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GLIAL CELL-LINE DERIVED NEUROTROPHIC FACTOR IS ESSENTIAL FOR ELECTROCONVULSIVE SHOCK-INDUCED NEUROPROTECTION IN AN ANIMAL MODEL OF PARKINSON'S DISEASE

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Abstract—Sustained motor improvement in human patients with idiopathic Parkinson's disease has been described following electroconvulsive shock (ECS) treatment. In rats, ECS stimulates the expression of various trophic factors (TFs), some of which have been proposed to exert neuroprotective actions. We previously reported that ECS protects the integrity of the rat nigrostriatal dopaminergic system against 6-hydroxydopamine (6-OHDA)-induced toxicity; in order to shed light into its neuroprotective mechanism, we studied glial cell-line derived neurotrophic factor (GDNF) levels (the most efficient TF for dopaminergic neurons) in the substantia nigra (SN) and striatum of 6-OHDA-injected animals with or without ECS treatment. 6-OHDA injection decreased GDNF levels in the SN control animals, but not in those receiving chronic ECS, suggesting that changes in GDNF expression may participate in the ECS neuroprotective mechanism. To evaluate this possibility, we inhibit GDNF by infusion of GDNF function blocking antibodies in the SN of 6-OHDA-injected animals treated with ECS (or sham ECS). Animals were sacrificed 7 days after 6-OHDA infusion, and the integrity of the nigrostriatal system was studied by tyrosine hydroxylase immunohistochemistry and Cresyl Violet staining. Neuroprotection observed in ECS-treated animals was inhibited by GDNF antibodies in the SN. These results robustly demonstrate that GDNF is essential for the ECS neuroprotective effect observed in 6-OHDA-injected animals. **© 2011 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: neuroprotection, 6-OHDA, GDNF, Parkinson's disease, astrocytic reaction, electroconvulsive.

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized histopathologically by the loss of dopaminergic neurons of the substantia nigra (SN) and the concomitant loss of dopamine (DA) in the target striatum. Administration of 6-hydroxydopamine (6-OHDA) is one of the most studied animal model of PD. The injection of this neurotoxin in the medial forebrain bundle of rats selectively destroys dopaminergic neurons of the SN and their projection axons (Ungerstedt, 1968; Dauer and Przedborski, 2003). This model has been extensively used to search for candidate neuroprotective treatments (Hyman et al., 1991; Korsching, 1993; Kearns et al., 1997; Shults et al., 2000; Vernon et al., 2005), including strategies as sustained overexpression of trophic factors (TFs) by recombinant vectors or genetically modified cells (Choi-Lundberg et al., 1997; Bilang-Bleuel et al., 1997; Mandel et al., 1997; Kozlowski et al., 2000; Shults et al., 2000; Hurelbrink and Barker, 2004), or direct intracerebral infusion of TFs (Spina et al., 1992; Arenas et al., 1995; Kearns et al., 1997). However, neuromodulation-induced expression of endogenous TFs levels has been poorly studied as a potential neuroprotectant.

The oldest and most widely available neuromodulation technique is electroconvulsive therapy, and its animal equivalent electroconvulsive shock (ECS) prevents neuronal death induced by adrenalectomy (Mascó et al., 1999), status epilepticus in rats (Kondratyev et al., 2001), as well as in huntingtin mutant mice (Mughal et al., 2011). Moreover, we recently demonstrated that chronic ECS treatment prevents DA neuron loss induced by 6-OHDA toxic injury, protects the nigrostriatal pathway (assessed by FluoroGold retrograde labeling), and reduces motor impairment in this model of PD (Anastasía et al., 2007). The mechanism of ECS neuroprotection is unknown, but several publications suggest that synthesis and release of TFs may have an essential role in this effect (Duman and Vaidya, 1998; Fujikilow et al., 2010; Anastasía et al., 2007). ECS alters the expression of TFs and their receptors in several brain regions (Newton et al., 2003; Altar et al., 2004). For instance, ECS regulates endogenous levels of brain-derived neurotrophic factor (BDNF) (Nibuya et al., 1995; Angelucci et al., 2002; Newton et al., 2003; Kim et al., 2010), nerve growth factor (Follesa et al., 1994; Angelucci et al., 2002, 2003; Kondratyev et al., 2002; Newton et al., 2003; Conti et al., 2009), fibroblast growth factor-2 (Kim et al., 2010; 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 hippocampus, cortex, and striatum.

It is very well established that GDNF has neuroprotective and regenerative effects to dopaminergic function in animal models of PD, making this TF a good candidate for

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Abbreviations: AP, anterior–posterior; BDNF, brain-derived neurotrophic factor; DA, dopamine; ECS, electroconvulsive shock; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; GFRalpha-1, GDNF family receptor a1pha 1; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NMDA, *N*-methyl-D-aspartic acid; PD, Parkinson's disease; SN, substantia nigra; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata; TF, trophic factor; TH, tyrosine hydroxilase; 6-OHDA, 6-hydroxidopamine.

