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Regular Article

Blueberry- and spirulina-enriched diets enhance striatal dopamine recovery and induce a rapid, transient microglia activation after injury of the rat nigrostriatal dopamine system

Ingrid Strömberg [a,*], Carmelina Gemma [b,c], Jennifer Vila [c], Paula C. Bickford [b,c,d]

[a] Department of Integrative Medical Biology, Umeå University, S 901 87 Umeå, Sweden
[b] James A Haley Veterans Administration Medical Center, Tampa, FL 33612, USA
[c] Center of Excellence for Aging and Brain Repair, FL 33612, USA
[d] Department of Neurosurgery, University of South Florida, Tampa, FL 33612, USA

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Abstract

Neuroinflammation plays a critical role in loss of dopamine neurons during brain injury and in neurodegenerative diseases. Diets enriched in foods with antioxidant and anti-inflammatory actions may modulate this neuroinflammation. The model of 6-hydroxydopamine (6-OHDA) injected into the dorsal striatum of normal rats, causes a progressive loss of dopamine neurons in the ventral mesencephalon. In this study, we have investigated the inflammatory response following 6-OHDA injected into the striatum of adult rats treated with diet enriched in blueberry or spirulina. One week after the dopamine lesion, a similar size of dopamine degeneration was found in the striatum and in the globus pallidus in all lesioned animals. At 1 week, a significant increase in OX-6- (MHC class II) positive microglia was found in animals fed with blueberry- and spirulina-enriched diets in both the striatum and the globus pallidus. These OX-6-positive cells were located within the area of tyrosine hydroxylase (TH) -negativity. At 1 month after the lesion, the number of OX-6-positive cells was reduced in diet-treated animals while a significant increase beyond that observed at 1 week was now present in lesioned control animals. Dopamine recovery as revealed by TH-immunohistochemistry was significantly enhanced at 4 weeks postlesion in the striatum while in the globus pallidus the density of TH-positive nerve fibers was not different from control-fed lesioned animals. In conclusion, enhanced striatal dopamine recovery appeared in animals treated with diet enriched in antioxidants and anti-inflammatory phytochemicals and coincided with an early, transient increase in OX-6-positive microglia.

Keywords: Neuroinflammation; Microglia; Parkinson's disease; Regeneration; Antioxidants

Introduction

Dopamine neurons in ventral mesencephalon are known to be vulnerable to injury, and this concern relates specifically to dopamine neurons in the substantia nigra. For instance, selective neuronal death of dopamine neurons in the substantia nigra may result in chronic neurological disorders like Parkinson's disease (Kanazawa, 2001). The slow, progressive loss of dopamine neurons is considered to be due, at least in part, to oxidative stress caused by generation of reactive oxygen species (ROS).

Injection of the neurotoxin 6-hydroxydopamine (6-OHDA) into the striatum induces a slow progressive degeneration of dopamine neurons in the substantia nigra (Sauer and Oertel, 1994). It is known that 6-OHDA is easily oxidized to ROS, and it has been suggested that 6-OHDA at least in part induces apoptosis (Choi et al., 1999; Cohen and Heikkila, 1974; Lotharius et al., 1999). As a result of the dopaminergic insult, proinflammatory cytokines such as tumor necrosis factor alpha (TNFα) are increased (Mladenovic et al., 2004; Mogi et al., 1999). Moreover, using the 6-OHDA lesion model, it has been suggested that the neuronal death per se does not induce production of proinflammatory cytokines (Depino et al., 2003). Elevated levels of proinflammatory cytokines are found also in the brain of parkinsonian patients

* Corresponding author. Fax: +1 46 90 786 6608.
E-mail address: ingrid.stromberg@histocel.umu.se (I. Strömberg).

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(Mogi et al., 1995; Mogi et al., 1994), and the production of proinflammatory cytokines has been localized to microglial cells (Loddick and Rothwell, 1999; Mogi et al., 1994). Thus, the 6-OHDA lesion is accompanied by microglia activation (Depino et al., 2003). Indeed, it is believed that neuroinflammation plays a critical role in the loss of dopamine neurons in Parkinson's disease.

Epidemiological studies demonstrate that consumption of a Mediterranean diet is beneficial to health (de Lorgeril et al., 1994; Trichopoulou et al., 2003). The Mediterranean diet is rich in fruits and vegetables, which contain high levels of vitamin C, vitamin E, flavonoids, and carotenoids (Willet et al., 1995). In animal experiments, it has been documented that blueberry, spinach, or spirulina-enriched diets may prevent age-related declines in the cerebellar noradrenergic receptor function (Bickford et al., 2000; Bickford et al., 1999). Furthermore, diets supplemented with antioxidants such as vitamin E, strawberry, or spinach increase dopamine release in the striatum and retard some age-related motoric declines (Joseph et al., 1999; Joseph et al., 1998; Martin et al., 2000). Another compound with antioxidative effects that has been used for thousands of years is ginseng. Data provided show that ginseng both in mice and rats has neuroprotective actions on injury of the dopamine system (Van Kampen et al., 2003).

Antioxidant supplementation improves cell-mediated immunity and has immunomodulatory effects (Meydani, 1999). However, little is known how injury of the dopamine system that may cause an inflammatory response will be affected by diet enriched in antioxidants. Therefore, this study was conducted to study the inflammatory response following injury of the nigrostriatal dopamine system by injection of 6-hydroxydopamine (6-OHDA) into the striatum of rats fed with diet enriched in antioxidants i.e. blueberry or spirulina. The MHC class II response as determined by OX-6-immunoreactive microglial cells was used as marker for inflammation.

Materials and methods

Animal treatment

Three-month-old F344 male rats obtained from Harlan Industries were placed onto either a NIH-31 (control) diet or NIH-31 diet supplemented with 2% blueberry or 0.1% spirulina (n = 5 for each group of animals and time point). Rats remained on these diets for the entire study. Twenty-eight days following the initiation of the diets, animals were anesthetized with isoflurane, placed in a stereotaxic frame, and unilaterally injected with 6-hydroxydopamine (6-OHDA) into the dorsal striatum using the coordinates 1.0 mm anterior and 3.0 mm lateral to the bregma, and 4.5 mm below the dura. Each animal was injected with 20 μg 6-OHDA in saline containing 0.5 M ascorbic acid (4 μl infused over 4 min), and the cannula was left in place for 2 min following infusion. Sham injections consisted of vehicle. Seven days or 4 weeks following the lesion, rats were euthanized for perfusion and immunohistochemistry.

