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Enriched environment protects the nigrostriatal dopaminergic system and induces astroglial reaction in the 6-OHDA rat model of Parkinson's disease

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Abstract

Enriched environment (EE) is neuroprotective in several animal models of neurodegeneration. It stimulates the expression of trophic factors and modifies the astrocyte cell population which has been said to exert neuroprotective effects. We have investigated the effects of EE on 6-hydroxydopamine (6-OHDA)-induced neuronal death after unilateral administration to the medial forebrain bundle, which reaches 85–95% of dopaminergic neurons in the substantia nigra after 3 weeks. Continuous exposure to EE 3 weeks before and after 6-OHDA injection prevents neuronal death (assessed by tyrosine hydroxylase staining), protects the nigrostriatal pathway (assessed by Fluorogold retrograde labeling) and reduces motor impairment. Four days after 6-OHDA injection, EE was associated with a marked increase in glial fibrillary acidic protein staining and prevented neuronal death (assessed by Fluoro Jade-B) but not partial loss of tyrosine hydroxylase staining in the anterior substantia nigra. These results robustly demonstrate that EE preserves the entire nigrostriatal system against 6-OHDA-induced toxicity, and suggests that an early post-lesion astrocytic reaction may participate in the neuroprotective mechanism.

Keywords: 6-hydroxydopamine, astrocytes, enriched environment, neuroprotection.

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Parkinson's disease (PD) is an age-related neurodegenerative disorder characterized by a progressive loss of dopamine neurons in the nigrostriatal system (Dauer and Przedborski 2003). Administration of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle (MFB) results in selective death of dopaminergic neurons in the midbrain, nearly total depletion of DA in the ipsilateral striatum, denervation supersensitivity of the post-synaptic striatal DA receptors, and a characteristic turning behavior in response to both D-amphetamine and apomorphine (Ungerstedt 1968; Dauer and Przedborski 2003). This model has been extensively used to search for putative neuroprotectants (Hyman *et al.* 1991; Kearns *et al.* 1997; Shults *et al.* 2000; Peterson and Nutt 2008).

The etiology of PD and factors affecting its progression are still not well understood, but epidemiological data suggest that lifestyle may influence these (Olanow and Tatton 1999). For example, the risk of PD might be influenced by educational achievement or occupation (Frigerio *et al.* 2005) as well as by physical exercise (Chen *et al.* 2005; Thacker *et al.* 2008). Both intellectual stimulation and physical activity can be modeled in experimental settings by the use of enriched environments (EE). EE is defined as a sustained and progressive increase in cognitive and sensorimotor stimuli with aggregated voluntary physical activity

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Abbreviations used: BDNF, brain-derived neurotrophic factor; EE, enriched environment; FJB, Fluoro Jade-B; FG, Fluorogold; GDNF, glia cell-line derived neurotrophic factor; GFAP, glial fibrillary acidic protein; MFB, medial forebrain bundle; OHDA, 6-hydroxydopamine; PD, Parkinson's disease; SN, substantia nigra; SNpc, substantia nigra pars compacta; TFs, trophic factors; TH, tyrosine hydroxylase.

and complex social interactions. Abundant experimental evidence shows that EE is beneficial in animal models of schizophrenia, amyotrophic lateral sclerosis, epilepsy, stroke, and Huntington's and Alzheimer's disease (Nithianantharajah and Hannan 2006; Laviola *et al.* 2008). EE also induces neuroprotection against MPTP toxicity to mice (Bezard *et al.* 2003; Faherty *et al.* 2005) and improves motor function after unilateral 6-OHDA injection in rats (Jadavji *et al.* 2006). The mechanism of EE neuroprotection is unknown, but numerous data suggest that synthesis and release of trophic factors (TFs) may play a crucial role (Nithianantharajah and Hannan 2006).

Enriched environment alters the expression of TFs and their receptors in several brain areas (Pham *et al.* 1999; Ickes *et al.* 2000; van Praag *et al.* 2000; Gobbo and O'Mara 2004; Spires *et al.* 2004) and induces astrogliogenesis (Steiner *et al.* 2006). In the nigrostriatal system, EE-housed animals show increased brain-derived neurotrophic factor (BDNF) expression in the striatum (Bezard *et al.* 2003; Turner and Lewis 2003) and glia cell-line derived neurotrophic factor (GDNF) mRNA in the substantia nigra (SN) (Faherty *et al.* 2005).

In response to almost any kind of CNS injury, at different times post-injury, astrocytes change their appearance and undergo a characteristic hypertrophy of their cellular processes, referred to as reactive astrocytes, astrocytic reaction, or astrogliosis. One of the hallmarks of this phenomenon is the up-regulation of the intermediate filament protein glial fibrillary acidic protein (GFAP). Astrogliosis is associated with synthesis and secretion of endogenous TFs (Schwartz and Nishiyama 1994; Chadi and Gomide 2004; Escartin and Bonvento 2008). It has been suggested that astrocytes exert a neuroprotective action *in vitro* and *in vivo* (Liberto *et al.* 2004; Kipp *et al.* 2006; Escartin and Bonvento 2008). Following unilateral 6-OHDA injections, a partially protective response is triggered by ipsilateral astrocytes detected by expression of GFAP (Gomide *et al.* 2005a,b; Henning *et al.* 2008) and striatal astrogliogenesis (Aponso *et al.* 2008), although the opposite finding (i.e., a decrease in newborn astrocytes) has also been reported (Steiner *et al.* 2006).

We recently demonstrated that electroconvulsive shock protects the nigrostriatal system from 6-OHDA-induced degeneration, possibly by increasing endogenous GDNF level (Anastasía *et al.* 2007). Likewise, an orally active trophic factor inducer exerts a neuroprotective role in the nigrostriatal system (Visanji *et al.* 2008). Thus, modulation of endogenous protective or repair mechanisms, such as increasing neuronal or glial protective factors, appears a promising strategy against degeneration of dopaminergic neurons in PD.

In this study, we tested whether EE housing protects the dopaminergic nigrostriatal system against 6-OHDA neurotoxicity. We present strong evidence that EE maintains anatomical and functional integrity in the nigrostriatal system and provide evidence suggesting that protection is mediated by astrocyte activation in the SN.

