

Protection of dopaminergic neurons by electroconvulsive shock in an animal model of Parkinson's disease

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Abstract

Electroconvulsive shock (ECS) improves motor function in Parkinson's disease. In rats, ECS stimulates the expression of various factors some of which have been proposed to exert neuroprotective actions. We have investigated the effects of ECS on 6-hydroxydopamine (6-OHDA)-injected rats. Three weeks after a unilateral administration of 6-OHDA, 85–95% nigral dopaminergic neurons are lost. Chronic ECS prevented this cell loss, protect the nigrostriatal pathway (assessed by FluoroGold retrograde labeling) and reduce motor impairment in 6-OHDA-treated animals. Injection of 6-OHDA caused loss of expression of glial cell-line derived neurotrophic factor

(GDNF) in the substantia nigra. Chronic ECS completely prevented this loss of GDNF expression in 6-OHDA-treated animals. We also found that protected dopaminergic neurons co-express GDNF receptor proteins. These results strongly suggest that endogenous changes in GDNF expression may participate in the neuroprotective mechanism of ECS against 6-OHDA induced toxicity.

Keywords: 6-hydroxydopamine, electroconvulsive shock, glial cell-line derived neurotrophic factor, neuroprotection, Parkinson, substantia nigra.

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Parkinson's disease (PD) is a progressive neurodegenerative disorder, which shows as its most prominent neuropathological change a loss of dopamine (DA) containing neurons in the nigrostriatal system (Dauer and Przedborski 2003). One of the most studied animal models of PD is the injection of 6-hydroxydopamine (6-OHDA) unilaterally into the medial forebrain bundle of rats, which selectively destroy the dopaminergic axons and cells bodies of the substantia nigra (SN) pars compacta (Ungerstedt 1968; Dauer and Przedborski 2003). This model has been extensively used to search for putative neuroprotectants (Hyman *et al.* 1991; Korsching 1993; Kearns *et al.* 1997; Shults *et al.* 2000; Vernon *et al.* 2005), including sustained over-expression of trophic factors using recombinant vectors or genetically modified cells (Bilang-Bleuel *et al.* 1997; Mandel *et al.* 1997; Kozlowski *et al.* 2000; Shults *et al.* 2000; Hurelbrink and Barker 2004), as well as intracerebral infusion of trophic factors (Spina *et al.* 1992; Arenas *et al.* 1995; Kearns *et al.* 1997).

Chronic electroconvulsive shock (ECS) alters expression of trophic factors and their receptors (Newton *et al.* 2003; Altar *et al.* 2004), and prevents neuronal death induced by adrenalectomy (Mascó *et al.* 1999) and by status epilepticus (Kondratyev *et al.* 2001). The mechanism of ECS neuroprotection is unknown, but several data suggest that synthesis and release of trophic factors may have a crucial role (Duman and Vaidya 1998). Chronic ECS regulates endogenous levels of brain-derived neurotrophic factor (BDNF) (Nibuya *et al.* 1995; Angelucci *et al.* 2002;

Newton *et al.* 2003), nerve growth factor (Follesa *et al.* 1994; Angelucci *et al.* 2002, 2003; Kondratyev *et al.* 2002; Newton *et al.* 2003), fibroblast growth factor-2 (Follesa *et al.* 1994; Gwinn *et al.* 2002; Kondratyev *et al.* 2002; Newton *et al.* 2003) and glial cell-line derived neurotrophic factor (GDNF) (Angelucci *et al.* 2002) in brain regions including the hippocampus, cortex, and striatum.

Glial cell-line derived neurotrophic factor rescues damaged dopaminergic neurons in culture and in animal models of PD more efficiently than other neurotrophins (Kriegstein 2004; Sun *et al.* 2005). GDNF acts through a multireceptor complex composed by the GDNF family receptor α -1 (GFR α -1) and a transmembrane c-Ret receptor tyrosine

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Abbreviations used: 6-OHDA, 6-hydroxydopamine; BDNF, brain-derived neurotrophic factor; DA, dopamine; ECS, electroconvulsive shock; FG, FluoroGold; GDNF, glial cell-line derived neurotrophic factor; GFR α -1, GDNF family receptor α -1; PD, Parkinson's disease; SDS, sodium dodecyl sulfate; SN, substantia nigra; TH, tyrosine hydroxylase.

kinase that triggers intracellular signaling (Saarna and Sariola 1999). GDNF promotes survival and neurite growth, and increases cell size of dopaminergic neurons *in vitro* (Lin *et al.* 1993; Ding *et al.* 2004) and *in vivo* (Tomac *et al.* 1995; Kearns *et al.* 1997). Also, GDNF rescues dopaminergic neurons in experimental PD models including 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine toxicity in mice (Tomac *et al.* 1995) and monkeys (Gash *et al.* 1996; Kordower *et al.* 2000), as well as 6-OHDA toxicity (Björklund *et al.* 1997; Kearns *et al.* 1997) and axonal transection (Beck *et al.* 1995) in rats. However, intraputamin administration of GDNF in patients with PD has failed to show consistent clinical benefits (Gill *et al.* 2003) in spite of a case report showing dopaminergic sprouting (Love *et al.* 2005). Lack of efficacy may be related to poor diffusion of GDNF protein out of the infusion canula, developing of anti-GDNF antibodies, or other unknown effects. Thus, strategies of neurostimulation leading to modulation of endogenous GDNF may be of additional benefit.

We tested if ECS treatment protects dopaminergic neurons in the 6-OHDA neurotoxicity model. We present evidence that ECS prevents 6-OHDA toxicity to dopaminergic neurons. Additionally, we studied expression of GDNF protein in pertinent areas. We found a strong correlation between neuroprotection and GDNF/GDNF receptors endogenous level modifications induced by ECS treatment.