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PD therapeutic research (Rangasamy et al., 2010). Indeed, GDNF rescues damaged dopaminergic neurons in culture and in animal models of PD more efficiently than other TFs (Krieglstein, 2004; Sun et al., 2005). Transduction of GDNF signaling depends on GDNF family receptor a1pha 1 (GFRalpha1) and the receptor tyrosine kinase Ret (Saarma and Sariola, 1999). These receptors are expressed in dopaminergic neurons (Trupp et al., 1996; Sarabi et al., 2001; Smith et al., 2003; Anastasía et al., 2007). GFRalpha1 is a glycosylphosphatidylinositol-anchored receptor for GDNF, which associates with transmembrane proteins, such as Ret or the cell adhesion molecule NCAM (neural cell adhesion molecule), for intracellular transmission of the GDNF signal (Saarma and Sariola, 1999; Ibáñes, 2010).

GDNF/GFRalpha1/RET signaling promote survival and neurite growth of dopaminergic neurons *in vitro* (Lin et al., 1993; Ding et al., 2004) and *in vivo* (Tomac et al., 1995a; Kearns et al., 1997). Furthermore, GDNF rescues dopaminergic neurons in experimental PD models, including l-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity in mice (Tomac et al., 1995a) and monkeys (Gash et al., 1996; Kordower et al., 2000), in addition to 6-OHDA toxicity (Björklund et al., 1997; Kearns et al., 1997) and axonal transection (Beck et al., 1995) in rats. In spite of the strength of the experimental evidence, intraputaminal administration of GDNF in patients with PD failed to show consistent clinical benefits (Gill et al., 2003; Love et al., 2005; Rangasamy et al., 2010). This variability of efficacy may be related to poor diffusion of GDNF protein out of the infusion cannulae, 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 recently reported a correlation between ECS-induced neuroprotection and increased GDNF levels after ECS treatment. Moreover, protected dopaminergic neurons of the SN maintain normal expression of GDNF receptor complex (Anastasía et al., 2007). In this work we present immunohistochemical data showing a GDNF increase in ECS-treated animals after 6-OHDA, confirming previous reported biochemical analysis (Anastasía et al., 2007). These results suggest that GDNF may participate in ECS neuroprotective effect. Thus, we tested this hypothesis directly. We present evidence that functional inhibition of GDNF reverses ECS-induced neuroprotective effects.

EXPERIMENTAL PROCEDURES

Animals

Adult male Wistar rats (Instituto Ferreyra, Cordoba, Argentina) weighing 180/250 g were housed with a 12-h 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). All efforts were made to minimize the number of animals used and their suffering.

Experimental design

To assess the effect of ECS on GDNF expression in the SN and striatum, animals were randomly assigned in four groups to compare the effects of ECS vs. sham ECS treatment in control (6-OHDA− ECS−, 6-OHDA− ECS+) or postlesion (6-OHDA+ ECS−, 6-OHDA+ ECS+) conditions. Animals received daily ECS (ECS+) or sham ECS (ECS−) for 2 days before and 13 days after unilateral 6-OHDA (or vehicle) administration. Rats were sacrificed 24 h after the last shock (day 14 after 6-OHDA) by transcardiac perfusion for GDNF immunohistochemical analysis.

The same experimental group assignments (6-OHDA− ECS−, 6-OHDA− ECS+, 6-OHDA+ ECS−, 6-OHDA+ ECS+) were used to study early astrocytic reaction and the impact of GDNF inhibition on nigrostriatal dopaminergic neuron degeneration. Animals received daily ECS (ECS+) or sham ECS (ECS−) for 2 days before and 6 days after unilateral 6-OHDA (or vehicle) administration and were sacrificed 24 h after the last shock (day 7 after 6-OHDA) by transcardiac perfusion, and processed for glial fibrillary acidic protein (GFAP, astrocytic marker) and tyrosine hydroxilase (TH) immunohistochemistry, or Cresyl Violet staining.

For GDNF blockade, we designed an inhibitory approach infusing well-characterized function-blocking GDNF antibodies (R&D Systems cat#AB-212-NA, Minneapolis, MN, USA) (Oo et al., 2003) in the SN, as described later in this section (Fig. 3A).

ECS treatment

Bilateral ECS was administered once a day via electrodes placed in the supra-orbital zone (pulse width: 0.1 ms; shock duration: 0.2 s; intensity: 40 mA) using a rodent ECS Unit 7801 (Ugo Basile, Comerio, Italy). Seizures evoked with these parameters were tonic-clonic (involving tonic forelimb and hind limb extension). Sham ECS animals received the same handling and contact with the electrodes, but no current was applied. Animals were excluded if motor tonic-clonic seizures lasting 10–15 s were not observed (approximately 10%).