Materials and methods

Animals

Adult male Wistar rats (Instituto Ferreyra, Córdoba, Argentina) weighing 100/130 g were housed with a 12-hour light/dark cycle with water and food *ad libitum*. Experiments were performed according to the standard set by the guide for the care and use of laboratory animals (National Academy Press 1996).

Experimental design

Animals were randomly assigned in two groups: standard condition or EE housing for 21 days; on day 21 animals received unilateral injections of 6-OHDA (or vehicle) in the MFB and were returned to their previous housing condition until being killed 4 or 21 days after the 6-OHDA administration:

Animals killed 21 days after 6-OHDA injection were assessed for the following: (i) percentage of tyrosine hydroxylase (TH) immunostained neurons in the substantia nigra pars compacta (SNpc), (ii) the integrity of the nigrostriatal fibers by retrograde labeling of SNpc neurons after injection of Fluorogold (FG) in the striatum, and (iii) motor D-amphetamine-induced (rotatory) behavior.

Animals killed 4 days after 6-OHDA injection were assessed for the following: (i) neurodegeneration of SNpc neurons by Fluoro Jade-B (FJB) staining, (ii) percentage of TH immunostained neurons in the SNpc, (iii) neuron number in the SNpc by cresyl violet staining, (iv) nigral neuron number assessed by FG labeling, and (v) expression of GFAP protein levels in the SNpc and striatum.

Integrity of nigrostriatal fibers was assessed in animals maintained in EE or standard condition for 21 days before and 14 days after receiving a 6-OHDA injection. On day 14 post-lesion, animals were bilaterally injected with FG in the striatum and returned to their respective housing.

Animals were killed 7 days later to allow sufficient retrograde labeling of SNpc neurons. Another methodology to determine nigral dopaminergic neuron number is labeling these neurons by retrograde transport of FG (bilaterally injected in the striatum) 1 week before the 6-OHDA. Four days after the toxin administration, animals were killed and processed for FG immunohistochemistry.

Enriched environment housing

The enriched cage (95 cm × 60 cm × 70 cm, two floors) contained tubes, shelves, ramps, stairs, and miscellaneous 'toys' (hard plastic balls, cubes, cones, and sticks) that were changed twice a week to continuously encourage exploration of the environment. The complexity (number of objects) of the housing facility was increased progressively: every 2 days, two to four objects were added to the environment. Ten days after housing animals in the EE, complexity was maximal but the positions of the objects continued to be changed every 2 days. Ten to twelve 1-month-old rats (100–130 g) rats were housed together to allow social interactions. Free running wheels were provided but individual animal activity was not monitored. The standard condition (control condition) consisted of

45 cm × 30 cm × 18 cm cages without objects or running wheels, housing three animals per cage.

6-hydroxydopamine injection

Anesthetized rats (Ketamine 55 mg/kg + Xylazine 11 mg/kg, i.p.; König, Buenos Aires, Argentina) were placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) and injected with 6-OHDA (4 μL over 8 min, 2 μg/μL in 0.02% ascorbic acid in 0.9% NaCl; Sigma, St. Louis, MO, USA) in the MFB [from bregma: anterior-posterior (AP): −1.8 mm; lateral (L): +2 mm; and ventral (V): −8.1 mm] (Paxinos and Watson 1986); the cannula was removed 5 min later. Sham-operated animals received the vehicle. All surgeries for a given experiment were carried out in the same session by a blinded operator.

Amphetamine-induced rotation test

Behavioral testing was performed 3 weeks after 6-OHDA. After acclimation to an individual cage, D-amphetamine (5 mg/kg in 0.9% NaCl, i.p.; Sigma) was administered, and the total number of full ipsilateral turns (to the left in our case) was recorded for 60 min. Twenty four hours after the rotational test, animals were perfused for TH immunohistochemistry.

Retrograde labeling with Fluorogold to study the nigrostriatal pathway

Rats received bilateral FG injections (0.5 μL, 2% FG; Fluorochrome, Denver, CO, USA) into the striatum (from bregma: AP: +1 mm; L: ±3 mm; and V: −5.0 mm). Retrograde labeling of SN neurons was assessed by immunohistochemistry 1 week later.

Retrograde labeling with Fluorogold to study nigral neuron number

One week before the unilateral 6-OHDA injection, rats received bilateral FG injections as described previously. Then, the animals were injected with 6-OHDA and killed 4 days after the toxin administration. FG labeling was assessed by immunohistochemistry.

Immunohistochemistry and analysis

Anesthetized animals (400 mg/kg chloral hydrate, i.p.; Parafarm, Buenos Aires, Argentina) were transcardially perfused with saline (glucose 0.4%, sucrose 0.8%, NaCl 0.8%; Sigma) followed by paraformaldehyde (4% in 0.36% sodium borate, 0.1% boric acid with 44 mg/L of sodium sulfite; Sigma), and brains removed, post-fixed, cryoprotected, and sectioned in a cryomicrotome (36 μm). Free-floating coronal sections were blocked (5% bovine serum albumin; Sigma, or non-fat dry milk), incubated with primary antibody (anti-TH 1 : 1000 for 24 h at 4°C; Chemicon, Temecula, CA, USA; or anti-FG 1 : 1600 for 72 h at 4°C, Chemicon), followed by secondary biotin-conjugated antibody (2 h at 20–25°C 1 : 250, Sigma), and developed with streptavidin peroxidase (2 h at 20–25°C 1 : 250, Sigma) using diaminobenzidine (Sigma) as the chromagen. Bright field images were captured digitally (Eclipse TE2000-U microscope, Digital Sight DS-U1 camera, Nikon, Tokyo, Japan).

Counts of FG-labeled and TH-positive cells in the SNpc were performed as previously described (Sauer and Oertel 1994). TH-immunostained cells in the SNpc (lateral to the medial terminal nucleus of the accessory optic tract) were counted bilaterally in six sections/animal (−4.52, −4.8, −5.2. −5.6, −6.04, and −6.3 mm from bregma) (Paxinos and Watson 1986). Neuronal survival 21 days after the 6-OHDA in this area was expressed as the percentage of TH immunoreactive neurons on the lesioned side, with respect to the contralateral intact side; this approach was chosen to avoid methodological biases because of interindividual differences, and is widely used to assess the extent of 6-OHDA-induced lesion in the SNpc 3 weeks post-lesion, where the expression of TH was highly correlated with the number of surviving neurons (Kirik *et al.* 2000; Paul *et al.* 2004). To further ensure the absence of bias in cell counting, the operator was blinded to the treatment group during the analysis.