Materials and methods

Animals

Adult male Wistar rats weighing 180/250 g were housed with a 12 h light/dark cycle with water and food *ad libitum*. Experiments were performed in keeping with the guide for the care and use of laboratory animals (National Academy Press).

Experimental design

Animals received unilateral injections of 6-OHDA in the medial forebrain bundle and we studied the effect of chronic administration of ECS on: (i) survival of tyrosine hydroxylase (TH) immunostained neurons, (ii) motor (rotatory) behavior, (iii) the integrity of the nigrostriatal pathway, (iv) expression of GDNF protein levels in the nigrostriatal regions of lesioned and unlesioned animals, and (v) the degree of co-expression of GDNF receptors Ret-GFR α -1 in dopaminergic neurons.

(i) To evaluate the survival of dopaminergic neurons, animals received either ECS or sham-ECS for 2 days before receiving an injection of 6-OHDA, and daily ECS or sham-ECS for 7, 14, or 21 days after the injection. Animals were killed 1 day after the last ECS. In one experiment the animals were killed on day 22 after receiving ECS on days -2 to 7.

(ii) Using the same protocol of ECS or sham-ECS described in (i), motor behavior was assessed following apomorphine injection on day 21 after 6-OHDA injection, to assess supersensitivity-dependent rotation.

(iii) Integrity of nigrostriatal fibers was assessed in animals receiving either ECS or sham-ECS for 2 days before receiving an injection of 6-OHDA, and daily ECS or sham-ECS for 14 days after the injection (a protocol resulting in increased neuronal survival after 6-OHDA toxicity with the active treatment). One day after the last ECS, animals were bilaterally injected with FluoroGold (FG) in the striatum and killed 7 days later to allow sufficient retrograde labeling of SN neurons.

(iv) To assess the effect of ECS on GDNF protein expression, normal animals received daily ECS or sham-ECS for either 1 or 3 days (acute ECS), or over periods of 7 or 14 days (chronic ECS). Tissue for western blots was collected 24 h after the last seizure. Once the baseline effect of ECS on GDNF protein expression was established, we evaluated the levels of GDNF expression, and ret/GFR α -1 expression in dopaminergic neurons; animals were injected with either 6-OHDA or vehicle after 2 days of either ECS or sham-ECS, and continued receiving the same treatment (ECS or sham-ECS) for two more weeks. All animals were killed 24 h after the last shock and processed for western blot analysis or for immunohistochemical colocalization analysis.

ECS treatment

Bilateral ECS was administered once a day via corneal electrodes (200 pulses/s; pulse width: 0.1 ms; shock duration: 0.2 s; 40 mA) using an ECS Unit 7801 (Ugo Basile, Comerio, Italy). Seizures evoked with these parameters were consistently maximal (involving tonic forelimb and/or hind limb extension) provided the electrode tips were wet prior to application. Sham-ECS animals received the same handling and contact with the electrodes, but no current. Animals were excluded (approximately 10%) if motor clonic-tonic seizures lasting 10–15 s were not observed. Preliminary experiments comparing the protective effect of one versus three daily ECS treatments revealed no difference on neuronal survival; we therefore designed all the experiments with the least effective dose (one ECS daily).

6-hydroxydopamine injection

Anesthetized rats (400 mg/kg chloral hydrate, i.p.) were placed in a Kopf stereotaxic apparatus and injected with 6-OHDA (4 μ L over 8 min, 2 μ g/ μ L in 0.2% ascorbic acid in 0.9% NaCl; Sigma, St Louis, MO, USA) in the medial forebrain bundle (from bregma: AP: -1.5; L: 1.8; and V: -8.3) (Paxinos and Watson 1986); the canula was removed 5 min later. Sham-operated animals received vehicle. All surgeries for a given experiment were performed on the same session by a blind operator.

Apomorphine-induced rotation test

Behavioral testing was performed 2 or 3 weeks after 6-OHDA (24 h after the last ECS). After acclimation to an individual cage, apomorphine (1 mg/kg in 0.7% NaCl, s.c.) (Apokinin Rontag, Paris, France) was administered, and the total number of full turns recorded for 60 min. Twenty-four hours after the rotational test animals were perfused for TH immunohistochemistry.

Retrograde labeling with FluoroGold

Two weeks after unilateral 6-OHDA injection, rats received bilateral FG injections (0.3 μ L, 2% FG; Fluorochrome, Denver, CO, USA) into the striatum (from bregma: AP: 3.0; L: \pm 1.8; and V: -5.0).

Retrograde labeling of SN neurons was assessed by immunohistochemistry 1 week later.

Immunohistochemistry and analysis

Anesthetized animals (400 mg/kg chloral hydrate, *i.p.*) were transcardially perfused (4% paraformaldehyde in phosphate-buffered saline), and the brains removed, post-fixed, cryoprotected, and sectioned (36 μ m). Free-floating coronal sections were blocked (5% bovine serum albumin), incubated with primary antibody (24 h at 4°C, anti-TH, 1 : 500, Boehringer Mannheim (Mannheim, Germany), or anti-FG, 1 : 1600; Chemicon (Temecula, CA, USA), followed by secondary biotin-conjugated antibody (1 h at 21 \pm 1°C, 1 : 1000 to 1 : 200; Sigma), and developed with streptavidin-peroxidase (1 : 100 to 1 : 200; Sigma) using diaminobenzidine as the chromagen.

Counts of FG-labeled and TH-positive cells in the SN were performed as previously described (Sauer and Oertel 1994). TH-immunostained cells in the SN pars compacta (lateral to the medial terminal nucleus of the accessory optic tract) were counted bilaterally in every sixth section (6 sections/animal). Neuronal survival 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 inter-individual differences and is widely used to assess the extent of 6-OHDA-induced lesion in the SN (Kirik *et al.* 2000; Paul *et al.* 2004). To further ensure the absence of bias in cell counting, the operator was blind to the treatment group during the analysis.