6-OHDA injection

Anesthetized rats (ketamine 55 mg/kg+xylazine 11 mg/kg, i.p.; Konig, Buenos Aires, Argentina) were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) and injected with 6-OHDA (Sigma, St. Louis, MO, USA; 4 μl over 8 min, 2 μg/μl in 0.9% NaCl with 0.02% ascorbic acid) in the medial forebrain bundle [from bregma: anterior–posterior (AP): −1.8 mm; lateral: +2 mm; and ventral: −8.1 mm] (Paxinos and Watson, 2007); the cannulae was removed 5 min later. Sham-operated animals received only the vehicle. All surgeries for a given experiment were carried out in the same session by a operator who was blind to the treatment groups.

Cannulae implantation and GDNF antibodies infusion

To study the effects of endogenous GDNF in the substantia nigra pars compacta (SNpc) using anti-GDNF blocking function antibodies (Figs. 3 and 4), rats were implanted (under anesthesia, ketamine 55 mg/kg+xylazine 11 mg/kg, i.p.; Konig) with a 22-gauge guide cannulae 3 mm above the SN at the following coordinates from bregma: AP: −5.2 mm; lateral: +2.2 mm; and ventral: −5.0 mm (Paxinos and Watson, 2007). In the same surgical procedure, 6-OHDA was infused in the medial forebrain bundle, as described previously. Following removal of the 6-OHDA infusion cannulae, the nigral guide cannulae and a screw placed in the parietal bone were fixed to the skull with dental acrylic. After surgery, animals received Gentamicin (Genta G, Laboratorio Vetue, Santa Fe, Argentina). Anti-GDNF blocking function antibodies (R&D Systems cat#AB-212-NA; 2 μg/3 μl in each injection) were infused immediately after the surgery and on days two and four following 6-OHDA administration (Fig. 3A) through an infusion cannulae (30 gauge) that passed the end of the guide cannulae by 3 mm, targeting the SNpc (AP: −5.2 mm; lateral: +2.2 mm; ventral: −8.0 mm) (Paxinos and Watson, 2007). Infusions were performed at a rate of 1 μl/min with a Hamilton precision pump. The cannulae were left *in situ* 2 min after infusion to allow the liquid to penetrate