Tyroxine hydroxalase immunoreactive cell counts 4 days post-lesion were assessed in the same manner although the number of cells may not reveal the actual nigral dopaminergic neuron number because phenotype (TH expression) was lost early after 6-OHDA (see Results).

For double-labeling immunohistochemistry, mouse anti-TH antibody (24 h at 4°C, 1 : 1000, Chemicon) was combined with anti-GFAP antibody (24 h at 4°C, 1 : 5, Immunostar, Inc, Hudson, WI, USA) followed by appropriate fluorescent secondary antibodies (Alexa Fluor 568 anti-rabbit IgG, 1 : 200 or Alexa Fluor 488 anti-mouse IgG, 1 : 200; Molecular Probes, Carlsbad, CA, USA). Negative controls without primary antibodies were performed for each immunodetection. Sections were assessed blindly under fluorescence microscopy (Eclipse TE2000-U, Nikon). Images were captured digitally (Digital Sight DS-U1, Nikon). After TH/GFAP double-labeling immunohistochemistry, SNpc was identified precisely by stacking the TH images onto GFAP-labeled images and delimiting the region of interest based on the TH staining (white line). GFAP gray level was analyzed by densitometry in the region of interest (SNpc) (Image J 1.34j; US National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997–2008).

Fluoro Jade-B staining

The number of degenerating neurons 4 days after 6-OHDA was assessed by labeling with FJB (Histo-Chem Inc, Arkansas, KN, USA) according to the published protocol (Schmued and Hopkins 2000). Labeled neurons were counted in six different sections of the SN. Epifluorescent images were captured digitally (Eclipse TE2000-U microscope, Digital Sight DS-U1 camera, Nikon). The number of FJB+ cells was obtained by averaging three sections for the anterior SNpc and posterior SNpc. Quantification did not aim to estimate the total number of injured neurons in a given brain area but to quantitatively compare the degree of injury among different treatments.

Cresyl violet staining

Briefly, 36-μm sections were mounted in alcohol–gelatin solution on gelatinized slides, dried overnight at 37°C, and hydrated to distilled water. Slides were then incubated in cresyl violet acetate 0.5% (Sigma) for 3 min, differentiated in 70% alcohol, dehydrated, cleared in xylene, and coverslipped.

Statistical analysis

After data transformation by the arc cosine of the square root of the ratio, a Student's *t*-test was performed for statistical comparison between number of TH-labeled neurons 21 days after the 6-OHDA.

For behavioral data statistical analysis, a one-way ANOVA with Dunnett's *post hoc* test analysis was performed. Other comparisons were performed by the non-parametric Kruskal–Wallis test. Results are provided as the mean ± SEM.

Results

Enriched environment housing promotes survival of dopaminergic neurons in the substantia nigra

The effect of EE on dopaminergic neuronal loss in the SNpc following unilateral injection of 6-OHDA was examined at 21 days. In standard housed animals, neuronal loss was nearly complete by this time (survival = 9.62 ± 3.82% of neurons of the contralateral side) (Fig. 1a–c), consistent with published data (Dowd *et al.* 2005). EE-housed animals had remarkably less neuronal loss (survival = 55.76 ± 7.48% of neurons of the contralateral side) (Fig. 1a–c). Qualitative assessment of TH staining in the striatum was consistent with a protective effect of EE on dopaminergic fibers (Fig. 1d), suggesting that the neuroprotection was not restricted to the neuronal soma. This observation was specifically tested with retrograde labeling (see below in the following result section). There were no significant differences in ipsilateral/contralateral ratios between the six different sections.