For double labeling immunohistochemistry, mouse anti-TH antibody (24 h at 4°C, 1 : 500, Boehringer Mannheim), was combined with either rabbit anti-GFR α -1 antibody (72 h at 4°C, 1 : 75, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or rabbit anti-c-Ret antibody (72 h at 4°C, 1 : 100, Genex BioScience, Hayward, CA, USA), and followed by appropriate fluorescent secondary antibodies (Alexa Fluor 488 anti-rabbit IgG, 1 : 100, Molecular Probes, Carlsbad, CA, USA; or Alexa Fluor 568 anti-mouse IgG, 1 : 200, Molecular Probes). Negative controls without primary antibodies were performed for each immunodetection. Sections were assessed blindly under confocal microscopy (LSM 5 Pascal, Zeiss, Oberkochen, Germany).

Western blot analysis

Animals were killed by decapitation. Brains were removed, regional samples dissected from each hemisphere and frozen. Samples were homogenized in ice-cold ristocetin-induced platelet agglutination buffer with protease inhibitors (150 mmol/L NaCl, 50 mmol/L Tris, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS), with freshly added 100 μ g/mL phenylmethylsulfonyl fluoride, 2 μ g/mL aprotinin, 5 μ g/mL leupeptin, 1 μ g/mL pepstatin, 1.12 mg/mL EDTA, and 183.9 μ g/mL sodium orthovanadate, and 21 mg/mL NaF), centrifuged, assessed for protein concentration by colorimetry (Bradford method), and run in an SDS-polyacrylamide gel electrophoresis. Blots were transferred to 0.2 μ m nitrocellulose membrane, blocked with dry milk (5% in Tris-buffered saline with 0.1% Tween 20), and incubated in primary antibodies (24 h, anti-GDNF, 1 : 200, RDI, Concord, MA, USA; anti-TH 1 : 500, Boehringer Mannheim; anti-tubulin 1 : 9000; Sigma), followed by biotin-conjugated

secondary antibodies (1 : 500; Sigma) and streptavidin-peroxidase (1 : 500; Sigma). Bands were visualized by enhanced chemiluminescence. Blots were scanned and digitized, and densitometry was performed using image analysis software (Image J 1.34j, NIH, Bethesda, MD, USA). Quantization was normalized to total tubulin for each individual sample.

Statistical analysis

Paired Student's *t*-test was performed for statistical comparison between paired immunohistochemical data. Where appropriate, one-way or two-way ANOVA with Dunnett's *post hoc* test analysis was performed. Results are provided as the mean \pm SEM.

Results

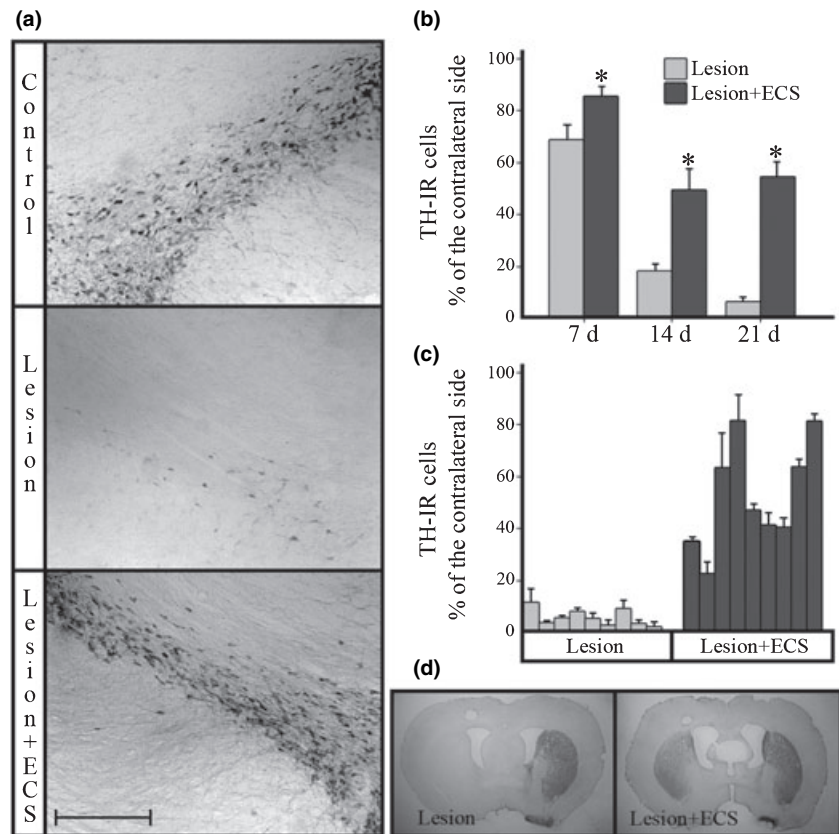
ECS treatment promotes survival of dopaminergic neurons in the substantia nigra

The effect of ECS on the progression of dopaminergic neuronal loss in the SN following unilateral injections of 6-OHDA was examined at 7, 14, and 21 days. In control (sham-ECS) animals, neuronal loss was evident but slight 7 days after injection, became marked by 2 weeks, and was nearly total by 3 weeks (6.5% \pm 0.98 of contralateral side). These findings are consistent with other published reports (Dowd *et al.* 2005). Daily administration of ECS resulted in significantly less neuronal loss at all assessed times (Fig. 1a–c). However, if ECS was discontinued after 7 days and the animals were allowed to survive until day 21 the protective was lost (survival was respectively: 6-OHDA alone = 22.9 \pm 2.3; 6-OHDA+7 days of ECS = 20.1 \pm 2.6). Qualitative assessment of TH staining in the striatum was consistent with a protective effect of ECS on dopaminergic terminals (Fig. 1d), suggesting that the neuroprotection was not restricted to the neuronal soma. This observation was specifically tested with retrograde labeling (see below).