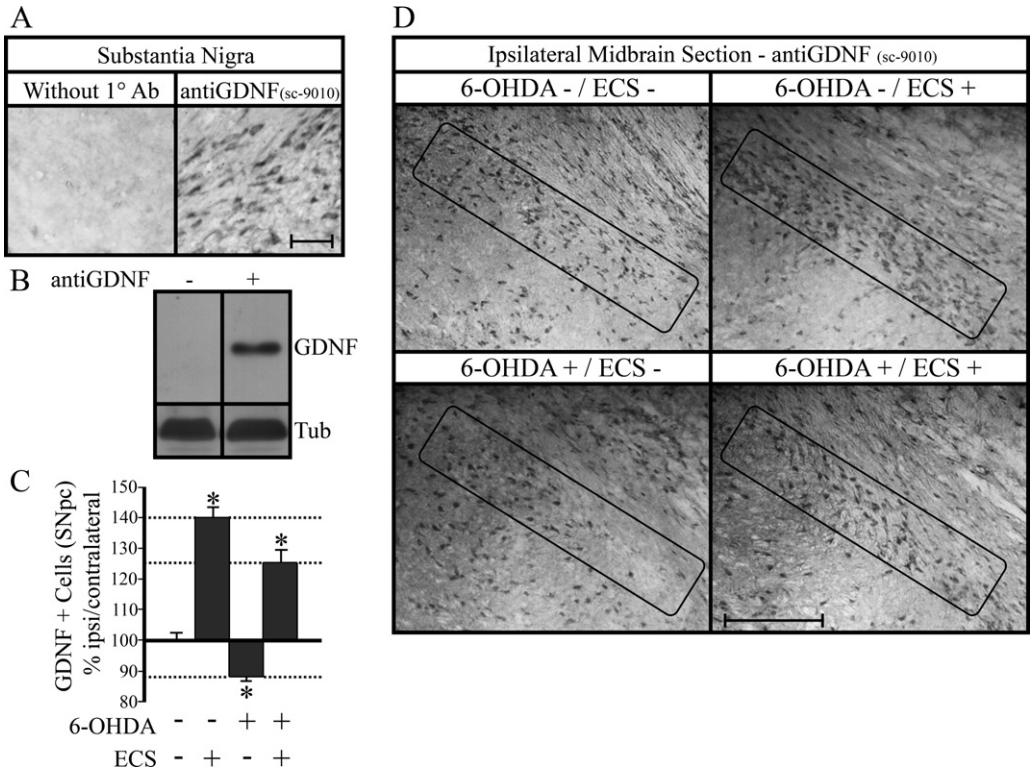

Fig. 1. ECS increases GDNF expression in 6-OHDA-injected animals. (A) Brain sections (through the SN) labeled with Santa Cruz anti-GDNF (catalog number sc-9010) and appropriate secondary antibodies, and a control staining where the primary antibody was omitted. (B) SN lysates were electrophoresed in SDS-PAGE followed by Western blot. The membranes were incubated with anti-GDNF antibody (1:200, RDI, Concord, MA, USA), followed by the corresponding secondary antibody with horseradish peroxidase, and ECL. Control where the primary antibody was omitted is shown. Tubulin was used as a loading control. (C) Number of GDNF+ cells in the SNpc after vehicle (6-OHDA−) or 6-OHDA (6-OHDA+) injection, with sham ECS (ECS−) or with ECS (ECS+) treatment. Baseline=100% corresponding to the 6-OHDA− ECS− control. (D) Microphotographs of rat midbrain sections (ipsilateral-injected side) stained for GDNF. Region of interest for GDNF+ cell count (black box, SNpc) was defined by stacking the following section stained with TH antibody. SNpc, substantia nigra pars compacta. Bars represent mean±SEM. * $P<0.05$. $n^{6\text{-OHDA}-\ \text{ECS}-}=4$; $n^{6\text{-OHDA}-\ \text{ECS}+}=4$; $n^{6\text{-OHDA}+\ \text{ECS}-}=5$; $n^{6\text{-OHDA}+\ \text{ECS}+}=5$. Panel (A) scale bar: 100 μm. Panel (D) scale bar: 300 μm.

the tissue. Histological examination of infusion cannulae placements was performed by analyzing Cresyl Violet-stained sections. Only the data from animals with the cannulae located in the intended site were included in the final analysis. Infusion sites of each animal analyzed are shown in Fig. 3B.

Immunohistochemistry and analysis

Anesthetized animals (400 mg/kg chloral hydrate, i.p.; Parafarm, Buenos Aires, Argentina) were transcardially perfused with glucose 0.4%, sucrose 0.8%, and 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). Brains were removed, postfixed, cryoprotected, and sectioned in a cryomicrotome (36 μm). Free-floating coronal sections were blocked (5% bovine serum albumin, or non-fat dry milk), incubated with primary antibody (anti-GDNF Santa Cruz cat# sc-9010 or RDI anti-GDNF cat# RDI-GDNFabrP 1:200 72 h at 4 °C; anti-TH 1:1000 over night at 4 °C, Chemicon, Temecula, CA, USA), followed by secondary biotin-conjugated antibody (2 h at 20–25 °C 1:250, Sigma), streptavidin peroxidase (2 h at 20–25 °C 1:250, Sigma), and developed using diaminobenzidine (Sigma) as the chromogen. Bright field images were captured digitally (Eclipse TE2000-U microscope, Digital Sight DS-U1 camera, Nikon, Tokyo, Japan).

Counts of GDNF+ and TH+ cells in the SNpc were performed as previously described (Sauer and Oertel, 1994), and after unbiased intensity and size selection criteria. A gray level threshold was selected for all the microphotographs of all different treatments and staining. After this selection, ImageJ particle analysis plugin was used to set the lower and higher size of pixels to be considered as a countable object (cell). Both selection thresholds were arbitrarily defined, but maintained rigorously for all the images. Finally, using ImageJ cell, counter plugin cells were counted in each image. TH- or GDNF-immunostained cells in the SNpc were counted bilaterally in six sections for each animal (−4.52, −4.8, −5.2. −5.6, −6.04, and −6.3 mm from bregma) (Paxinos and Watson, 2007). GDNF+ cell count was performed in the SNpc, region of interest defined in a contiguous section stained for TH (Fig. 1D). TH+ and GDNF+ cell numbers in the SNpc were expressed as the percentage of positive cells on the lesioned side with respect to the contralateral intact side; this approach is widely used (Kirik et al., 2000; Paul et al., 2004; Anastasía et al., 2007) and was chosen to avoid methodological biases caused by interindividual differences. In the striatum, GDNF level was analyzed by densitometry (Image J 1.34j; US National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997–2008). To verify the anti-GDNF primary antibodies specificity, a series of brain sections through the SN were incubated with or without two different antibodies (Santa Cruz anti-GDNF sc-9010 or RDI anti-GDNF RDI-GDNFabrP) followed by the appropriate secondary biotinylated antibodies and the avidin-peroxidase complex. Both primary antibodies displayed specific GDNF labeling (as shown for Santa Cruz anti-GDNF in Fig. 1A). As the signal–background ratio of the Santa Cruz antibody was higher than the RDI antibody, we quantify GDNF using the former.