Immunohistochemistry

Rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused transcardially with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in phosphate buffer. The brains were dissected and postfixed in paraformaldehyde for 12 h, after which they were transferred into 30% sucrose in phosphate buffer for at least 16 h. Cryostat sections (14 or 40 μm) were processed for indirect immunohistochemistry. Antibodies used were directed against tyrosine hydroxylase (TH; Pel-Freez, Arkansas, USA; 1:300 anti-rabbit or DiaSorin, Stillwater, USA; 1:1 500 anti-mouse), OX-6 (Serotec, Hamar, Norway, 1:200), Iba1 (Wako Chemicals GmbH, Neuss, Germany, 1:1 000), and glial fibrillary acidic protein (GFAP; Sigma, Sweden, 1:100,) and were diluted in PBS containing 0.3% Triton-X. Incubations were performed for 48 h at 4°C. After washing, the sections were incubated in Alexa 488 and Alexa 594 secondary antibodies (Molecular Probes, Leiden, The Netherlands) for 1 h at room temperature. After additional rinsing, the sections were mounted in 90% glycerin in PBS. Double labeling of TH and OX-6, TH and GFAP, or OX-6 and Iba1 was performed. Cell counts were performed on 40 μm thick sections processed for TH-immunohistochemistry using Vectastain ABC system (Elite ABC kit, Vector Laboratories). Sections were rinsed in Tris-buffered saline (TBS) after being incubated in primary antibodies as described above, followed by incubation in biotinylated secondary antibody solution for 30 min and after additional rinsing treatment with Vectastain Elite ABC reagent. After rinsing, sections were treated with 3,3′-diaminobenzidine to visualize the reaction. Sections were then mounted in DPX. Evaluation was performed using image analysis and Improvision software and for unbiased stereological cell counts using StereoInvestigator software (MicroBrightfield, VT).

Cell counts and statistics

The TH-negative zone surrounding the injection site in the striatum was calculated as the radius perpendicular from the injection track to the most distal part of the striatum showing TH-negativity. Measurements were performed over 5 sections and means for each brain was calculated. Means from each brain was used for one-way ANOVA giving the means of different treatments and time points. Cell counts of OX-6-positive microglia cells in the striatum and the globus pallidus were performed using a standardized frame. OX-6-positive cell counts were performed on 5 sections for each brain within this frame. The density of TH-positive nerve fibers and GFAP-positive astroglia was calculated from images captured with a CCD camera (ProgRes C14, Jenoptik, Jena) using NIH image software on binary images and expressed as mean grey density. The density of TH-immunoreactivity was measured over globus pallidus and GFAP-positivity at the injection site in 5 sections per brain. Means for each brain and antibody were then processed for calculations using one-way ANOVA. All measurements were performed on blind coded slices.

One-way ANOVA followed by Fisher post hoc analysis was used to analyze differences between groups and time points. Stereological cell counts of TH-positive neurons were performed within the nigral area. The histological material was viewed with a Nikon Eclipse 600 microscope and quantified using Stereo Investigator software (Version 6 MicroBrightField, Colchester, VT). The substantia nigra pars compacta was outlined. Neurons in the substantia nigra were counted using the optical fractionator method of unbiased stereological cell counting techniques (West et al., 1991). Optical dissectors were cubes 100 × 100 × 10 μm spaced in a systematic random manner 100 μm apart and offset from the section surface by 5 μm. The sampling was optimized to sample 300 counted cells per animal with error coefficients less than 0.07. Each counting frame was placed at an intersection of the lines forming a virtual grid (160 × 1600 μm), which was randomly generated and randomly placed by the software within the outlined structure. TH labeled neurons were counted using a 60× oil lens (n.a. 1.4) and were included in the measurement only when they came to focus within the dissector (dissector height with 20 μm average thickness of mounted sections was 30 μm, thickness was measured at random intervals throughout every section and estimated by the program).

Results

Effects of dopamine denervation in the striatum

In the striatum, TH-immunohistochemistry revealed at 1 week postinjection a TH-negative area surrounding the injection site in all animals that had received 6-OHDA while

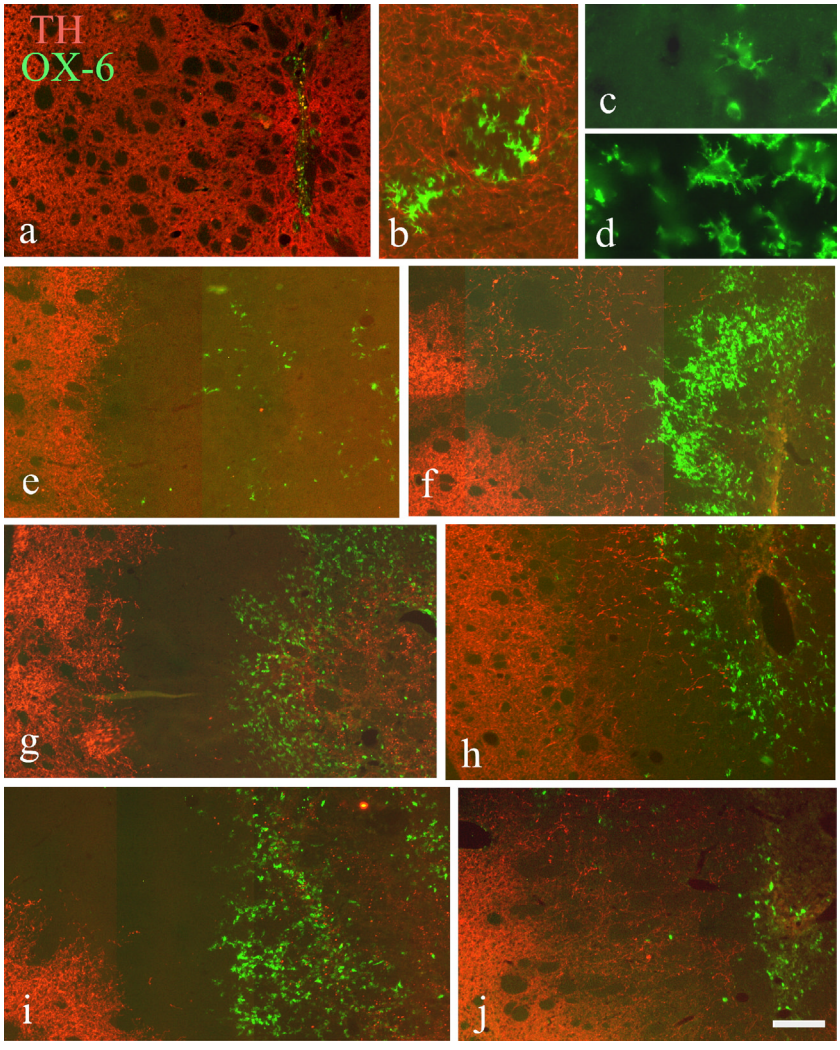

Fig. 1. TH- (red) and OX-6- (green) immunoreactivity in the striatum of either sham (a) or 6-OHDA (b–j) lesioned animals treated with control diet (a–c, e, f), blueberry-enriched diet (d, g, h), or spirulina-enriched diet (i, j) at 1 week (c–e, g, i) or 4 weeks (a, b, f, h, j). The size of the TH-negative zone was similar in all lesioned animals at 1 week after the lesion (e, g, i). A rapid increase in OX-6-positive microglia was found in all animals at 1 week, while in animals fed with blueberry or spirulina, this increase was much more pronounced (e, g, i). At 4 weeks after the lesion, this increase of OX-6-positive microglia was reversed in animals fed with blueberry or spirulina-rich diet (h, j) while it was now enhanced in lesioned animals treated with control diet (f). OX-6-positive microglia found in rats fed with blueberry or spirulina-rich diet were enlarged with many processes (d) at 1 week as compared to OX-6-immunoreactive microglia found in control-fed lesioned animals (c). At 4 weeks postlesion, OX-6-positive microglia were often found in myelinated fiber bundles posterior to the injection sites in control-fed lesioned animals (b). Scale bar: a, e–j = 200 μm; b = 50 μm; c, d = 25 μm.