ECS preserve the nigrostriatal pathway after 6-OHDA injection

Preservation of striatal TH staining could be associated with the protection of nigrostriatal dopaminergic fibers or with sprouting of surviving terminals. To distinguish between these alternatives, we carried out retrograde FG labeling experiments in 6-OHDA-injected animals treated with either ECS or sham-ECS. To control for technical and inter-individual variations, all animals received striatal bilateral injections of the tracer. Striatal sections were taken from each brain for FG staining to confirm the injection site (Fig. 2b). Rats received 2 days of ECS prior to injection of 6-OHDA and daily ECS for 14 days following the toxin; FG was injected on day 14 and retrograde labeling was assessed 1 week later. Very little FG immunostaining was observed in the SN of lesioned and sham-ECS-treated animals, compared with the contralateral unlesioned side (Fig. 2a and c),

Fig. 1 Protection of the nigral dopaminergic neurons and terminals fibers by electroconvulsive shock (ECS). (a) Microphotographs of rat midbrain sections stained for tyrosine hydroxylase immunohistochemistry. Top panel is a section from the contralateral intact midbrain of lesioned animals, middle panel is a section from 6-hydroxydopamine (6-OHDA)-injected animals treated with sham-ECS, and bottom panel is a section from 6-OHDA-injected animals treated with ECS for 21 days. (b) Time course of dopaminergic neurons survival in the substantia nigra after 6-OHDA injection (lesion) in rats treated with ECS (dark gray) or sham-ECS (light gray). Panel (c) exhibits the mean value (six sections per animal) for each of the animals of the 21 days time point of panel (b). Panel (d) low-power photomicrographs of rat frontal sections stained with tyrosine hydroxylase 21 days following 6-OHDA injection after receiving daily sham-ECS (left panel) or active ECS (right panel). Bars represent mean \pm SEM, $n = 9$ per group ($*p < 0.05$). TH-IR, tyrosine hydroxylase immunoreactive. Scale bar: 300 μ m.



whereas in lesioned and ECS-treated animals the number of retrogradely labeled neurons was reduced by only approximately 35% in the ECS-treated lesioned rats whereas loss of labeled cells was approximately 85% in non-ECS-treated lesioned rats (Fig. 2a and c). Similar results were observed with DiI (not shown).

ECS prevents rotational behavior in 6-OHDA-treated animals

Protection of dopaminergic neurons had a clear functional impact, as no denervation supersensitivity was observed in ECS-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 assessed the functional impact of ECS neuroprotection in lesioned animals with apomorphine-induced rotational behavior at 2 and 3 weeks. Sham-ECS (control) animals turned 11.6 ± 1.8 and 13.9 ± 2.7 times/min at 14 and 21 days, respectively. ECS significantly prevented rotational behavior (Fig. 3), and most animals were behaviorally normal. If daily ECS treatments were discontinued after 7 days and 6-OHDA-injected rats allowed to survive until day 21, behavioral protection was not observed (10.3 ± 2.7 times/min). Qualitatively, behavioral manifestations of dopaminergic deficits such as hair bristle, hyperphagia, or trembling, that were prominently present in

6-OHDA-injected rats, were indistinguishable between control and ECS-treated lesioned animals.

ECS alters regional concentrations of GDNF in the brain of normal rats

In normal rats, acute ECS treatment did not significantly change GDNF protein levels in SN or striatum (Fig. 4a and b), but chronic daily ECS significantly increased GDNF protein in SN at 7 and 14 days ($54.1 \pm 14.2\%$ and $73.2 \pm 9\%$, respectively), when compared with sham-ECS (Fig. 4a). GDNF levels were not altered in striatum of the same animals (Fig. 4b).

ECS still causes an increase in GDNF protein expression in 6-OHDA-lesioned animals to the same extent that it does in non-lesioned animals

Injection of 6-OHDA significantly decreased GDNF expression in SN 14 days later, compared with vehicle-injected animals. When daily ECS was administered to 6-OHDA-injected animals, GDNF levels were similar to those observed in vehicle-injected rats treated with ECS (Fig. 4c). Neither 6-OHDA injection nor ECS treatment altered GDNF expression in striatum (Fig. 4d). GDNF protein expression in the hippocampus was not modified in any of the studied conditions (data not shown). To verify the effectiveness of the 6-OHDA lesion, TH protein was measured by western