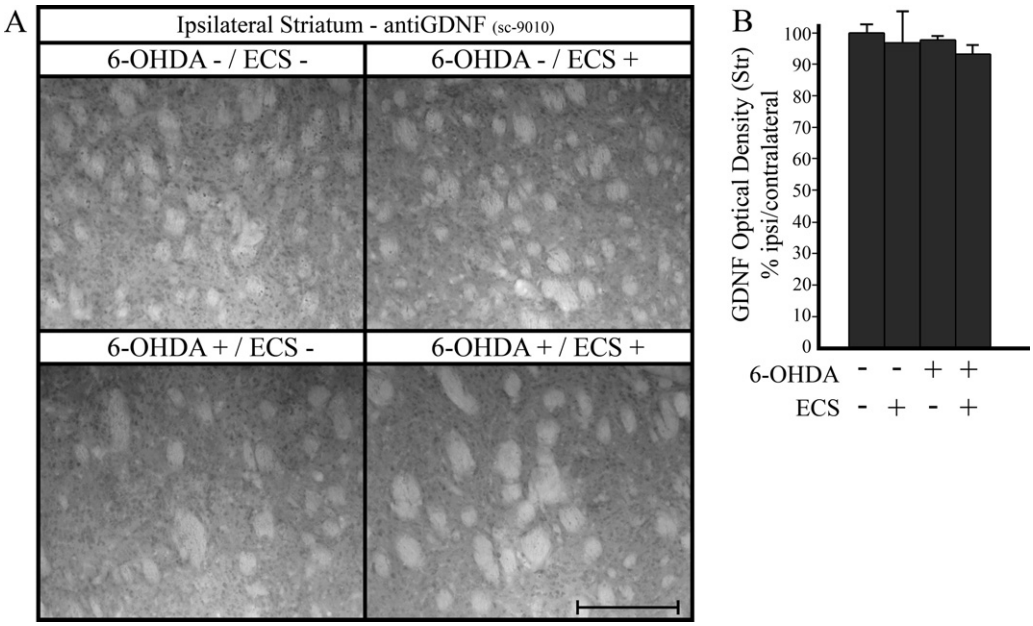

Fig. 2. GDNF expression is unaffected in the striatum following 6-OHDA injection and/or ECS treatment. (A) Microphotographs of striatal sections (ipsilateral-injected side) stained for GDNF. (B) GDNF gray level analyzed by densitometry in the striatum after vehicle (6-OHDA–) or 6-OHDA (6-OHDA+) injection, with sham ECS (ECS–) or with ECS (ECS+) treatment. Str, striatum. Bars represent mean±SEM. $n^{6\text{-OHDA}-\ \text{ECS}-}=4$; $n^{6\text{-OHDA}-\ \text{ECS}+}=4$; $n^{6\text{-OHDA}+\ \text{ECS}-}=5$; $n^{6\text{-OHDA}+\ \text{ECS}+}=5$. Scale bar: 300 μm.

To study GFAP levels, the following antibodies were used: anti-GFAP polyclonal antibody (1:1000 over night at 4 °C, Chemicon), anti-TH monoclonal antibody (1:1000 over night at 4 °C, Chemicon), followed by fluorescent secondary antibodies (Alexa Fluor 568 anti-rabbit IgG, 1:200; Alexa Fluor 488 anti-mouse IgG, 1:200; Molecular Probes, Carlsbad, CA, USA). Sections were assessed under fluorescence microscopy (Eclipse TE2000-U, Nikon) and images were captured digitally (Digital Sight DS-U1, Nikon). After TH and GFAP 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 (Fig. 5A, white line). GFAP level was analyzed by densitometry (ImageJ) in the SNpc and the striatum.

To further ensure absence of bias in cell counting or densitometry analysis, sections were coded, and the operator was blind to treatment groups. Negative controls without primary antibodies were performed for each immunodetection.

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

Statistical comparisons were performed by the non-parametric Kruskal–Wallis test. Results are provided as the mean±SEM.

RESULTS

ECS causes an increase in GDNF protein expression in 6-OHDA-injected animals

ECS treatment increased the number of GDNF+ cells in SNpc of vehicle-injected animals, whereas 14 days after 6-OHDA injection a significant decrease in GDNF+ cell number in SNpc was observed. Furthermore, when daily ECS was administered to 6-OHDA-injected animals, the number of GDNF+ cells was increased in the SNpc (Fig. 1C, D). GDNF expression in striatum was unaffected by either 6-OHDA or ECS (Fig. 2A, B). These results are in agreement with previous results from our laboratory using Western blot protein detection (Anastasía et al., 2007). To verify the effectiveness of the 6-OHDA lesion, TH+ cells in the SNpc were counted as well. As we reported previously (Anastasía et al., 2007), 6-OHDA injection significantly reduced TH cell number in the SNpc, and treatment with ECS prevented the decrease (data not shown).

Infusion of GDNF antibodies in the SNpc prevents ECS-induced TH+ cells protection

The changes in endogenous GDNF levels induced by ECS (Fig. 1 and Anastasía et al., 2007), strongly suggest that this TF may play a role in ECS-induced neuroprotection. To address this hypothesis directly, anti-GDNF function blocking antibodies (Oo et al., 2003) were injected stereotaxically in the SNpc of 6-OHDA (or vehicle)-injected animals receiving ECS (or sham ECS). Animals were sacrificed 7 days after the neurotoxic injection, when the neuroprotective effect of ECS is already detectable (Anastasía et al., 2007).

