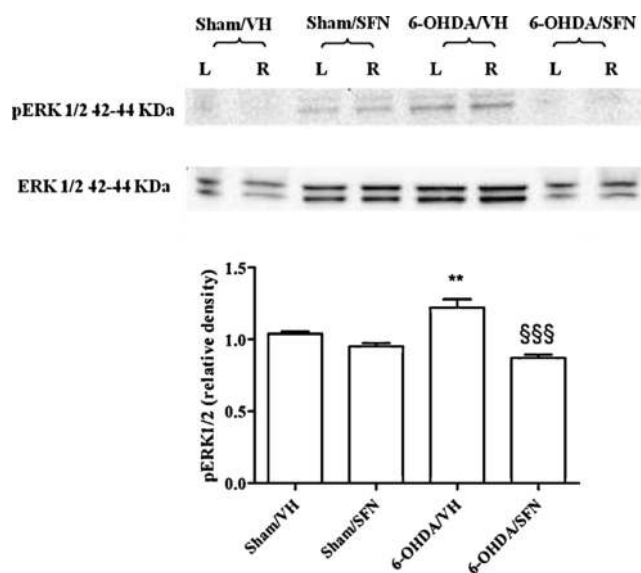
Fig. 9. Effect of SFN on phosphorylation of ERK 1/2 in 6-OHDA lesioned mice. Phospho-Thr202/Thr204-ERK1 and phospho-Thr202/Thr204-ERK2 were determined by Western blotting. Top: representative images of the protein expression. We detected pERK1/2 and total ERK at 42, 44 kDa. Bottom: quantitative analysis of the Western blot results for the pERK1/2 levels. SFN significantly decreased phosphorylation of ERK1/2 in SN samples. Values are expressed as mean of fold increase versus respective contralateral intact site $\pm$ SEM ($n = 10$) (**$p < 0.01$, Sham/VH vs. 6-OHDA/VH, §§§$p < 0.001$, 6-OHDA/VH vs. 6-OHDA/SFN; ANOVA, post hoc test Bonferroni).

apomorphine-induced contralateral rotation is a reliable marker for the nigrostriatal dopamine depletion. We report here an appreciable decrease in the number of induced rotations in lesioned mice treated with SFN compared to lesioned mice treated with VH, which could be closely linked to the significant increase in TH positive cells that we found in the SN of the same group of animals. In rotarod, performance is measured by the duration that an animal stays up on the drum and this task provided a rich source of quantitative information on walking movements (Whishaw et al., 2008). It was observed that the mean time on the rotating drum was less in 6-OHDA lesioned mice compared to the SFN treated group, suggesting the efficacy of SFN to enhance the coordination and equilibrium of lesioned animals. Our findings are in accordance with earlier studies carried out, where motor deficits in Parkinsonian models were attenuated by compounds with antioxidant activity (Ahmad et al., 2005; Chaturvedi et al., 2006; Khan et al., 2012). Even more interesting, Chen et al. (2011) demonstrated that SFN could ameliorate motor performance in a model of early brain injury after experimental subarachnoid hemorrhage.

Toxicity of 6-OHDA is due to its pro-oxidant activity. The toxin undergoes rapid auto-oxidation in the extracellular space, promoting a high rate of reactive oxygen species formation (Hanrott et al., 2006), which is associated with activation of the apoptotic process (Jeon et al., 1999; Ouyang and Shen, 2006). Many lines of evidence point to a significant contribution of apoptotic cell death after 6-OHDA injection into the striatum (Martí et al., 2002; Mladenović et al., 2004). Evidence from human PD brain tissue also suggests that degenerating DA neurons undergo an apoptotic process and activate the caspase cascade (Hartmann et al., 2000; Mogi et al., 2000; Tatton, 2000). In the present study, 6-OHDA injection significantly increased DNA fragmentation and caspase-3 activity in the SN, whereas the two parameters were dramatically lower in the mice treated with SFN, suggesting that SFN could abort the apoptotic signaling pathway. Our results are consistent with previous findings (Chen et al., 2011), that showed the capacity of

SFN treatment to repress cortical apoptosis in a model of subarachnoid hemorrhage and this resulted in a significant amelioration of functional outcome, as shown by rotarod performance. Interestingly, the increase that we observed in DNA fragmentation due to 6-OHDA injection is considerably higher than the increase of caspase-3 activity, probably because other mechanisms of death, as well as apoptosis, are implicated in the neurodegeneration. Moreover, we demonstrated that SFN protected against 6-OHDA-induced loss of TH+ neurons in the SN and also increased TH expression after 6-OHDA injection. However, its stronger antiapoptotic effect, in terms of DNA fragmentation and caspase activation, compared to the increase of TH+ neurons could suggests that SFN cytoprotective activity may be also ascribed to its ability to modulate the effects of neighboring non-dopaminergic neurons or glial cells.