no reduction in TH-immunohistochemistry was found after sham injection (Fig. 1). The TH-negative zone was surrounded by a high density of TH-immunoreactive nerve fibers and thus the delineation between TH-positive and TH-negative areas was abrupt. The TH-negative area as measured by the radius from the injection site to the presence of TH-positive nerve fibers in a medial-to-lateral axis in the striatum after the lesion was approximately of the same size in all animals at 1 week after the lesion except for that seen in the sham-injected animals (Fig. 2a). Four weeks after the lesion, the TH-negative zone was significantly reduced in animals treated with blueberry- and spirulina-enriched diets as compared to those treated with control diet. The TH-negative zone was also significantly smaller at 4 weeks in blueberry and spirulina-fed animals than that measured at 1 week ($P < 0.01$ in blueberry-fed animals and $P < 0.001$ in spirulina-fed animals, $n = 5$ for each treatment). However, no significant reduction of the TH-negative radius was measured in the control diet-fed animals at 4 weeks. In animals treated with the antioxidant-rich diet, the area close to the TH-negative zone was no longer a sharp delineation between TH-negative and TH-positive areas but more characterized by a sparse nerve fiber network including intensive fluorescence and thick nerve endings, indicating a process of recovery.

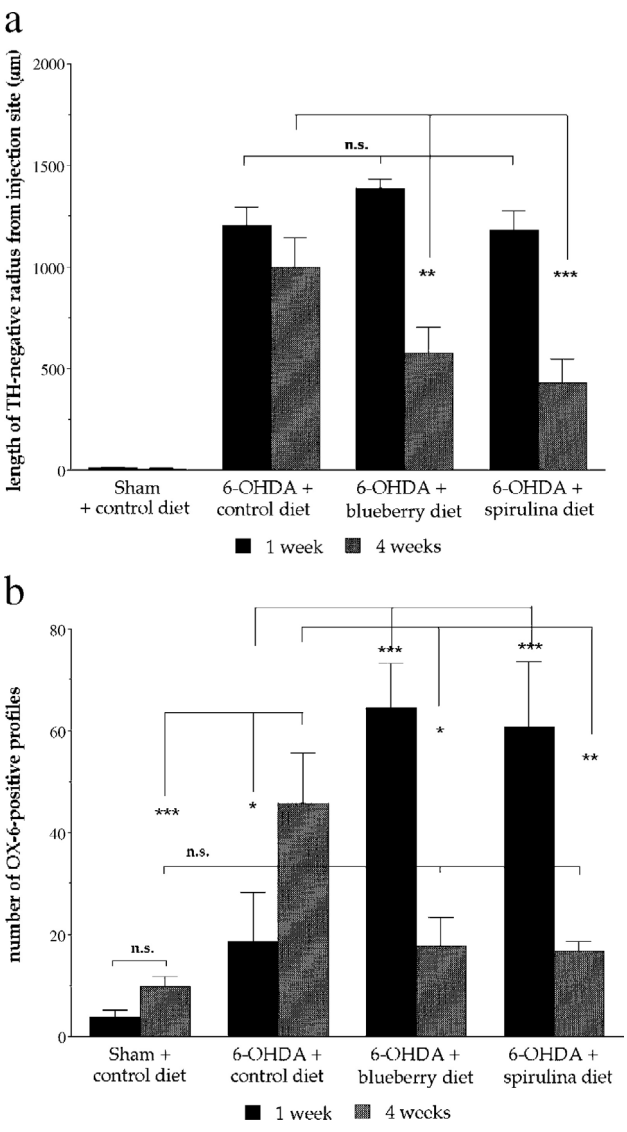

Fig. 2. Graphs showing the TH-negative zone in the striatum (a) and the number of OX-6-positive microglial cells in the TH-negative zone (b). The TH-negative zone was measured as the radius from the injection site to the rim of TH-immunoreactivity within the lesioned striata. Measurements show that the TH-negative zone was similar in all lesioned animals at 1 week after the lesion, while a significant reduction is found at 4 weeks after the lesion in animals treated with blueberry or spirulina-enriched diets (a). The number of OX-6-positive microglia was significantly increased at 1 week in animals with antioxidant-enriched diet (b). This increase was reversed at 1 month while at this time point a significant increase in number of OX-6-positive cells was found in the striata of control-fed lesioned animals. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, $n = 5$ for each group.

OX-6-immunohistochemistry showed positive cells in all injected striata, while control sides of all animals were OX-6-negative. However, only few OX-6-positive microglia were found in sham-injected animals, and those were located within the injection track (Fig. 1a). In animals that had received 6-OHDA injections, microglial cells were found at a small distance from the needle track but still within the TH-negative area leaving an OX-6-negative zone surrounding the needle track at 1 week after the lesion (Fig. 1). The number of OX-6-positive cells was moderately increased 1 week after the 6-OHDA injection in animals fed with control diet when comparing to sham-injected rats (Fig. 2b). Interestingly, a significant increase of OX-6-positive cells was found at 1 week in animals fed with blueberry- and spirulina-enriched diet ($P < 0.001$, $n = 5$, Fig. 2b). Four weeks after the lesion, the number of OX-6-positive microglial cells counted in blueberry and spirulina diet-fed animals was returned to values found in sham-injected animals. In lesioned animals treated with control diet, significantly increased number of OX-6-positive cells was found at 1 month (Figs. 1 and 2b). In areas posterior to the TH-negative zone, OX-6-positve cells were found in the myelinated fiber bundles crossing the striatum (Fig. 1b).