6-OHDA injection resulted in significant TH+ cell loss in the SNpc, and ECS treatment prevented this loss, confirming our published results (Fig. 3C; Anastasía et al., 2007). Moreover, ECS *per se* did not alter the number or intensity of TH+ cells in the SNpc of unlesioned animals

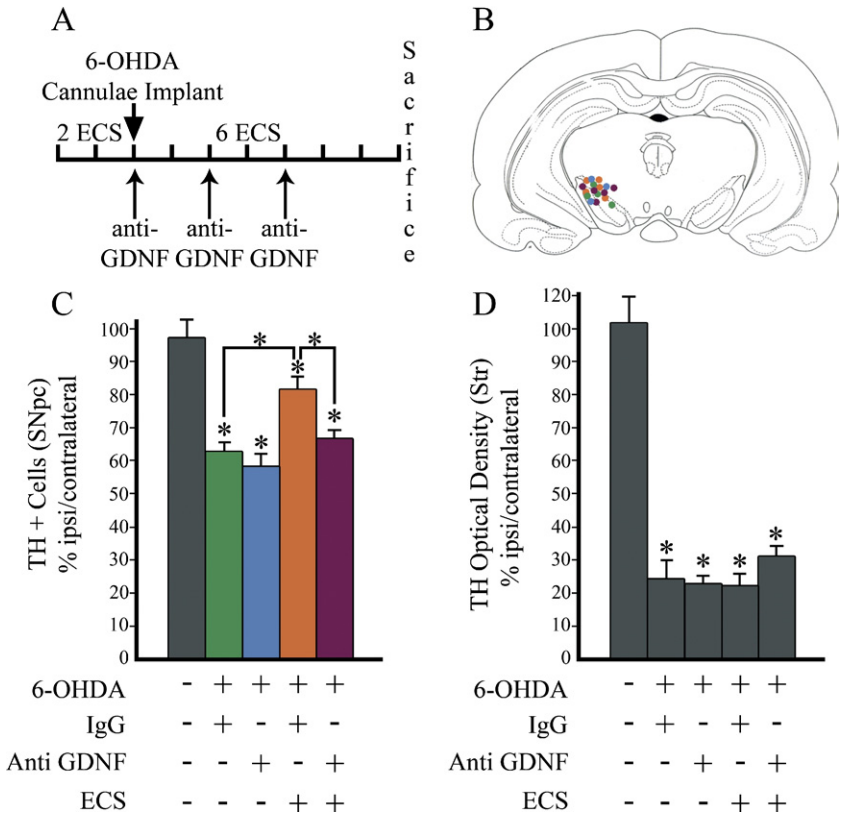

Fig. 3. Infusion of GDNF antibodies in the SNpc prevents ECS-induced TH+ cells protection. (A) Animals received daily ECS (ECS+) or sham ECS (ECS−) for 2 days before and 6 days after the unilateral 6-OHDA administration. These animals were implanted with cannulae in the SN on the day of 6-OHDA administration, and infused with anti GDNF function blocking antibodies (or control IgGs) on day 0, 2, and 4 following 6-OHDA administration. Animals were sacrificed 24 h after the last shock (day 7 after 6-OHDA). (B) Cannulae position assessed by Cresyl Violet staining. Each dot represents the position of the cannulae in one animal, and different colors were designated for each treatment as shown in panel (C). (C) TH+ cell count in the SNpc 7 days after 6-OHDA. Gray bar represents control animals injected with vehicle and treated with sham ECS (n=3); all the other experimental groups received 6-OHDA and the following treatments: (green bar) IgGs infusions (n=4); (blue bar) anti GDNF function blocking antibodies infusions (n=3); (orange bar) IgGs infusion and ECS treatment (n=5); (purple bar) anti GDNF function blocking antibodies infusions and ECS treatment (n=5). In all the experimental groups TH+ cells were counted bilaterally in six sections/animal, and there were no significant differences in ipsilateral/contralateral ratios between the different sections. (D) TH gray level analyzed by densitometry in the striatum. SNpc, substantia nigra pars compacta; Str, striatum; TH, tyrosine hydroxylase. Bars represent mean±SEM. * $P<0.05$, compared to the control bar. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

(data not shown). Intranigral injection of three doses of function blocking anti-GDNF antibody (2 μg/injection, Fig. 3A) in 6-OHDA-injected animals abolished TH+ cells protection effect of ECS, but the administration of unspecific goat IgGs had no effect (Fig. 3C). Anti-GDNF injection or goat IgGs by themselves did not affect TH+ cell loss in 6-OHDA-lesioned animals (Fig. 3C), suggesting that the anti-GDNF administration protocol chosen did not exacerbate 6-OHDA neuronal death *per se*. TH+ cells were counted bilaterally in six sections for each animal, and there were no significant differences in ipsilateral/contralateral ratios between the different sections.

Quantitative assessment of TH staining in the striatum showed a marked decrease following 6-OHDA, regardless of ECS or IgGs administration (Fig. 3D). We carefully studied the infusion cannulae position using Cresyl Violet staining. There was no significant difference between the anti-GDNF or IgGs infusion cannulae position in the different treatments (Fig. 3B).