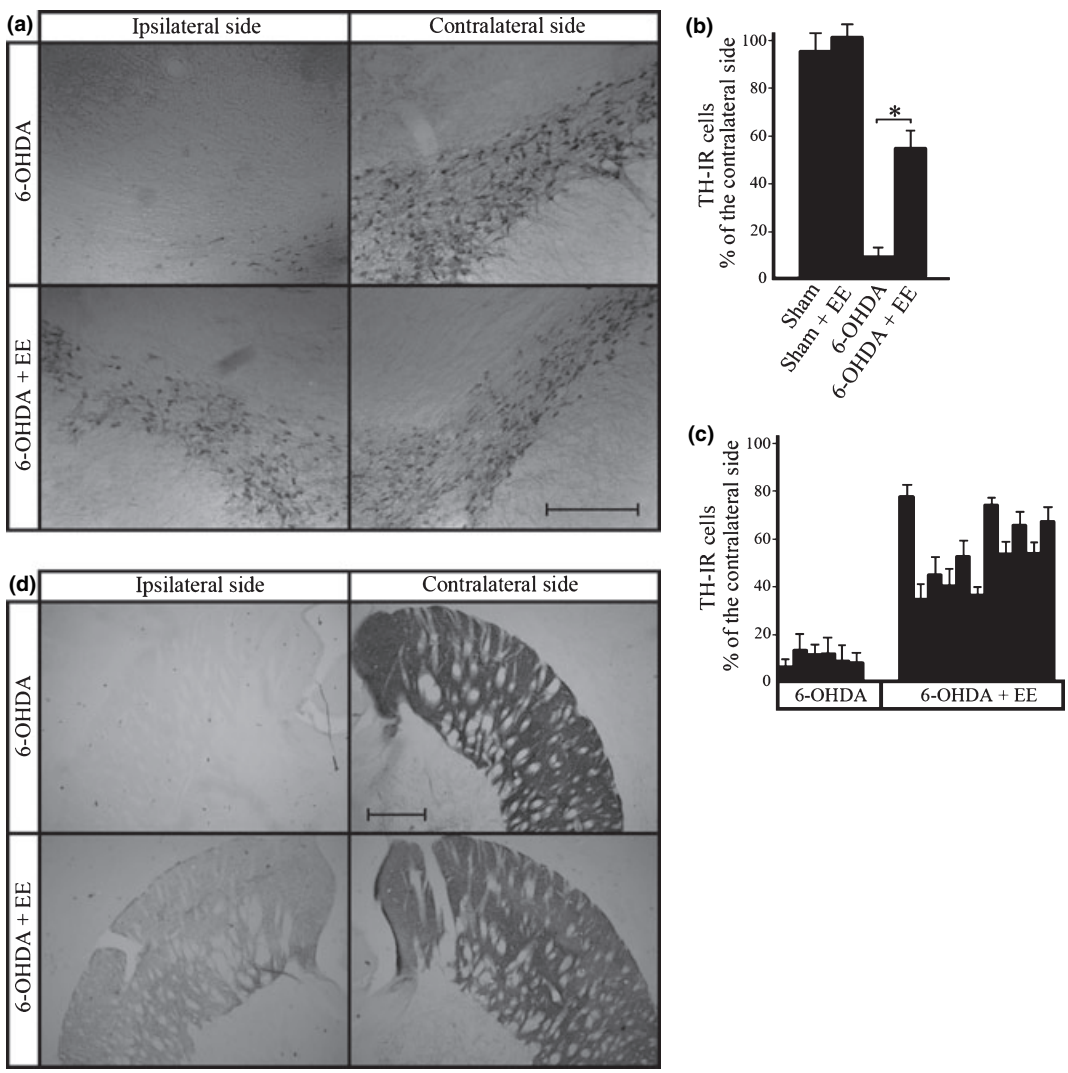


Fig. 1 Protection of the nigral dopaminergic neurons and terminal fibers by enriched environment (EE). (a) Microphotographs of rat midbrain sections stained for tyrosine hydroxylase (TH) immunohistochemistry 21 days post-lesion. (b) Dopaminergic neuron survival in the substantia nigra 21 days after the 6-OHDA injection (lesion) in rats housed in EE (6-OHDA + EE) or in standard condition (6-OHDA). Panel (c) exhibits the mean value (six sections per animal) for each of the animals of panel (b). Panel (d) shows photomicrographs of rat striatal sections stained with TH 21 days following 6-OHDA injection after EE (6-OHDA + EE) or standard housing (6-OHDA). Bars represent mean ± SEM. Sham, $n = 3$; Sham + EE, $n = 3$; 6-OHDA, $n = 6$; 6-OHDA + EE, $n = 11$ ($^*p < 0.05$). TH-IR: tyrosine hydroxylase immunoreactive. Panel (a) scale bar: 300 μm. Panel (d) scale bar: 0.5 mm.

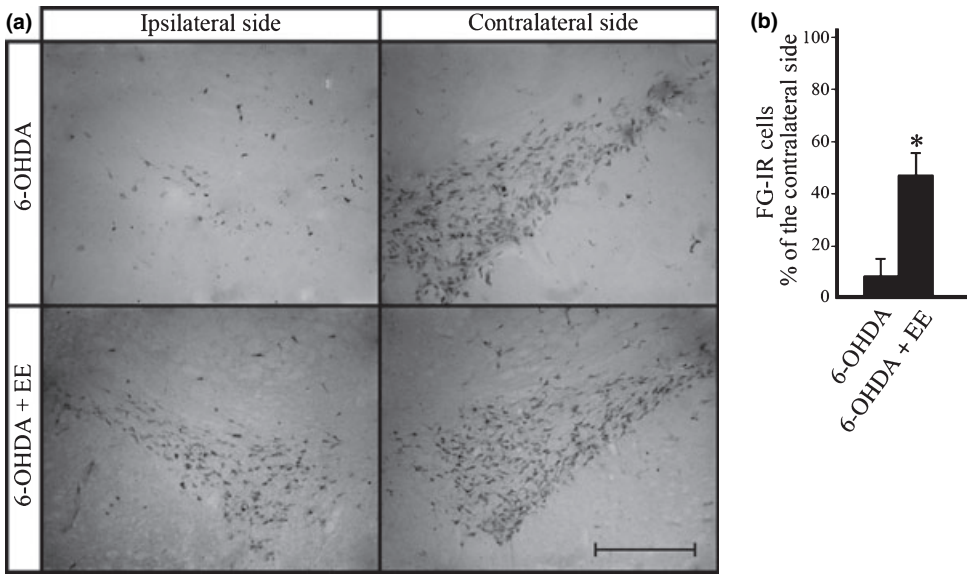


Fig. 2 Structural protection of the nigrostriatal pathway by enriched environment (EE). (a) Microphotographs of rat midbrain sections stained for FG immunohistochemistry 21 days post-lesion. (b) Percentage of FG-stained neurons in the substantia nigra of rats 21 days after 6-OHDA and housed in EE (6-OHDA + EE) or standard conditions (6-OHDA). Bars represent mean ± SEM, $n = 5$ per group ($^*p < 0.05$). FG-IR, Fluorogold immunoreactive. Scale bar: 300 μm.

EE housing preserves the nigrostriatal pathway after 6-OHDA injection

Dopaminergic neurons of the SNpc project to the striatum and the loss of this innervation underlies functional failure in the 6-OHDA model of Parkinson's disease. To assess the integrity of the nigrostriatal pathway, we carried out retrograde FG labeling experiments in 6-OHDA injected animals maintained either in EE or standard housing. To control for technical and interindividual variation, all animals received striatal bilateral injections of the tracer and the results were normalized to the unlesioned contralateral side. Retrograde labeling assessed 1 week after injection of the tracer and 21 days after 6-OHDA injection showed profound loss of FG-labeled cells in the SNpc of control (standard housed) animals (7.95 ± 4.76% of the contralateral side) (Fig. 2a and b). EE resulted in remarkable preservation of retrogradely labeled neurons (47.25 ± 5.08% of the contralateral side) (Fig. 2a and b), indicating substantial protection of nigrostriatal fibers. Vehicle injection (sham animals) did not modify the number of FG-stained neurons (ipsilateral compared with contralateral side, data not shown).