In response to 6-OHDA toxicity, which is mainly associated with oxidative stress, GSH is the major defense in the brain due to its ability to buffer free radicals. It has been demonstrated that the brain and in particular dopaminergic neurons are especially vulnerable to oxidative damage (Zafar et al., 2003; Sánchez-Iglesias et al., 2009). Overproduction of free radicals, such as superoxide and peroxynitrite, causes an imbalance in the redox environment of cells, leading to eventual apoptotic cell death. An important contribution to the protective role of GSH is provided by several antioxidant enzymes, such as GR and GST. GSH is regenerated by redox recycling, in which GSSG is reduced to GSH by GR with a consumption of one NADPH. GST catalyses the detoxification of oxidized metabolites of catecholamines and reduces the short-lived membrane lipid hydroperoxides, that break down to yield secondary electrophiles responsible for amplifying the oxidative damage (Hayes et al., 2005). All of the antioxidant defenses are inter-related and a disturbance in one may involve the balance in all. For instance, a reduction in the level of GSH may impair H_2O_2 clearance and promote hydroxyl radical formation, the most toxic radical to the brain, leading to a higher oxidant load and further oxidative stress (Dringen, 2000). In this study, 6-OHDA injection caused an overproduction of free radicals which, in turn, decreased GSH levels and GST and GR activities in the SN. Administration of SFN in the sham group did not significantly alter the basal level of GSH and its related enzymes. However, following the oxidative stress induced by 6-OHDA, SFN restored the level of nigral GSH and the activities of GST and GR, suggesting that SFN increases antioxidant potential in the brain and helps it to fight against 6-OHDA-induced oxidative damage. It has been well established that molecular mechanisms involving the 'indirect antioxidant' activity of SFN are dependent upon the activation of the transcription factor Nrf2, which regulates the expression of a battery of genes that constitute the so-called phase-II response. An increasing number of studies have suggested that Nrf2 activation is a novel neuroprotective pathway that confers resistance to a variety of oxidative stress-related neurodegenerative insults like 6-OHDA; for instance, Jakel et al. (2007) demonstrated that the dopamine neurons of Nrf2 knockout mice are more vulnerable to neurotoxins compared with wild-type mice.

It has been shown that Nrf2 is related to several kinase pathways in various tissues, including MAPK (Owuor and Kong, 2002). In particular, we focused our interest on the extracellular signal regulated protein kinases (ERK1/2), which are emerging as important regulators of neuronal responses to both functional and pathologic stimuli. Mechanisms underlying ERK1/2-mediated neuronal death are only beginning to emerge. Oxidative stress generated by ROS is often linked to an activation of the ERK1/2 pathway. Indeed, ERK1/2 activation appears to be mediated by redox mechanisms in both acute neuronal injuries (Alessandrini et al., 1999) and in models of neurodegeneration (Kuperstein and

Yavin, 2002). Phospho-ERK1/2 is also increased in SN neurons of patients with PD and other Lewy body diseases, and the midbrains of these patients show elevated ERK activity (Zhu et al., 2002). Both transgenic animal studies (Noshita et al., 2002) and cell culture studies (Kulich and Chu, 2003) suggest that inhibition of ERK1/2 signaling comprises an important mechanism by which antioxidants confer protection. Moreover, De Bernardo et al. (2004) demonstrated that GSH depletion by L-buthionine-(S,R)-sulfoximine induced selective neuronal cell death in co-cultures of neuron/glia via a free-radical producing a cascade which activates ERK pathways. In our model of neurodegeneration, we observed an increase in SN phospho-ERK1/2 levels after 6-OHDA lesion and more importantly we found that SFN could inhibit ERK activation, suggesting the involvement of MAPK pathway in its mechanisms of neuroprotection. Interestingly, as ERK has both physiological and pathological roles (Subramaniam and Unsicker, 2010), we propose that SFN treatment represents a specific way to inhibit pathological ERK signaling without affecting physiologically important ERK activation, as shown by the administration of SFN in the sham group that did not significantly alter the basal level of phospho-ERK1/2.

5. Conclusions

Growing evidence suggests that neuroprotective strategies against oxidative stress are becoming more important in neurodegenerative disease research, in particular in PD. However, in our opinion, a molecule that has only antioxidant activity is not sufficient to be an effective therapeutic compound that could act against neuronal degeneration. In summary, we report here that our experimental model produces the characteristic alterations in the behavioral pattern of 6-OHDA model, which is further confirmed by the biochemical and histological alterations in the various classical parameters used in characterizing PD and PD-related disorders. Taken together, our results indicate that SFN protects dopaminergic neurons, showing antiapoptotic properties which might delay the onset or at least slow down the progression of PD. SFN protected 6-OHDA induced apoptosis via blocking DNA fragmentation and caspase-3 activation. At the same time, motor impairments seen in this mouse model of PD are partially and significantly reduced. While the exact mechanism/s underlying the neuroprotective effects of SFN merit further investigation, based on our findings, we hypothesize that it may be wholly or in part due to its ability to enhance GSH and its related enzymes and also to its ability to counteract ERK1/2 activation induced by 6-OHDA. Overall, our study suggests the potential efficacy of SFN for prevention of PD by the modulation of oxidative stress and apoptotic machinery, thus providing a promising profile which would allow efficacy in a wide range of neurodegenerative diseases.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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