Survival of SNpc neurons induced by ECS after 6-OHDA requires GDNF

Three weeks after 6-OHDA injection, dopaminergic neuron survival and the expression of TH are highly correlated (Kirik et al., 2000; Paul et al., 2004). However, TH immunoreactive cell counts at early postlesion times may not reveal the actual nigral dopaminergic neuron number because TH expression may be lost without neuronal death (Anastasía et al., 2009).

Thus, to investigate if the reduction in TH-stained neurons observed 7 days after 6-OHDA (Fig. 3) was due to changes in TH expression or neuronal loss, we measured the nigral neuron number using Cresyl Violet staining. We observed a significant reduction of Cresyl Violet cell number only 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, 2007) 7 days after the toxic insult (Fig. 4A, B). In the posterior SNpc (defined arbitrary as −5.6, −6.04, and −6.3 mm sections AP from bregma)

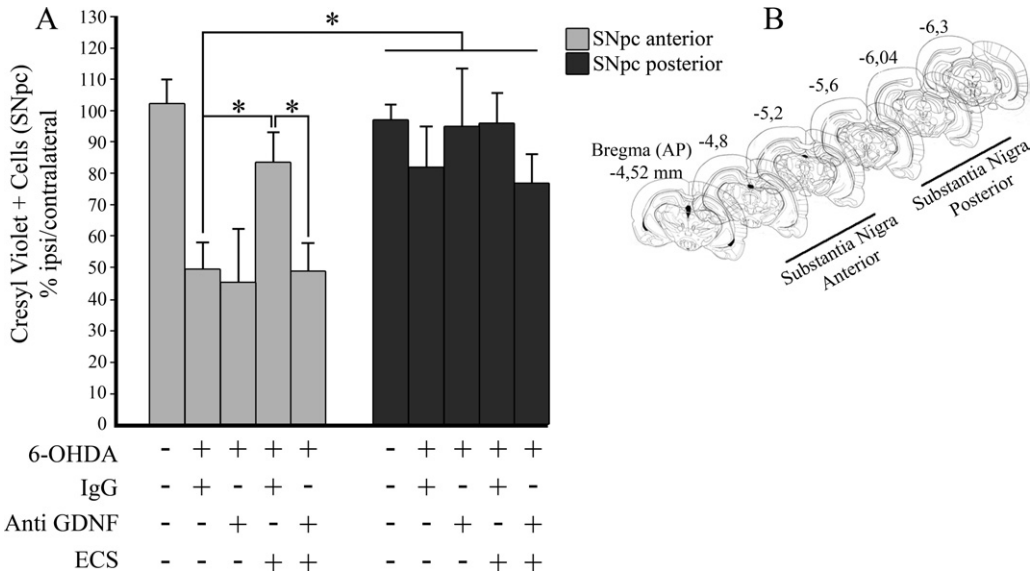

Fig. 4. Survival of SNpc neurons induced by ECS after 6-OHDA requires GDNF. (A) Cresyl Violet+ cell count in the SNpc 7 days after 6-OHDA administration. Number of Cresyl Violet+ cells was obtained by averaging three sections for the anterior SNpc and three sections for the posterior SNpc. (B) Representative brain coronal section pictures showing an arbitrary division in anterior and posterior SN. SNpc, substantia nigra pars compacta; AP, anterior–posterior axis. Bars represent mean±SEM. * $P<0.05$. $n^{6\text{-OHDA}-\ IgG-\ ECS-}=3$; $n^{6\text{-OHDA}+\ IgG+}=4$; $n^{6\text{-OHDA}+\ antiGDNF+}=3$; $n^{6\text{-OHDA}+\ IgG+\ ECS+}=5$; $n^{6\text{-OHDA}+\ antiGDNF+\ ECS+}=5$.

(Paxinos and Watson, 2007), virtually no Cresyl Violet cell loss were detected (Fig. 4A, B). Notably, this was consistent with the reported pattern of degenerating nigral neurons assessed with FluoroJade-B marker 4 days after a 6-OHDA injection (Anastasía et al., 2009). Moreover, a similar dopaminergic neurons degeneration pattern was described in the MPTP primate model of PD (Freundlieb et al., 2006).

ECT reduced 6-OHDA-induced Cresyl Violet cell loss in the anterior SNpc (Fig. 4A). This result clearly demonstrated that the neuroprotective effect of ECT became evident shortly after exposure to 6-OHDA. Intranigral injection of anti-GDNF antibody in ECS-treated and 6-OHDA-injected animals resulted in a suppression of Cresyl Violet+ cells neuroprotection in the anterior SNpc, as compared with IgGs injected condition (Fig. 4A).

Taken together, TH and Cresyl Violet results demonstrate that ECT induces dopaminergic neuron protection in the anterior SNpc. Moreover, TH phenotype is modified by 6-OHDA administration and ECS treatment in the posterior SNpc 7 days after the toxic injury, whereas no significant Cresyl Violet cell loss is evident at this time point in our lesion model. These results confirm previous data on this dissociation (Anastasía et al., 2009).