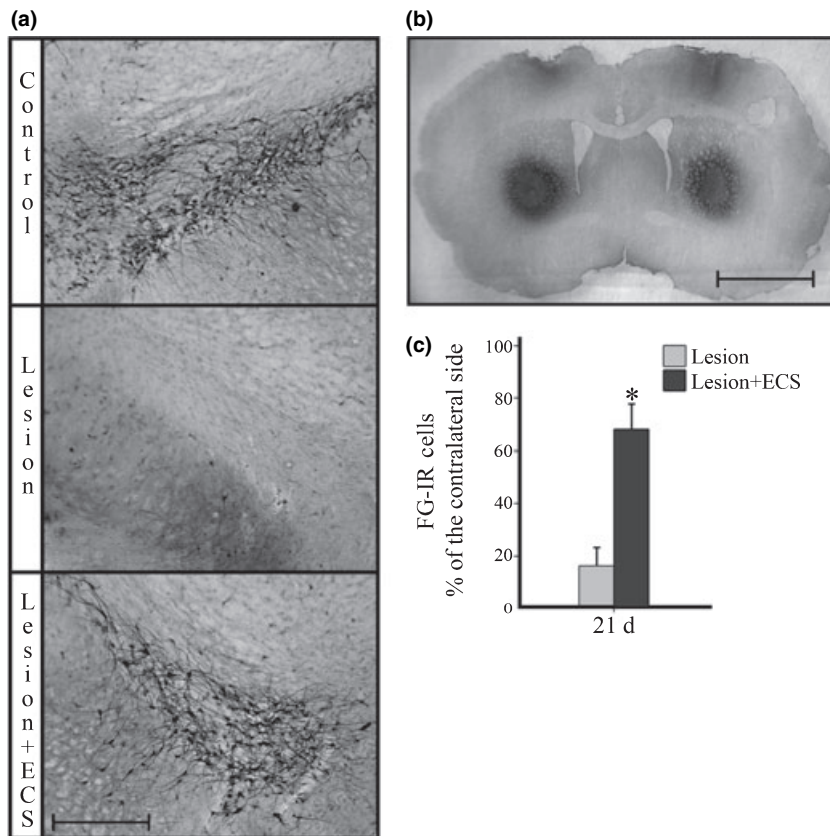


Fig. 2 Structural protection of the nigrostriatal pathway by electroconvulsive shock (ECS). (a) Microphotographs of rat midbrain sections stained for FluoroGold immunohistochemistry. Top panel is a section from the contralateral intact midbrain of lesioned animals, middle panel is a section from 6-hydroxydopamine (6-OHDA)-injected animals treated with sham-ECS, and bottom panel is a section from 6-OHDA injected animals treated with ECS for 21 days. (b) Microphotographs of rat striatal sections stained for FluoroGold immunohistochemistry showing the retrograde marker bilateral injection site. (c) Percentage of FluoroGold stained neurons in the substantia nigra of rats 21 days after 6-OHDA and treated with ECS (dark gray) or sham-ECS (light gray). Bars represent mean \pm SEM, $n = 6$ per group ($*p < 0.05$). FG-IR, FluoroGold immunoreactive. Scale bars (a): 300 μ m; (b): 300 μ m.

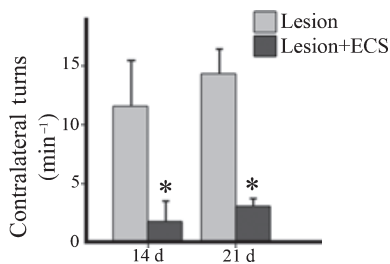


Fig. 3 Functional protection of the nigrostriatal system by electroconvulsive shock (ECS). Results of apomorphine-induced rotation test 14 and 21 days after 6-hydroxydopamine (6-OHDA) injection. Bars represent mean \pm SEM, $n = 9$ per group ($*p < 0.05$).

blot in both SN and striatum in all the experimental groups. 6-OHDA injection significantly reduced TH expression in both regions (Fig. 4c and d). Treatment with ECS prevented the decrease in TH protein.

Dopaminergic neurons express Ret receptor and the binding protein GFR α -1

Expression of the transduction mechanism (c-Ret and GFR α -1) is a requirement for GDNF action. We analyzed, by confocal microscopy, co-localization of GDNF receptor proteins with TH in different experimental conditions. In sham-operated animals, virtually all TH⁺ neurons co-

expressed GFR α -1 (Fig. 5, upper panels) and c-Ret (Fig. 6, upper panels); few cells were found labeled with TH and not for c-Ret (Fig. 6). Predictably, many cells negative for TH expressed both receptors. Injection of 6-OHDA induced nearly total loss of TH⁺ after 14 days, but expression of GFR α -1 (Fig. 5, middle panels) and c-Ret (Fig. 6, middle panels) was unaltered in surviving TH⁺ neurons. On the other hand, ECS-treated 6-OHDA-injected animals showed strong TH⁺ labeling and also double immunostaining for both GFR α -1 (Fig. 5, lower panels) and c-Ret (Fig. 6, lower panels) that was indistinguishable from vehicle-treated, sham-operated animals (Figs 5 and 6, upper panels).

Discussion

We have shown that chronic ECS can prevent 6-OHDA-induced degeneration of dopaminergic neurons in the SN of adult rats, preserve nigrostriatal projections, and most importantly improve dopaminergic function. Following unilateral 6-OHDA injection, treatment with apomorphine results in turning contralateral to the lesion, reflecting DA receptor up-regulation secondary to denervation of the striatum (Ungerstedt and Arbuthnott 1970). Chronic ECS treatment prevented rotational behavior induced by apomorphine (Fig. 3). Our findings are consistent with previ-