EE reduces rotational behavior in 6-OHDA-treated animals

Unilateral motor deficits caused by 6-OHDA are not easily detectable in rats unless drugs acting on dopaminergic neurons or receptors accentuate a side bias that can be measured. We used amphetamine-induced rotational behavior to assess the functional impact of EE neuroprotection 21 days after 6-OHDA injection. Standard-housed lesioned animals turned 9.17 ± 1.75 times/min. EE housing significantly reduced rotational behavior (3.17 ± 1.22 turns/min) (Fig. 3), with most animals behaving normally (i.e., no rotation in response to amphetamine). Lesioned (standard

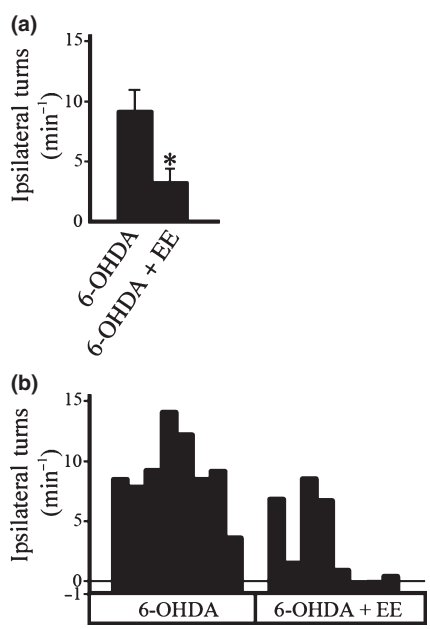


Fig. 3 Functional protection of the nigrostriatal system by enriched environment (EE). (a) Results of amphetamine-induced rotation test 21 days after 6-OHDA injection. (b) Rotation behavior of individual rats. Experimental groups: 6-OHDA (toxin injected and standard housing) and 6-OHDA + EE (toxin injected and EE housing). Bars represent mean ± SEM, $n = 8$ per group ($^*p < 0.05$).

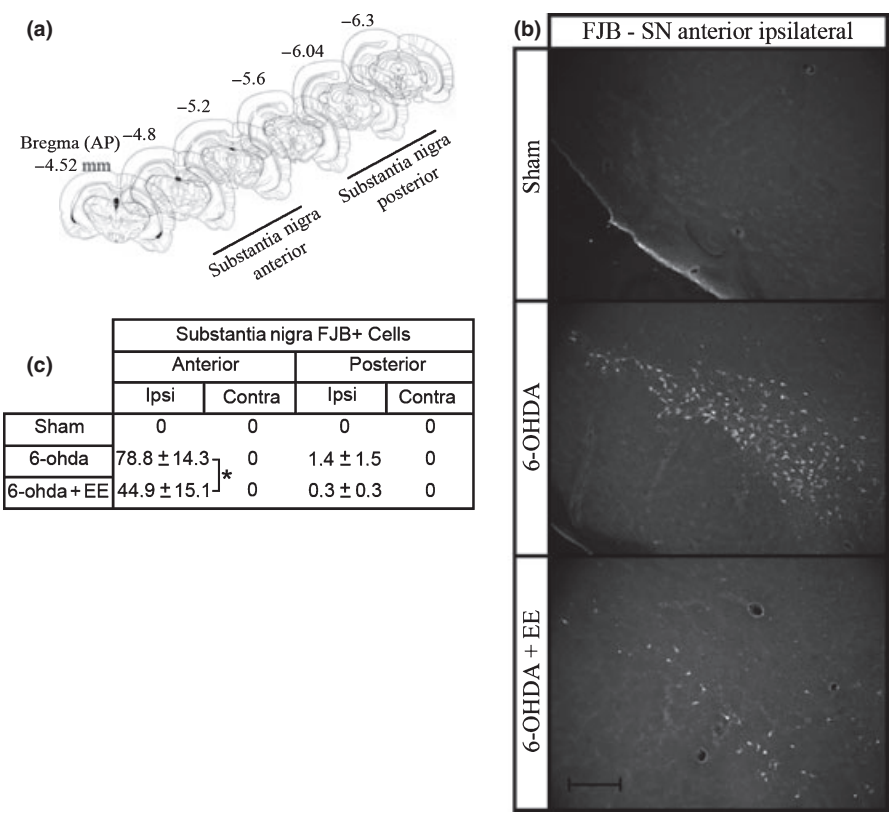
and EE housed) animals rotated less than 0.3 turns/min to the contralateral side. Vehicle injected animals (sham) did not rotate following amphetamine administration (data not shown). We did not find a correlation between ipsilateral turns and TH-labeled cell number as reported previously (Carman *et al.* 1991).

EE prevents early 6-OHDA-induced degeneration

In order to investigate the effect of EE on early neurodegenerative changes induced by 6-OHDA, animals were killed 4 days after the injection. FJB degenerating cells were only present in the most anterior part of the SNpc (defined arbitrarily as −4.52, −4.8, and −5.2 mm sections AP from bregma (Paxinos and Watson 1986) 4 days after the toxic insult (Fig. 4a–c). In the posterior SNpc (defined arbitrary as −5.6, −6.04, and −6.3 mm sections AP from bregma, (Paxinos and Watson 1986), virtually no cells were labeled for the degeneration marker (Fig. 4a and c). Notably, this was consistent with the known pattern of degeneration of dopaminergic neurons in the MPTP primate model of PD (Freundlieb *et al.* 2006). Housing in EE reduced 6-OHDA-induced degeneration in the anterior SNpc by half (Fig. 4a–c). This result clearly demonstrated that the neuroprotective effect of EE became evident shortly after exposure to 6-OHDA. We found no differences in the intensity of FJB stained cells among treatment (individual cell optical density quantification using ImageJ-NIH software, data not shown).

EE does not prevent a reduction in TH expression 4 days after 6-OHDA

Four days after 6-OHDA injection, the TH+ cell number was reduced in the ipsilateral SNpc (both anterior and posterior) which was not affected by housing in EE (Fig. 5a and d). We used two approaches to investigate if this reduction in TH-stained neurons reflected a change in expression of TH or neuronal loss. First, we estimated the nigral neuron number using cresyl violet staining. We found no differences in the number of neurons in the ipsilateral versus contralateral SNpc of 6-OHDA animals, whether



(c)

	Substantia nigra FJB+ Cells			
	Anterior		Posterior	
	Ipsi	Contra	Ipsi	Contra
Sham	0	0	0	0
6-ohda	78.8 ± 14.3 *	0	1.4 ± 1.5	0
6-ohda + EE	44.9 ± 15.1 *	0	0.3 ± 0.3	0

Fig. 4 Enriched environment (EE) prevents early neurodegeneration induced by 6-OHDA. (a) Representative brain coronal section drawing showing an arbitrary division in anterior and posterior substantia nigra (SN). (b) Rat midbrain sections of the ipsilateral anterior SN stained with the neurodegeneration marker Fluoro Jade-B (FJB). (c) Table showing anterior nigral neurodegeneration in contrast with an unstained posterior SN. Anterior values are the mean FJB+ cell number from the −4.52, −4.8, and −5.2 mm sections from bregma (±SEM); posterior values are the mean FJB+ cell number from the −5.6, −6.04, and −6.3 mm sections from bregma (±SEM). Experimental groups: sham (vehicle injected and standard housing), sham + EE (vehicle injected and EE housing), 6-OHDA (toxin injected and standard housing), and 6-OHDA + EE (toxin injected and EE housing) animals. $n = 5$ per group (*$p < 0.05$). Scale bar: 150 μm.