Iba1-immunoreactivity visualizes all microglial profiles, and thus much more Iba1-positive cells were found than OX-6-positive microglia. In all animals, the injection sites were marked by an increase in Iba1-positive microglia. In sham-operated animals, Iba1-immunoreactivity was concentrated to the injection site, while in lesioned animals, a sphere of increased density of Iba1-positive cells surrounding injection site had been formed (Fig. 3). This sphere with increased Iba1-immunoreactivity was located within the TH-negative area in the lesioned striatum. At 1 week postlesion, center portions of the striatum were densely packed with Iba1-positive microglia which displayed a rounded morphology while at more peripherally sites but still within the TH-negative area, Iba1-positive cells possessed processes (Fig. 3). These cells were also OX-6-immunoreactive, and found in animals fed with antioxidant-enriched diet (Fig. 3h). The density of Iba1-immunoreactive microglial cells was higher in areas for dopamine depletion than that seen on control sides. Thus, the Iba1-positve cell density was clearly reduced outside the area of OX-6-positive cells (Fig. 3e). Morphology

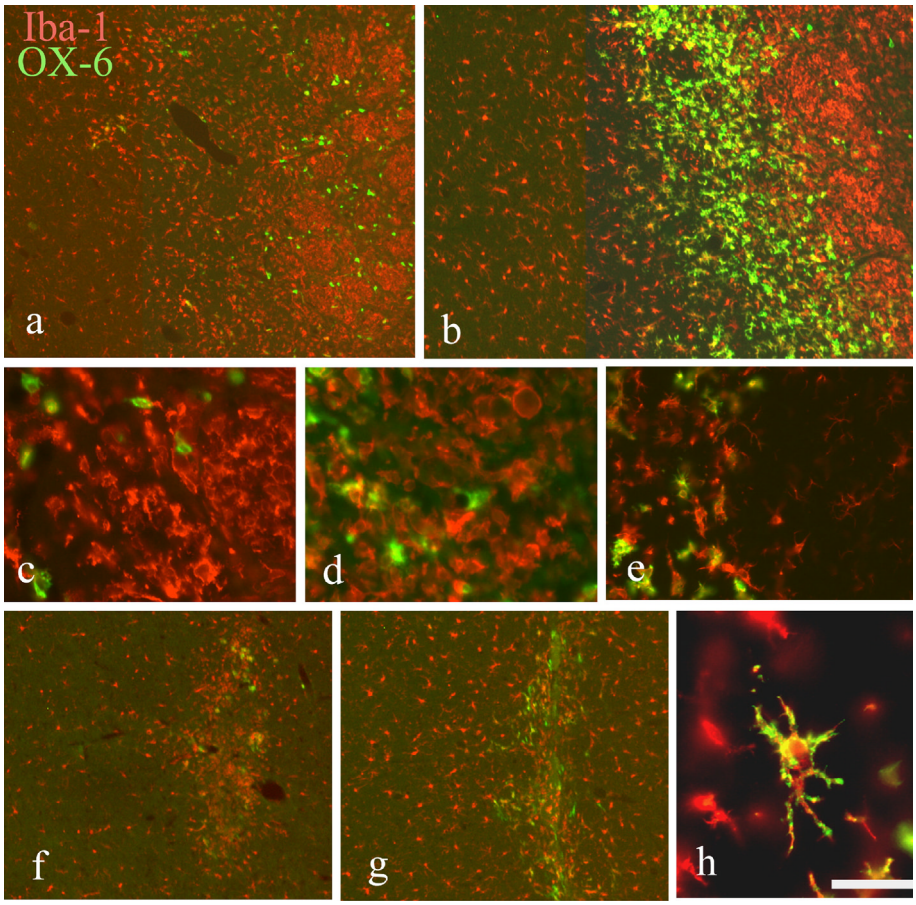

Fig. 3. Iba1- (red) and OX-6- (green) immunoreactivity in sham (f) or 6-OHDA lesioned (a–e, g, h) striata of animals fed with control diet (a, c, f), blueberry (b, d, e, h), or spirulina (g). At 1 week postlesion, dense Iba1-immunoreactivity was found close to the injection sites of control-fed (a) and blueberry-fed (b) animals (injection site to the right). Few OX-6-postive microglia were found in control-fed animals (a) while in blueberry-fed animals OX-6-postive cells were numerous (b). The pan-microglia marker Iba1 showed that positive microglia was rounded close to the injection sites in both control- (c) and blueberry- (d) fed animals and in the center portions of the lesions only few OX-6-positive microglia were found. OX-6-positive microglia was also Iba1-immunoreactive (h). The density of Iba1-immunoreactive cells was higher where also OX-6-positive microglia was found, i.e. within the TH-negative zone (see Fig. 1) such that a strict border was found between the dense and normal density of Iba1-positive cells (e). At 4 weeks after the lesion, Iba1-immunoreactivity did not differ in spirulina-fed animals (g) as compared to sham-injected controls (f). Scale bar: a, b, f, g = 200 μm; c and d = 50 μm; e = 40 μm; h = 25 μm.

of Iba1-positive microglia showed larger cell bodies and shorter processes than those Iba1-positive microglia that was located in the striatal areas remote from the dopamine lesion. Thus, microglia located within the TH-negative zone appeared activated while microglia in areas not hit by the dopamine depletion appeared resting. The numerous rounded Iba1-immunoreactive microglia seen in center portions of the lesion appeared larger in animals fed with antioxidants. These cells had disappeared in all animals at 1 month (Figs. 3f, g). At this time point, a reduction in Iba1-positive microglial profiles was found in animals fed with antioxidants, while in control diet-fed lesioned animals, Iba1-immunoreactivity was still intense.

The injection into the striatum caused increased density of GFAP-immunoreactivity in the injected striata. GFAP-immunoreactive cells were concentrated to the immediate vicinity of the needle track and did not leave any negative central zone as that seen with OX-6-immunoreactivity. GFAP-immunoreactivity induced by the injection showed the same density at injection site in all animals at 1 week after the lesion (Fig. 4). At 4 weeks after the dopamine depletion, a significant increase in GFAP-positive profiles was found in lesioned animals given control diet, while blueberry- and spirulina-treated animals showed no change compared to sham-injected animals or to the 1 week time point (Fig. 4).

Effects in the globus pallidus by dopamine denervation

A degeneration of TH-immunoreactive nerve fibers was found in the globus pallidus ipsilateral to the injected side in all lesioned animals independently of diet. Nerve fiber density was significantly reduced in all groups compared to sham-injected controls and no difference between diets was found. One month after the lesion, TH-positive nerve fiber density was not different between groups of 6-OHDA treatment, but TH-positive nerve fiber density in blueberry control diet and spirulina-fed animals was significantly reduced when comparing to sham-injected animals, while nerve fiber density in blueberry-fed animals was not significantly different from sham-