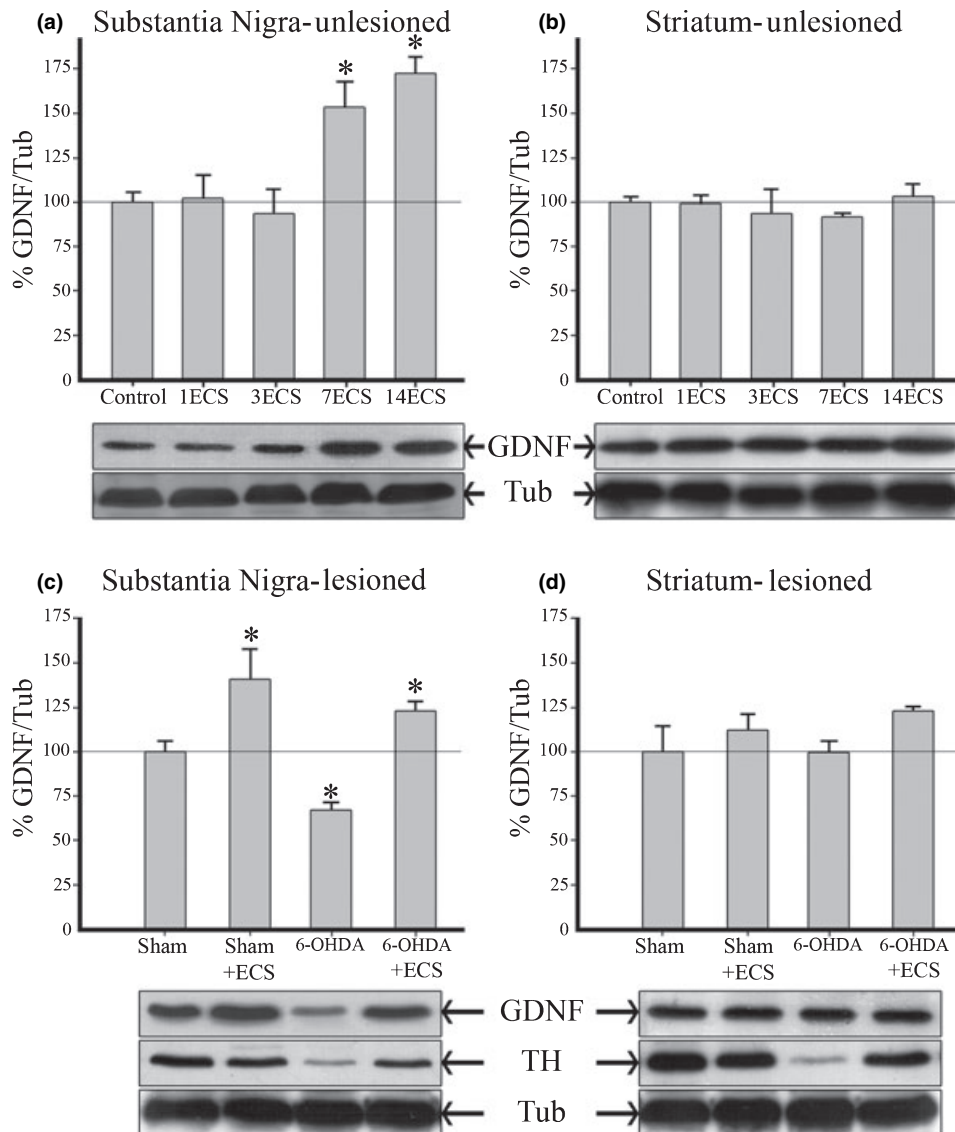


Fig. 4 Effects of 6-hydroxydopamine (6-OHDA) injection and electroconvulsive shock on glial cell-line derived neurotrophic factor (GDNF) protein expression. Bars represent mean \pm SEM of densitometry data normalized to tubulin. Values are normalized to sham-ECS animals. Representative western blots are shown at the bottom of each panel. (a) Displays protein levels in the substantia nigra (SN) of normal rats. Seven (7 ECS) or 14 (14 ECS), but not 1 (1 ECS) or 3 (3 ECS) days of ECS increased the expression of GDNF protein. (b) Displays densitometry results in the striatum of the same animals; no

significant changes were observed. (c) Displays protein levels in the SN 14 days after 6-OHDA (lesion) or vehicle (sham) injection. Injection of 6-OHDA resulted in decreased GDNF protein expression in the SN (light gray bar). ECS increased GDNF protein in vehicle-injected rats and in 6-OHDA-injected animals to the same degree. (d) Displays densitometry results in the striatum of the same animals; no significant changes were observed. Bars represent mean \pm SEM, $n = 4$ per group (*refer to a significant difference between the treatments and the vehicle (sham)-injected animals, $*p < 0.05$).

ously reported ECS protection of neuronal death induced by adrenalectomy (Mascó *et al.* 1999) and status epilepticus (Kondratyev *et al.* 2001) in other brain structures. Absence of TH immunoreactivity has been shown to correlate with dopaminergic neuronal death in this model (Carman *et al.* 1991). Furthermore, FG retrograde labeling of nigral neurons clearly demonstrates integrity of the axonal projections in the nigrostriatal pathway (Fig. 2). The possibility of loss of TH expression without neuronal death

after 6-OHDA at some of the time points studied was not addressed directly by our experimental design. Some disparity between the dopaminergic phenotype expression and neuronal death has been described in other experimental paradigms (Hagg and Varon 1993; Bowenkamp *et al.* 1996). However, because the number of cells that were FG+ was similar to the number of TH+ cells, this is an indication that the ECS treatment in the lesioned rats led to preservation in nigral cell number.