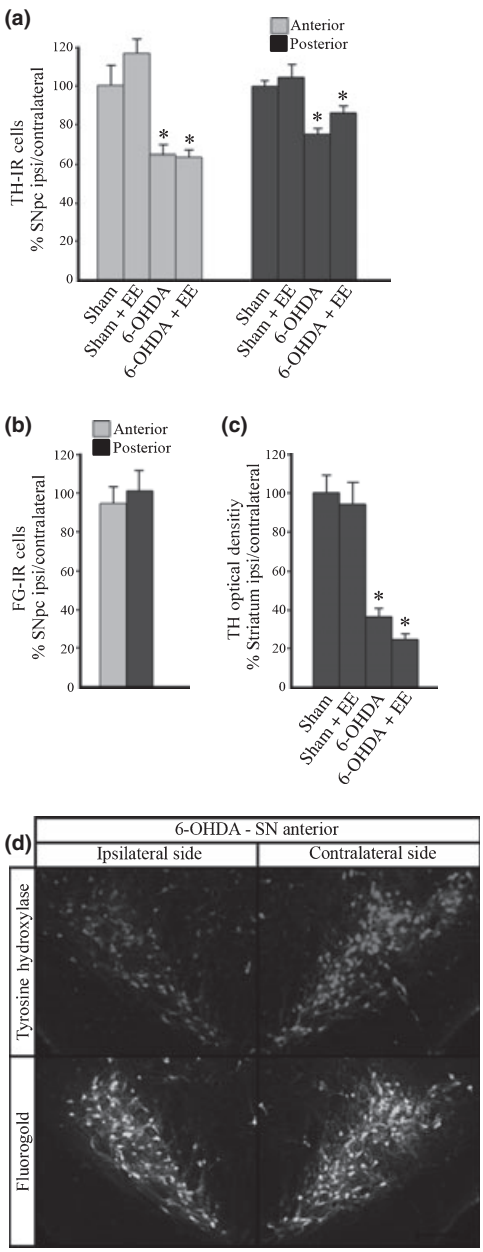


Fig. 5 Enriched environment (EE) does not prevent 6-OHDA-induced loss of tyrosine hydroxylase (TH) 4 days after the injection. (a) TH cells 4 days after the 6-OHDA administration in sham (vehicle injected and standard housing), sham + EE (vehicle injected and EE housing), 6-OHDA (toxin injected and standard housing), and 6-OHDA + EE (toxin injected and EE housing) animals. Number of FJB+ cells was obtained by averaging three sections for the anterior SNpc and three sections for the posterior SNpc. (b) 6-OHDA administration did not decrease FG-labeled neurons 4 days after the injection. (c) Striatal TH levels 4 days after 6-OHDA. (d) Microphotographs of rat anterior midbrain sections stained for TH and FG immunohistochemistry 4 days after the toxin injection. Bars represent mean ± SEM. $n = 5$ per group (*$p < 0.05$). TH-IR, tyrosine hydroxylase immunoreactive; FG-IR, Fluorogold immunoreactive. Scale bar: 100 μm.

housed in standard or EE conditions (data not shown). Also, there were no differences in cresyl violet staining between anterior and posterior SNpc (data not shown). Second, we identified neurons projecting to the striatum by FG retrograde labeling prior to 6-OHDA injection, and evaluated their survival 4 days after injection. In agreement with the cresyl violet experiment, we found no significant loss of FG-labeled neurons either in the anterior or in the posterior SNpc, regardless of the housing condition (Fig. 5b). In summary, 6-OHDA did not induce detectable neuronal loss in the SNpc 4 days after the toxin injection but resulted in significant reduction in the number of TH immunoreactive cells. Exposure to EE did not preserve the dopaminergic phenotype at this early time after 6-OHDA injection. Likewise, TH immunostaining decreased in the striatum 4 days after 6-OHDA injection, and EE did not affect this reduction (Fig. 5c).

Environmental enrichment induced astrocytic reaction in the anterior SNpc early after 6-OHDA lesion

In normal rats GFAP-labeled cells are present in the SN pars reticulata but rarely in the SNpc (Depino *et al.* 2003). Four days after 6-OHDA injection GFAP immunolabeling was unchanged in the SNpc of animals housed in the standard condition but markedly increased in the ipsilateral anterior SNpc of EE-housed animals (Fig. 6a and b). Neither EE in vehicle injected rats nor 6-OHDA by itself modified levels of GFAP immunostaining (Fig. 6b). In the striatum, GFAP levels were increased in all experimental groups compared with sham animals (Fig. 7a and b).

Discussion

We found that EE significantly reduced 6-OHDA degeneration of dopaminergic neurons in the SNpc of adult rats, preserved nigrostriatal projections, and most importantly, improved the dopaminergic function assessed by markedly decreasing the rotational behavior induced by amphetamine. Our findings are consistent with previously reported EE protection from neuronal death induced by MPTP (Bezard *et al.* 2003) and prevention of the motor deficit induced by 6-OHDA (Jadavji *et al.* 2006). Absence of TH immunoreactivity has been shown to correlate with dopaminergic neuronal death in this model at 3 weeks post-lesion (Carman *et al.* 1991). However, a disparity between dopaminergic phenotype expression and neuronal death has been reported in other experimental paradigms (Hagg and Varon 1993; Bowenkamp *et al.* 1996). Our results using FG retrograde labeling of nigral neurons clearly demonstrates the integrity of the axonal projections in the nigrostriatal pathway (Fig. 2). Furthermore, the number of FG+ cells was similar to the TH+ cell number, which indicates that the EE housing in the lesioned rats led to preservation of nigral cell numbers 21 days post-lesion.