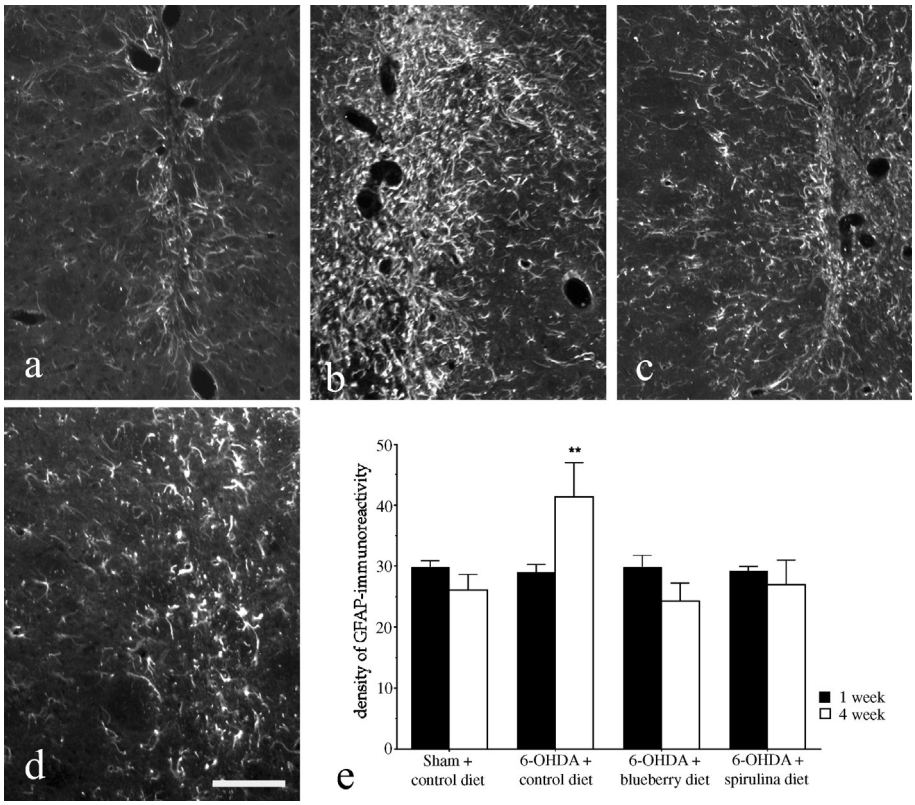

Fig. 4. GFAP-immunoreactivity in striata from sham (a) or 6-OHDA (b–d) -injected animals fed with control (a, b), blueberry (c), or spirulina (d) diets at 4 weeks after the lesion. An increase in density of GFAP-positive profiles was found in control-fed lesioned animals at 4 weeks ($P < 0.001$, $n = 5$), while animals treated with antioxidants did not differ from sham-injected controls (e). At 1 week after the injection, the density of GFAP-immunoreactivity was similar in all animals (e). Scale bar: a–d = 200 μm.

injected controls (Fig. 6a). Furthermore, a significant ($P < 0.001$, $n = 5$) increase in number of OX-6-positive microglia was found in the globus pallidus after dopamine lesions in animals treated with blueberry or spirulina at 1 week after the lesion (Fig. 6b). This increase was reversed at 4 weeks and no difference was found compared to sham-injected animals at this time point

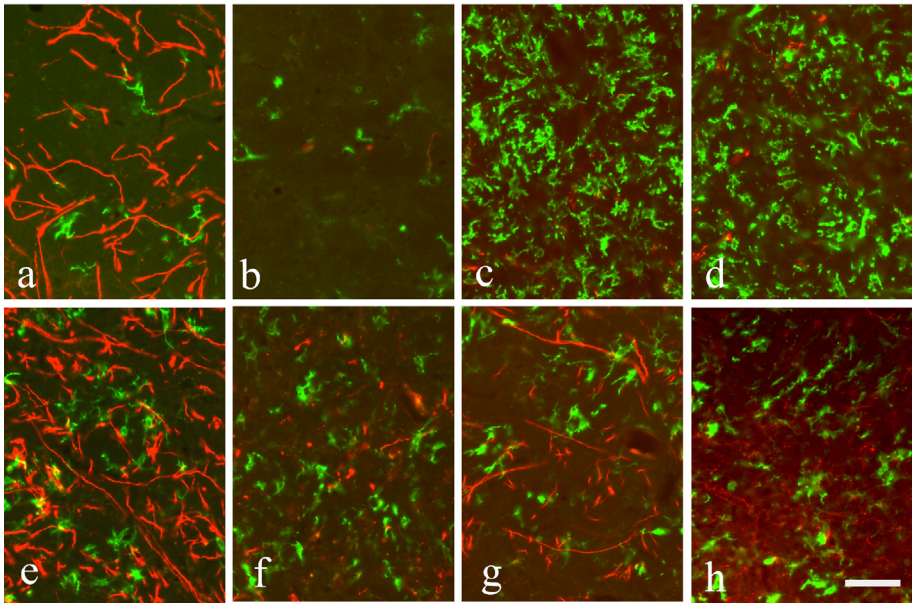

Fig. 5. TH (red) and OX-6 (green) in the globus pallidus of sham (a, e) or 6-OHDA (b–d, f–h) -injected animals treated with control diet (a, b, e, f), blueberry (c, g), or spirulina (d, h) at 1 week (a–d) or 4 weeks (e–h) after injection. TH-immunoreactivity was reduced in all animals given 6-OHDA at 1 week (b–d) while a recovery was found at 4 weeks in animals fed with blueberries (g). OX-6-positive profiles were dense in globus pallidus of animals fed with blueberry (c) and spirulina (d) at 1 week while in lesioned control animals (b) the number of OX-6-positive cells was similar to that found in sham-injected animals (a). At 4 weeks, the number of OX-6-positive cells was increased in control-fed lesioned animals (f), while in blueberry (g) and spirulina (h) treated striata, OX-6-positive cells did not differ from sham-injected controls (e). Scale bar: a–h = 200 μm.

(Figs. 5 and 6). However, in lesioned animals fed with control diet, a significant increase in OX-6-positive cells was found at 4 weeks compared to that measured at 1 week. The levels at 4 weeks in control diet-fed animals were not different from those measured in animals fed with antioxidant-enriched diet at 1 month (Fig. 6b). No OX-6-positive profiles were found in the control sides of any animals. In addition, no change in number of GFAP-positive astrocytes was found in globus pallidus ipsilateral to the injection in any animal.