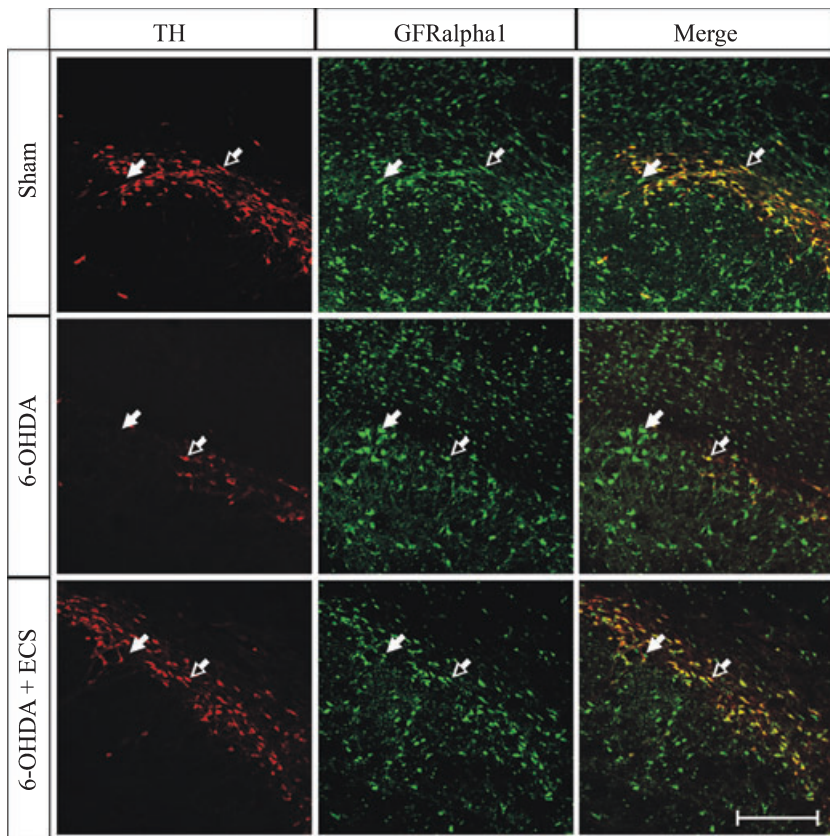


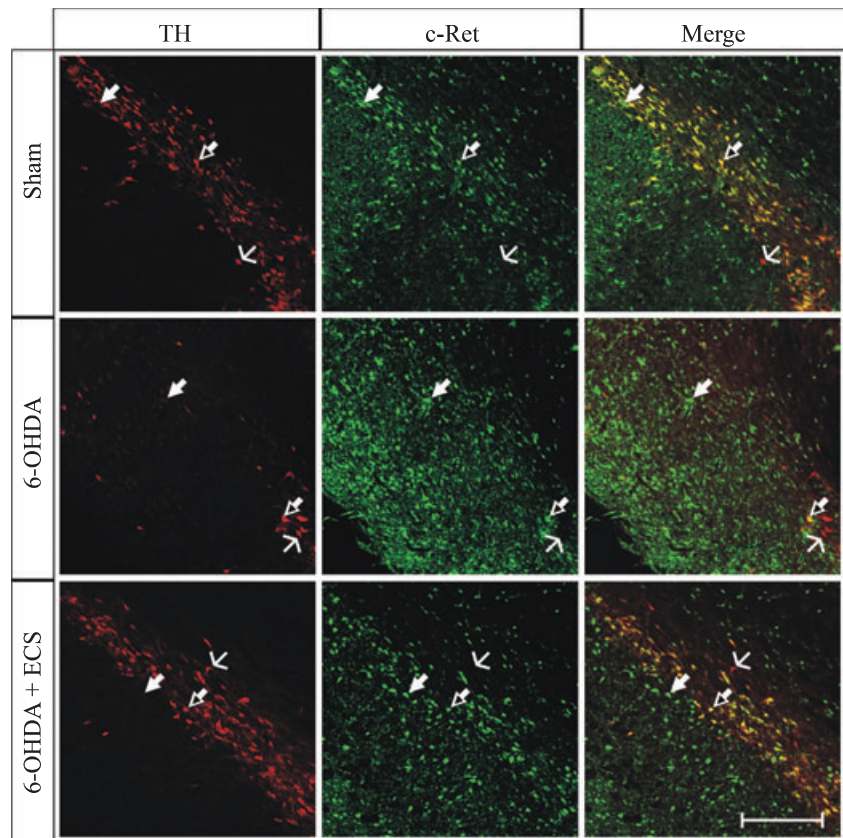
Fig. 5 Dopaminergic neurons co-express glial cell-line derived neurotrophic factor family receptor α -1 (GFR α -1). Rat midbrain sections were double-labeled with anti-tyrosine hydroxylase (TH, red fluorescence, left column) and anti-GFR α -1 (green fluorescence, middle column). Superimposed (merged) images are shown on the right column. Most neurons expressing TH co-express GFR α -1 co-receptor protein. Examples of dopaminergic neurons expressing GFR α -1 protein are marked with empty arrows. Examples of non-dopaminergic cells expressing GFR α -1 are marked with full arrows. Scale bar: 300 μ m.

Neuroprotective effects of ECS probably result from a confluence of factors. Amongst other effects, ECS-induced seizures are associated with glutamate release and the activation of NMDA glutamate receptors. In cultured dopaminergic neurons, NMDA activation has trophic effects (Isaacs *et al.* 1996), and subtoxic activation of NMDA receptors protects cultured cerebellar granule neurons by increasing synthesis and release of neurotrophins (Marini *et al.* 1998). A similar mechanism may be operative *in vivo*. GDNF promotes subsistence of damaged dopaminergic neurons more efficiently than other neurotrophins (Kriegstein 2004), and is a candidate for therapy in PD (Gill *et al.* 2003; Love *et al.* 2005). NMDA activation differentially regulates GDNF in rat striatum (Marco *et al.* 2002), and metabotropic glutamate receptor ligands are neuroprotective against 6-OHDA toxicity *in vivo* (Vernon *et al.* 2005). In our experiments, the injection of 6-OHDA significantly decreased GDNF expression in SN but not in striatum 2 weeks later (Fig. 4c and d). Our finding in the SN differs from unchanged levels found by Yurek and Fletcher-Turner (2001). On the other hand, the literature on the effect of 6-OHDA on striatal GDNF expression is not uniform. One publication found (as we did) no changes in striatal GDNF (Smith *et al.* 2003), whereas other reported an up-regulation of striatal GDNF mRNA (+1100%) (Zhou *et al.* 2000) and protein levels (+36%) (Yurek and Fletcher-Turner 2001).

One possible explanation for the differences is that while we report results of SDS-polyacrylamide gel electrophoresis and western blot analysis, other studies used either RT-PCR (Zhou *et al.* 2000) or ELISA (Yurek and Fletcher-Turner 2001).