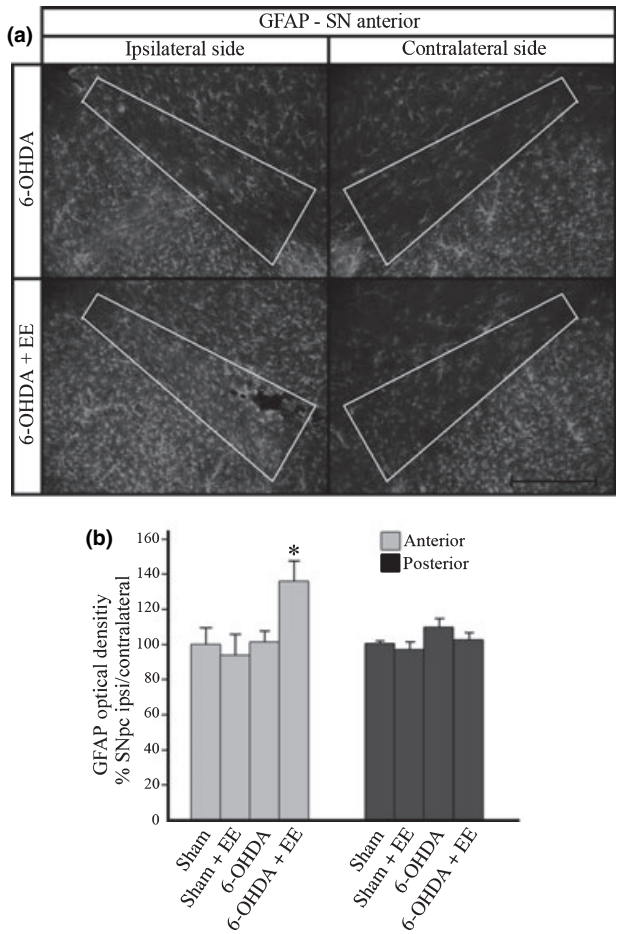


Fig. 6 Astrocytic reaction in the substantia nigra pars compacta (SNpc) 4 days after 6-OHDA. (a) Microphotographs of rat midbrain sections stained for glial fibrillary acidic protein (GFAP) immunohistochemistry 4 days after 6-OHDA injection. Experimental groups: 6-OHDA (toxin injected and standard condition housing) and 6-OHDA + EE (toxin injected and EE housing) animals. White line was drawn using TH immunostaining of the same section as reference, and limits the SNpc area. (b) GFAP optical density quantification 4 days post-toxin administration. Determinations were assessed in the anterior and posterior. Experimental groups: sham (vehicle injected and standard condition housing), sham + EE (vehicle injected and EE housing), 6-OHDA (toxin injected and standard housing), and 6-OHDA + EE (toxin injected and EE housing). Bars represent mean ± SEM. $n = 5$ per group (*$p < 0.05$). Scale bar: 300 μm.

We found for the first time that EE resulted in overall nigrostriatal dopaminergic system neuroprotection in the 6-OHDA neurodegeneration model (preservation of dopaminergic neurons and their striatal connections, as well as preservation of motor function 21 days after injection). On the other hand, exposure to EE for 7 weeks after 6-OHDA injection resulted in improved motor behavior assessed by the amphetamine-induced test, in agreement with our results but not neuroprotection of SNpc neurons (Steiner *et al.* 2006); these results suggest that EE can result in compensatory behavior rather than prevention of function loss, a possibility that cannot be excluded by our results. One obvious difference between our results and those reported by Steiner *et al.* (2006) is the time of onset of exposure to EE (21 days before 6-OHDA in our case, and after 6-OHDA injection in their report). EE also enhanced limb placement accuracy in skilled walking following 6-OHDA injection, with a trend towards protection of dopaminergic neurons in EE animals (Jadavji *et al.* 2006). Our experiments differed from this report in the protocol of 6-OHDA injection, feeding of EE animals, rat strain used (Long–Evans), and restricted use of female rats (Jadavji *et al.* 2006).

The present report contributes to the known beneficial effects of EE that have been found in animal models of schizophrenia, epilepsy, stroke, and Alzheimer's disease (Nithianantharajah and Hannan 2006; Laviola *et al.* 2008). EE benefits have also been observed in movement neurodegenerative disorder animal models. EE delays the onset of Huntington's disease in mice (van Dellen *et al.* 2000), and neuroprotection has been correlated with an entire rescue of BDNF levels in the hippocampus and striatum in this model (Spires *et al.* 2004). EE significantly improved motor performance in a model of amyotrophic lateral sclerosis (Stam *et al.* 2008).

Increased physical activity (voluntary or forced running), which is a part of the environmental enrichment, results in neuroprotection in a variety of experimental conditions including nigrostriatal system injury (Tillerson *et al.* 2003; Mabandla *et al.* 2004; Nithianantharajah and Hannan 2006; Petzinger *et al.* 2007; Yoon *et al.* 2007). Running is an interesting model of physical activity but EE housing includes complex features intended to mimic a natural habitat. Moreover EE allows comparing cognitive, sensorimotor, and social interaction effects with constraints on the brain function of impoverished rodent housing typically used in biomedical research.

Most studies have examined the behavioral and neurochemical effects of 6-OHDA administration after behavioral deficits first occur, which is typically more than 2 weeks after the injection, even though toxicity to dopaminergic neurons certainly begins almost immediately after the toxin administration. We studied the effects of 6-OHDA 4 days after its injection to assess early changes in dopaminergic neurons and their modifications by EE exposure. We found that neurodegeneration assessed by FJB (marker of neurodegeneration) 4 days after the 6-OHDA lesion was restricted to the anterior SN (Fig. 4a–c). Virtually, no cell damage was observed in the posterior SNpc (Fig. 4b). This is consistent with the known pattern of degeneration of dopaminergic neurons in the MPTP primate model of PD (Freundlieb *et al.* 2006) but it has not been described in the 6-OHDA model, most likely because of reliance on TH staining to assess neuronal loss or a lack of quantitative assessment of neuronal death when using FJB (Zuch *et al.* 2000). Differences