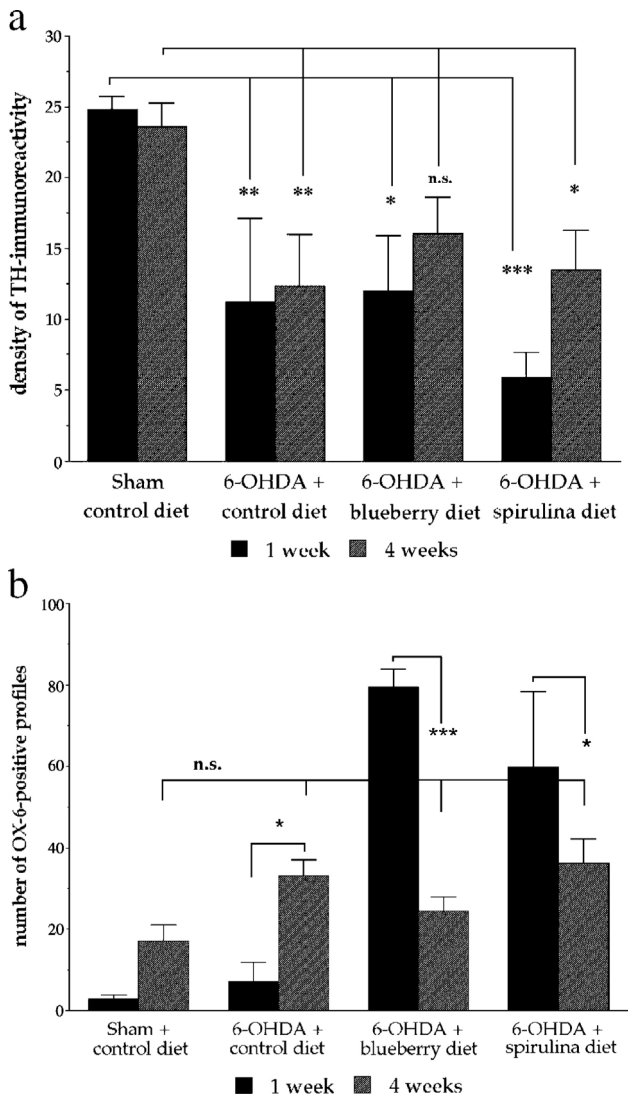

Fig. 6. The density of TH-immunoreactive nerve fibers in globus pallidus showed a significant reduction in all 6-OHDA lesioned animals at 1 week after the lesion when comparing to sham-injected controls (a). At 4 weeks, nerve fiber density had recovered in blueberry-fed animals as compared to sham-injected controls, but not when comparing to the other lesioned animals (a). There was no significant recovery between 1 and 4 weeks time points when comparing within the same treatment (a). The number of OX-6-positive cells in globus pallidus followed the same pattern as that seen in the striatum, i.e. a slight increase in dopamine lesioned control animals and a significant increase in animals that received blueberry or spirulina at 1 week (b). At 4 weeks, the increase in antioxidant-fed animals was reversed and not significantly different from sham-controls, while a significant increase was found in control-fed lesioned animals (b). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, n.s. not significant, $n = 5$ for each group.

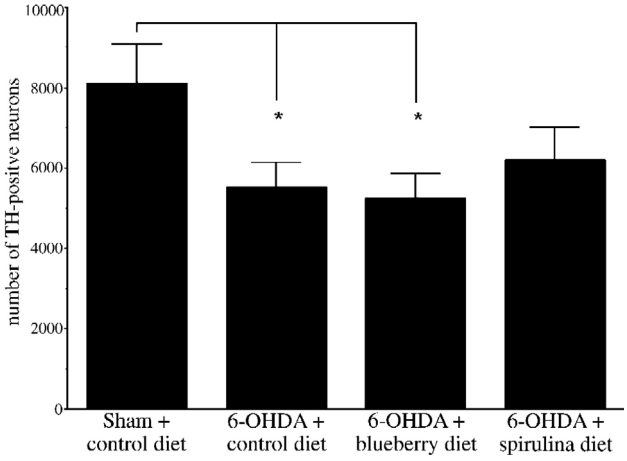

Fig. 7. Stereological cell counts of TH-positive neurons in the substantia nigra ipsilateral to the lesion or sham injection at 4 weeks after injection. The number of TH-positive neurons was reduced in all lesioned animals while a significance was found in control and blueberry-fed animals ($P < 0.05$ vs. sham control, $n = 5$).

Effects of striatal dopamine denervation in the substantia nigra

Cell counts of TH-positive neurons using stereology revealed a significant reduction in number of neurons in control- and blueberry-fed animals as compared to sham-injected animals at 4 weeks after the lesion ($P < 0.05$; Fig. 7). Treatment with spirulina resulted in fewer but not statistically different number of TH-immunoreactive neurons at 1 month. However, none of the lesioned animals differed individually.

Discussion

This study shows that diet enriched in phytochemicals with anti-inflammatory and/or antioxidant properties such as blueberry and spirulina causes a rapid but transient increase of OX-6-positive microglia in the striatum and the globus pallidus of intrastriatally injured animals, while the dopamine lesion per se did not differ in size at 1 week. Thus, 1 month after the lesion, the antioxidant-treated animals had normalized their OX-6-positive microglia levels while an increase was now shown in control-fed animals. At this time point, recovery of striatal TH-positive nerve fibers had occurred only in animals fed with blueberry and spirulina while a loss of dopamine neurons in the substantia nigra was obvious in all lesioned animals. The enhanced recovery of striatal dopamine innervation is in accordance with previous studies showing that other antioxidants in combination with dopamine lesions will preserve striatal dopamine levels (Datla et al., 2004). In addition, using ginseng to reduce oxidative stress promotes survival of ventral mesencephalic dopamine neurons after MPTP lesions (Van Kampen et al., 2003). Furthermore, blocking the cyclooxygenase (COX) pathway using COX-2 inhibitors will result in significant regeneration of TH-positive nerve fibers (Sánchez-Pernaute et al., 2004). One active compartment of *Spirulina platensis* is c-phycocyanin, which inhibits COX-2 (Reddy et al., 2000). Thus,

recovery of striatal TH-positive nerve fibers after treatment with antioxidants such as spirulina seen in this study has support from other reports.

Using the COX-2 inhibitor to rescue dopamine neurons from injury will result in fewer activated microglia (Du et al., 2001; He et al., 2001; Sánchez-Pernaute et al., 2004; Wu et al., 2002). While a clear increase using the pan-microglia marker Iba1 was found in all lesioned animals in the present study, a robust increase of OX-6-positive microglial cells early after the lesion was associated with enhanced striatal TH-positive recovery. Thus, the outcome was reversed from what was predicted. Interestingly, using the antisense to TNFα to supress OX-6 expression in microglia during the initial phase after dopamine lesion will result in larger dopamine depletions. In contrary, inhibiting TNFα and microglia activation at a later stage results in smaller dopamine lesions (Gemma et al., 2003). Thus, the rapid increase of activated microglia early after injury may be beneficial to neural repair. The reason for this is still unknown, but the rapid onset of OX-6-positive microglia in animals fed with antioxidant-rich diet might improve phagocytosis and prepare for a rapid onset of regeneration. These suggestions are not proven in this study; however, it has been shown in other brain areas that rapid and transient onset of microglia activation or adding microglia into an injured area may enhance neuronal survival and/or regeneration, while a long-term microglial reaction results in neuronal death (Hollerbach et al., 1998; Rabchevsky and Streit, 1997).

The rapid onset of OX-6-positive microglia in the globus pallidus was present in animals treated with the antioxidant-enriched diet, which is in accordance with what was found in the striatum. Obviously, the dopaminergic collaterals from the nigrostriatal pathway were also affected by the striatal lesion and resulted in dopamine denervation of the globus pallidus. Interestingly, the normalization of OX-6-positive microglia at 1 month after the lesion had effect on recovery of dopamine nerve fibers only in blueberry treated animals when comparing to sham-injected controls, although the effect was not convincing since nerve fiber density did not differ in any of the lesioned animals and no significant recovery was found between 1 and 4 week time points in any of the different treatments. Poor regeneration of globus pallidus has been noticed before. In grafting studies, graft-derived reinnervation avoids targeting the globus pallidus and instead forms nerve fiber bundles that pass peripherally to the globus pallidus rather than crossing through it (Strömberg et al., 1992, 2001). What reduces the capacity of globus pallidus to achieve regenerating dopamine nerve fibers is still unknown, but the robust onset of OX-6-positive microglia did not appear to promote regeneration in globus pallidus as that seen in the striatum.