Decreased GDNF expression in the SN may be due to loss of cells, decreased synthesis, excess degradation, or interruption of striatonigral retrograde transport. Whatever the case, ECS prevented the decrease in GDNF protein in SN of 6-OHDA-injected animals. Chronic ECS in intact animals increased GDNF protein expression in SN but not in striatum (Fig. 4a and b), and a similar pattern was found following 6-OHDA injection (Fig. 4c and d). ECS treatment induced a significant increase in GDNF protein expression only in the SN also in 6-OHDA-injected rats. Lack of change in striatal GDNF expression following ECS, in the face of strong protection of dopaminergic neurons, suggests that if neuroprotection is mediated by GDNF, it may be primarily the result of local regulation on the SN. Rapid transport of newly synthesized striatal GDNF to the SN is also a theoretical possibility that would account for the observed increase in striatal GDNF mRNA (Zhou *et al.* 2000). In the SN GDNF mRNA is below detection (Trupp *et al.* 1996) suggesting that the primary source of nigral GDNF is indeed retrograde transport from the striatum and/or other brain regions. Decreased GDNF

Fig. 6 Dopaminergic neurons co-express c-Ret. Rat midbrain sections were double labeled with anti-tyrosine hydroxylase (TH, red fluorescence, left column) and anti-c-Ret (green fluorescence, middle column). Superimposed (merged) images are shown on the right column. Most neurons expressing TH co-express c-Ret receptor protein. Examples of dopaminergic neurons expressing c-Ret protein are marked with empty arrows. Examples of non-dopaminergic cells expressing c-Ret are marked with full arrows. Examples of dopaminergic neurons not expressing c-Ret receptor protein are marked with open arrows. Scale bar: 300 μm .



expression in striatum of intact animals has also been reported following chronic ECS (Angelucci *et al.* 2002). As GDNF protein expression did not change in the hippocampus, and protein expression changes in the striatum are at the very least variable, it seems reasonable to conclude that the effects of ECS on GDNF protein expression are regionally specific.

We found, as have others, that dopaminergic neurons express GDNF receptor $\text{GFR}\alpha\text{-1}$ and c-Ret (Trupp *et al.* 1996; Araujo and Hilt 1998; Sarabi *et al.* 2001; Smith *et al.* 2003). Neuroprotective action of endogenous GDNF depends on the availability of its receptors after damage. Several groups have shown c-Ret and $\text{GFR}\alpha\text{-1}$ expression after 6-OHDA-lesioned SN. Thus, to suggest the participation of GDNF on its protective mechanism it was essential to show that the effect of ECS on GDNF expression was accompanied by the preservation of its receptor complex. Indeed, surviving dopaminergic neurons in the SN of 6-OHDA-injected animals treated with ECS also retained normal expression of the GDNF receptor complex (Figs 5 and 6). Thus, ECS-induced GDNF protein expression and release should increase trophic signaling in dopaminergic neurons. Recent results support the idea that c-Ret is not critical for the normal physiology of the SN in adult mice (Jain *et al.* 2006). However, c-Ret could act as part of a neuroprotective mechanism when these cells are injured.

Thus, chronic ECS resulted in anatomical and functional preservation of the entire nigrostriatal system. These findings may explain the sustained motor improvement in human patients with idiopathic PD following treatment with ECS (Shulman 2003; Fregni *et al.* 2005). Motor improvement following ECS therapy in patients with PD was reported in 1947 (Gallinek 1947), and confirmed in a double-blinded, controlled trial (Andersen *et al.* 1987). Many studies support this fact (Balldin *et al.* 1981; Rasmussen and Abrams 1991; Fall *et al.* 2000; Fregni *et al.* 2005). The latter, which involved patients without concomitant depression, showed sustained motor improvement (weeks to years) in two-thirds of the patients. The mechanism of this benefit is unknown. ECS changes binding of neurotransmitter receptors for DA (Fochtman *et al.* 1989; Andrade *et al.* 2002), norepinephrine (Kellar *et al.* 1981; Francis and Fochtman 1993), GABA (Green and Vincent 1987), glutamate (Smialowska *et al.* 2002), and serotonin (Gur *et al.* 2002). Yet, none of these receptor changes can easily account for the sustained motor benefit observed in patients with PD. We have shown that chronic ECS prevents 6-OHDA-induced toxicity to dopaminergic neurons, results in preserved axonal integrity, and prevents dopaminergic functional and behavioral impairment. These neuroprotective effects are correlated with sustained levels of GDNF protein expression in the SN, which are abolished

by the toxin, and preservation of the expression of the GDNF receptor complex in dopaminergic neurons. Our results may explain the well-known beneficial effects of electroconvulsive therapy on motor impairment in idiopathic PD and in neuroleptic-induced parkinsonism.

An inhibitory approach for GDNF (e.g. intracerebral infusion of short interference mRNA or blocking function antibodies), with all the technical difficulties that this type of experiments introduce, will be required to confirm endogenous GDNF neuroprotective effect.

Exposure to enriched environmental conditions and increased physical activity result in neuroprotection in a variety of experimental conditions, including nigrostriatal system injury (Bezard *et al.* 2003, Faherty *et al.*, 2005, Spires *et al.*, 2004, Steiner *et al.*, 2006, Jadavji *et al.* 2006). The mechanisms of these neuroprotective effects have not been completely elucidated, but likely involve modulation of neurotrophic factors, receptors, and proteins involved in signal transduction increased neurogenesis, and inhibition of apoptosis. Exposure to enriched environmental conditions in mice increased BDNF expression in the striatum (Bezard *et al.* 2003) and BDNF and GDNF expression in the hippocampus (Young *et al.*, 1999), and reduced spontaneous and toxic neuronal death (Bezard *et al.* 2003). Thus, ECS may promote neuronal protection by modulation of trophic factor expression mechanisms active in more physiological conditions, such as during exposure to enriched environments or increased exercise.

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