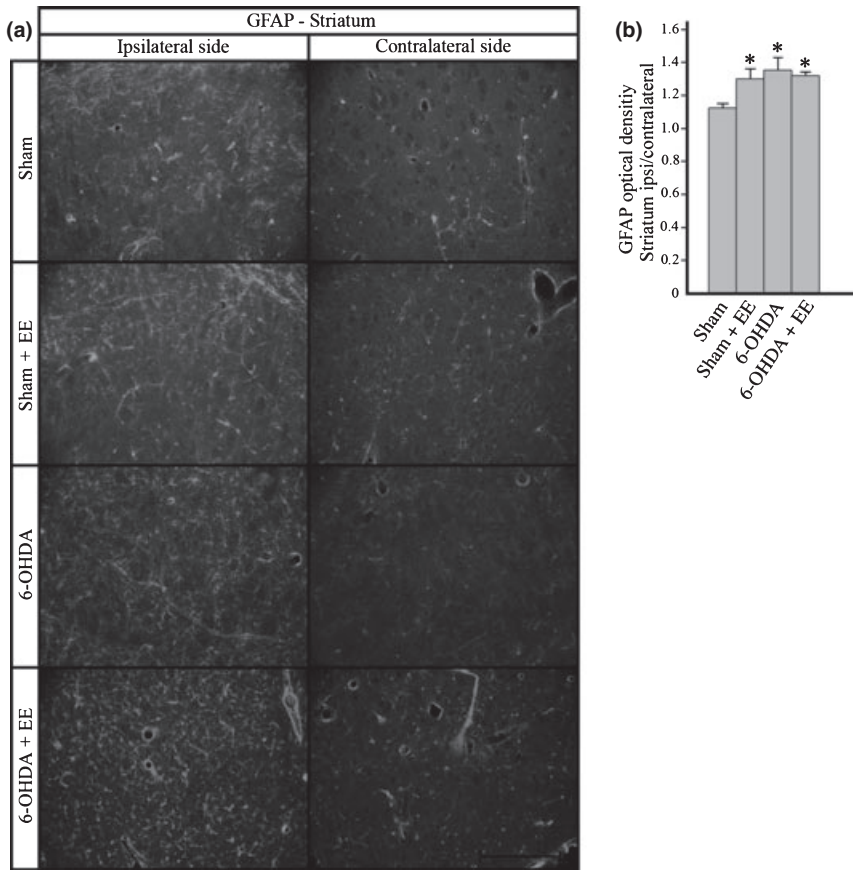


Fig. 7 Striatal astrocytic reaction 4 days after 6-OHDA. (a) Microphotographs of rat striatal sections stained for glial fibrillary acidic protein (GFAP) immunohistochemistry 4 days after the injection. Experimental groups: sham (vehicle injected and standard housing), sham + EE (vehicle injected and EE housing), 6-OHDA (toxin injected and standard housing), and 6-OHDA + EE (toxin injected and EE housing) animals. (b) GFAP optical density quantification 4 days post-toxin administration. Bars represent mean ± SEM. $n = 5$ per group (*$p < 0.05$). Scale bar: 300 μm.

described between anterior and posterior SN (Gibb and Lees 1991; Damier *et al.* 1999) could account for the different vulnerability observed. To exclude the possibility of parenchymal diffusion of the 6-OHDA, we injected 4 μL of India ink in two normal animals using the same coordinates as 6-OHDA and killed them 4 or 24 h after the administration. We found no ink in the anterior SN (data not shown). In addition, 6-OHDA induced similar reductions in TH immunoreactivity in anterior and posterior SNpc 4 days after injection, suggesting that differences in FJB staining are because of differential neuronal vulnerability (Fig. 5a and d). Reduction in TH immunoreactivity occurred in both standard condition and EE-housed animals (Fig. 5a and d), and is independent of any detectable neuronal death in the SNpc by cresyl violet or loss of nigrostriatal projection neurons labeled by retrograde FG staining (Fig. 5). Thus, loss of TH expression cannot be accounted for neuronal death when short post-injection times are analyzed. In summary, early after lesion, no FG+ cell loss was observed, although TH protein levels were decreased. Therefore, degenerating neurons (assessed by FJB) are still FG-positive, and they cannot account for neuronal loss.

The neuroprotective effects of EE probably result from a confluence of factors. Any neuroprotective or repair mechanism associated with exposure to EE should lead to micro-environmental changes present around the time of toxin administration. Activation of astrocytes has been proposed as one such change. We found, as others had reported (Depino *et al.* 2003), no evidence of reactive astrocytes in the SNpc of standard housed animals 4 days after injection of the toxin. On the other hand, an extraordinary and significant astroglial reaction was evident, restricted to the anterior SNpc of EE-housed animals at the same time post-toxin injection, and correlated with a 50% reduction in FJB-staining in the same region. Thus it appears that animals exposed to an EE have an increased ability to respond to the toxic injury where the most susceptible neuronal population is located. A similar synergic GFAP increase has been described in animals with ischemic hemispheric brain lesions that were forced to use the impaired limb (Schallert *et al.* 2000).

Reactive astrocytes probably participate in endogenous cell repair or neuroprotective mechanisms triggered at very early times following exposure to the toxin, possibly involving release of BDNF, GDNF, and nerve growth factor among many others (Nithianantharajah and Hannan 2006). Indeed, it is well known that astrocytes become reactive after brain injury (Teissmann *et al.* 2003; McGeer and McGeer 2008) but in the case of 6-OHDA injection the astrocytic response is detectable only at prolonged times after toxin

administration (Gordon *et al.* 1997; Gomide *et al.* 2005a,b; Henning *et al.* 2008). Typically, astroglial reaction consists of hypertrophy of their cellular processes and up-regulation of GFAP. Alternate explanations of the GFAP increase observed could be migration from surrounding areas (McGeer and McGeer 2008) (such as SN pars reticulata) to the SNpc, and/or astrogliogenesis (Aponso *et al.* 2008) but the latter finding has been contradicted (Lie *et al.* 2002).

It has been suggested that lifestyle influences the neurodegenerative processes and incidence of PD (Olanow and Tatton 1999; Elbaz and Moisan 2008). We now show that an experimental proxy for lifestyle, namely EE housing, exerts a neuroprotective effect on the nigrostriatal system in a commonly used rodent model of PD. Notably, the protective effect of EE correlates with targeted activation of glial response. Furthermore, EE can be used to search for molecular targets to facilitate development of novel therapeutic molecules that can be called 'enviromimetics' (McOmish and Hannan 2007).

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