The intrastriatal dopamine lesion model is characterized by a slow, progressive neurodegeneration of ventral mesencephalic dopamine neurons (Sauer and Oertel, 1994). Although it appears that the neurotoxic effect is maximal already at 48 h after the lesion (Mladenovic et al., 2004; Zuch et al., 2000), a significant loss of TH-positive neurons is not obvious before 6 days after the striatal lesion (Martí et al., 2002). At 4 weeks, there was a reduction in all lesioned animals except for those treated with spirulina diet. However, the spirulina-treated animals did not differ from the other lesioned animals and thus the antioxidant-rich diet did not appear to protect at the cellular level, but at the nerve terminal level as seen in the striatum. Since it has been shown that striatal 6-OHDA injection induces apoptosis in the nigral neurons, and that both TH and fluorogold prelabeling of nigral neurons disappear, it is likely that the nigral neurons undergo cell death (Emsley et al., 2001; Martí et al., 2002; Mladenovic et al., 2004; Rosenblad et al., 2000). Thus, the recovery of TH-positive nerve fibers seen in the striatum in animals treated with antioxidant-rich diet, was probably due to sprouting from non-injured dopamine neurons rather from regenerating rescued dopamine neurons or upregulation of lost TH synthesis.

The density of GFAP-positive astrocytes was calculated from the area of injection sites. Thus, the injection per se did cause an astrocytic reaction equal to that found in the lesioned animals independent of diet at 1 week after injection. However, when comparing the astrocytic levels remote from the injection sites but still ipsilateral to the lesion, an upregulation of reactive astrocytes after both 6-OHDA and MPTP lesions has previously been shown (Strömberg et al., 1986). However, increased density of GFAP had occurred at 1 month after the lesion in animals that received control diet compared to that measured in sham-injected controls and antioxidant treated 6-OHDA-injected animals. It is known that reactive astrocytes may produce proinflammatory cytokines such as TNFα (Dong, 2001). TNFα is toxic to dopaminergic neurons and thus might have prevented regeneration in control-fed animals (McGuire et al., 2001). Furthermore, TNFα mRNA levels are increased in the striatum after a dopamine lesion (Mladenovic et al., 2004). Interestingly, animals fed with diet enriched in antioxidants reduce their TNFα levels (Cartford et al., 2002), which might explain the dopaminergic regeneration within the striatum.

In summary, the size of the dopamine denervation was similar in all lesioned animals at 1 week, and therefore we may conclude that the dopamine recovery was enhanced in animals fed with blueberries or spirlina. Microglia was densely packed in the close vicinity to the injection site as seen by the presence of the pan-microglia marker Iba1, but microglia expressing OX-6 were significantly increased in the striatum as well as in the globus pallidus in animals on antioxidant-rich diet early after the lesion. At 4 weeks, the number of OX-6-positive cells was reversed, while at this time point, an increase was found in control diet-fed animals.

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References

Bickford, P., Shukitt-Hale, B., Joseph, J., 1999. Effects of aging on cerebellar noradrenergic function and motor learning: nutritional interventions. Mech. Ageing Dev. 111, 141–154.

Bickford, P., Gould, T., Briederick, L., Chadman, K., Pollock, A., Young, S., Shukitt-Hale, B., Joseph, J., 2000. Antioxidant-rich diets improve cerebellar physiology and motor learning in aged rats. Brain Res. 866, 211–217.

Cartford, M.C., Gemma, C., Bickford, P., 2002. Eighteen-month-old Fischer 344 rats fed a spinach-enriched diet show improved delay classical eyeblink conditioning and reduced expression of tumor necrosis factor α (TNFα) and TNFβ in the cerebellum. J. Neurosci. 2002, 2816–5813.

Choi, W.S., Yoon, S.Y., Oh, T.H., Choi, E.J., O'Malley, K.L., Oh, Y.J., 1999. Two distinct mechanisms are involved in 6-hydroxydopamine- and MPP^+-induced dopaminergic neuronal cell death: role of caspases, ROS, and JNK. J. Neurosci. Res. 57, 86–94.

Cohen, G., Heikkila, R.E., 1974. The generation of hydrogen peroxide, superoxide radical, and hydroxyl radical by 6-hydroxydopamine, dialuric acid, and related cytotoxic agents. J. Biol. Chem. 249, 2447–2452.

Datla, K.P., Bennett, R.D., Zbarsky, V., Ke, B., Liang, Y.-F., Higa, T., Bahorun, T., Aruoma, O.I., Dexter, D.T., 2004. The antioxidant drink "effective microorganism-x (EM-X)" pre-treatment attenuates the loss of nigrostriatal dopaminergic neurons in 6-hydroxydopamine-lesion rat model of Parkinson's disease. J. Pharm. Pharmacol. 56, 649–654.

de Lorgeril, M., Renaud, S., Salen, P., Monjaud, I., Mammelle, N., Martain, J.L., Guidollet, J., Touboul, P., Delaye, J., 1994. Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. Lancet 343, 1454–1459.

Depino, A.M., Earl, C., Kaczmarczyk, E., Ferrari, C., Besedovsky, H., del Rey, A., Pitossi, F.J., Oertel, W.H., 2003. Microglial activation with atypical proinflammatory cytokine expression in a rat model of Parkinson's disease. Eur. J. Neurosci. 18, 2731–2742.

Dong, Y., 2001. Immune function of astrocytes. Glia 36, 180–190.

Du, Y., Ma, Z., Lin, S., Dodel, R.C., Gao, F., Bales, K.R., Triarhou, L.C., Chernet, E., Perry, K.W., Nelson, D.L.G., Luecke, S., Phebus, L.A., Bymaster, F.P., Paul, S.M., 2001. Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. Proc. Natl. Acad. Sci. 98, 14669–14674.

Emsley, J.G., Lu, X., Hagg, T., 2001. Retrograde tracing techniques influence reported death rates of adult rat nigrostriatal neurons. Exp. Neurol. 168, 425–433.

Gemma, C., Catlow, B., Hudson, C., Bickford, P.C., 2003. Knockdown TNFa with antisense in 6-hydroxydopamine lesioned rats. Abstr. Am. Soc., 410.416.

He, Y., Appel, S., Le, W., 2001. Minocycline inhibits microglial activation and protects nigral cells after 6-hydroxydopamine injection into mouse striatum. Brain Res. 909, 187–193.

Hollerbach, E.H., Haas, C.A., Hildebrandt, H., Frotscher, M., Naumann, T., 1998. Region-specific activation of microglial cells in the rat septal complex following fimbria–fornix transection. J. Comp. Neurol. 390, 481–496.

Joseph, J.A., Shukitt-Hale, B., Denisova, N.A., Prior, R., Cao, G., Martin, A., Taglialatela, G., Bickford, P., 1998. Long-term dietary strawberry, spinach, or vitamin E supplementation retards the onset of age-related neuronal signal-transduction and cognitive behavioral deficits. J. Neurosci. 18, 8047–8055.

Joseph, J.A., Shukitt-Hale, B., Denisova, N.A., Bielinski, D., Martin, A., McEwen, J.J., Bickford, P.C., 1999. Reversal of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. J. Neurosci. 19, 8114–8121.

Kanazawa, I., 2001. How do neurons die in neurodegenerative diseases? Trends Mol. Med. 7, 339–344.

Loddick, S.A., Rothwell, N.J., 1999. Mechanisms of tumor necrosis factor alpha action on neurodegeneration: interaction with insulin-like growth factor-1. Proc. Natl. Acad. Sci. 96, 9449–9451.

Lotharius, J., Dugan, L.L., O'Malley, K.L., 1999. Distinct mechanisms underlie neurotoxin-mediated cell death in cultured dopaminergic neurons. J. Neurosci. 19, 1284–1293.

Martí, M.J., Saura, J., Burke, R.E., Jackson-Lewis, V., Jiménez, A., Bonastre, M., Tolosa, E., 2002. Striatal 6-hydroxydopamine induces apoptosis of nigral neurons in the adult rat. Brain Res., 185–191.

Martin, A., Prior, R., Shukitt-Hale, B., Cao, G., Joseph, J.A., 2000. Effects of fruits, vegetables, or vitamin E-rich diet on vitamins E and C distribution in peripheral and brain tissue: implications for brain function. J. Gerontol. 55, B144–B151.

McGuire, S.O., Ling, Z.D., Lipton, J.W., Sortwell, C.E., Collier, T.J., Carvey, P.M., 2001. Tumor necrosis factor a is toxic to embryonic mesencephalic dopamine neurons. Exp. Neurol. 169, 219–230.

Meydani, M., 1999. Dietary antioxidants modulation of aging and immune-endothelial cell interaction. Mech. Ageing Dev. 111, 123–132.

Mladenovic, A., Perovic, M., Raicevic, N., Kanazir, S., Rakic, L., Ruzdijic, S., 2004. 6-Hydroxydopamine increases the level of *TNFα* and *bax* mRNA in the striatum and induces apoptosis of dopaminergic neurons in hemi-parkinsonian rats. Brain Res. 996, 237–245.

Mogi, M., Harada, M., Riederer, P., Narabayashi, H., Fujita, K., Nagatsu, T., 1994. Tumor necrosis factor alpha (TNF-alpha) increases both in the brain and in the cerebrospinal fluid from Parkinsonian patients. Neurosci. Lett. 165, 208–210.

Mogi, M., Harada, M., Kondo, T., Narabayashi, H., Riederer, P., Nagatsu, T., 1995. Transforming growth factor β-1 levels are elevated in the striatum and in ventricular cerebrospinal fluid in Parkinson's disease. Neurosci. Lett. 193, 129–132.

Mogi, M., Togari, A., Tanaka, K.-I., Ogawa, N., Ichinose, H., Nagatsu, T., 1999. Increase in level of tumor necrosis factor (TNF)-α in 6-hydroxydopamine-lesioned striatum in rats without influence of systemic L-DOPA on the TNF-α induction. Neursci. Lett. 268, 101–104.

Rabchevsky, A.G., Streit, W.J., 1997. Grafting of cultured microglial cells into the lesioned spinal cord of adult rats enhances neurite outgrowth. J. Neurosci. Res. 47, 34–48.

Reddy, C.M., Bhat, V.B., Kiranmai, G., Reddy, M.N., Reddanna, P., Madyastha, K.M., 2000. Selective inhibition of cyclooxygenase-2 by C-phycocyanin, a biliprotein from *Spirulina platensis*. Biochem. Biophys. Res. Commun. 277, 599–603.

Rosenblad, C., Kirik, D., Björklund, A., 2000. Sequential administration of GDNF into the substantia nigra and striatum promotes dopamine neuron survival and axonal sprouting but not striatal reinnervation or functional recovery in the partial 6-OHDA lesion model. Exp. Neurol. 161, 503–516.

Sánchez-Pernaute, R., Ferree, A., Cooper, O., Yu, M., Brownell, A.-L., Isacson, O., 2004. Selective COX-2 inhibition prevents progressive dopamine neuron degeneration in a rat model of Parkinson's disease. J. Neuroinflammation 1, 6.

Sauer, H., Oertel, W.H., 1994. Progressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6-hydroxydopamine: a combined retrograde tracing and immunocytochemical study in the rat. Neuroscience 59, 401–415.

Strömberg, I., Björklund, H., Dahl, D., Jonsson, G., Sundström, E., Olson, L., 1986. Astrocyte responses to dopaminergic denervations by 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine as evidenced by glial fibrillary acidic protein immunohistochemistry. Brain Res. Bull. 17, 225–236.

Strömberg, I., Bygdeman, M., Almqvist, P., 1992. Target-specific outgrowth from human mesencephalic tissue grafted to cortex or ventricle of immunosuppressed rats. J. Comp. Neurol. 315, 445–456.

Strömberg, I., Törnqvist, N., Johansson, S., Bygdeman, M., Almqvist, P., 2001. Evidence for a target-specific outgrowth from subpopulations of grafted human dopamine neurons. Microsc. Res. Tech. 54, 287–297.

Trichopoulou, A., Costacou, T., Bamia, C., Trichopoulos, D., 2003. Adherence

to a Mediterranean diet and survival in a Greek population. N. Engl. J. Med. 348, 2599–2608.

Van Kampen, J., Robertson, H., Hagg, T., Drobitch, R., 2003. Neuroprotective actions of the ginseng extract G115 in two rodent models of Parkinson's disease. Exp. Neurol. 2003, 521–529.

West, M.J., Slomianka, L., Gundersen, H.G.J., 1991. Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. Anat. Rec. 231, 482–497.

Willet, W.C., Sacks, F., Trichopoulou, A., Drescher, G., Ferro-Luzzi, A., Helsing, E., Trichopoulos, D., 1995. Mediterranean diet pyramid: a cultural model for healthy eating. Am. J. Clin. Nutr. 61, 1402S–1406S.

Wu, D.C., Jackson-Lewis, V., Vila, M., Tieu, K., Teisman, P., Vadseth, C., Choi, D.-K., Ischiropoulos, H., Przedborski, S., 2002. Blockade of microglia activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. J. Neurosci. 22, 1763–1771.

Zuch, C.L., Nordstroem, V.K., Briedrick, L.A., Hoernig, G.R., Granholm, A.-C., Bickford, P.C., 2000. Time course of degenerative alterations in nigral dopaminergic neurons following a 6-hydroxydopamine lesion. J. Comp. Neurol. 427, 440–454.