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

In order to study a possible contribution of astrocytes to ECT-induced neuroprotection, we assessed the levels of the astrocytic marker GFAP early after 6-OHDA administration. In normal rats, GFAP-labeled cells are present in the substantia nigra pars reticulate (SNpr), but rarely in the SNpc (Anastasía et al., 2009; Depino et al., 2003). ECT treatment in vehicle-injected rats did not alter GFAP immunostaining in the SNpc (Fig. 5B). Seven days after 6-OHDA injection, GFAP immunolabeling was increased in the SNpc of animals (two folds in the anterior SNpc and by half in the posterior SNpc; Fig. 5A, B). Remarkably, we observed an extraordinary GFAP labeling increase in the anterior SNpc of ECT-treated animals 7 days after the 6-OHDA lesion. On the other hand, GFAP levels in the posterior SNpc were no further increased by ECT treatment after the toxin administration. In the striatum, GFAP levels were increased by 6-OHDA regardless of the ECS treatment (Fig. 6A, B).

DISCUSSION

The major finding of this work is that GDNF is necessary for ECS-induced neuroprotection of the nigrostriatal dopaminergic system. We have shown that GDNF is increased in the SNpc after chronic ECS treatment, and functional inhibition of GDNF prevents ECS-induced neuronal survival. Moreover we found that in the 6-OHDA lesion model we used, Cresyl Violet cell loss was limited to the anterior, but not posterior SNpc, while reduction of TH occurs in both areas. Finally, we established that 6-OHDA and ECS induces a synergistic increase in astrocytic reaction suggesting a possible participation of this cellular population in the neuroprotection described.

ECS improves neuronal survival in a variety of contexts (Mascó et al., 1999; Kondratyev et al., 2001; Mughal et al., 2011; Anastasía et al., 2007), but the mechanisms of such effect has not been established. ECS-induced seizures are associated with glutamate release and the activation of glutamate receptors: *N*-methyl-D-aspartic acid (NMDA) receptor activation may have trophic effects in cultured do-

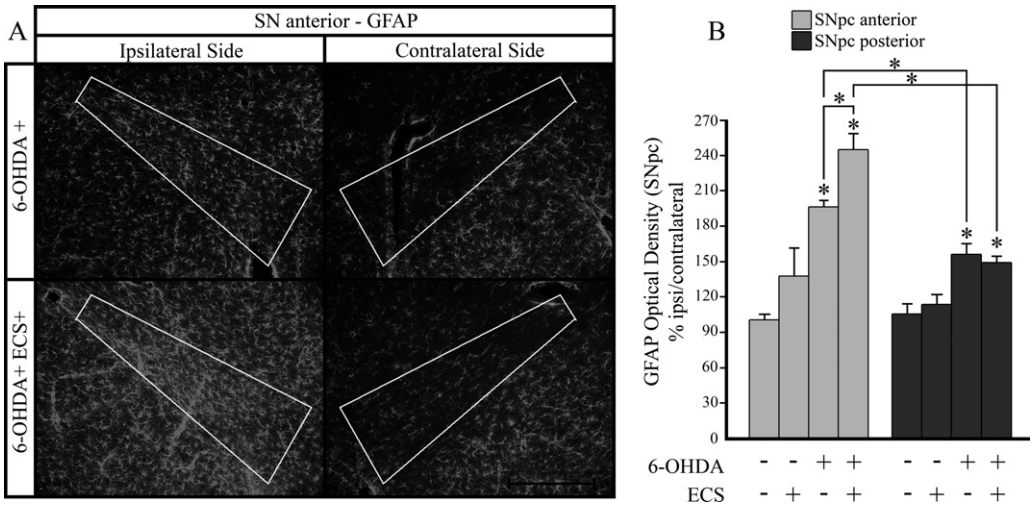

Fig. 5. ECT induced astrocytic reaction in the anterior SNpc early after 6-OHDA lesion. (A) Microphotographs of rat midbrain sections stained for glial fibrillary acidic protein (GFAP) 7 days after 6-OHDA injection. SNpc (white delimitated area) was identified by stacking the TH images onto GFAP-labeled images and delimiting the region of interest based on the TH staining. (B) GFAP optical density quantification obtained by averaging three sections for the anterior SNpc and three sections for the posterior SNpc. Animals received vehicle (6-OHDA−) or 6-OHDA (6-OHDA+) injection, with sham ECS (ECS−) or with ECS (ECS+) treatment. SNpc, substantia nigra pars compacta. Bars represent mean±SEM. * $P<0.05$. $n^{6\text{-OHDA}-\ \text{ECS}-}=3$; $n^{6\text{-OHDA}-\ \text{ECS}+}=5$; $n^{6\text{-OHDA}+\ \text{ECS}-}=5$; $n^{6\text{-OHDA}+\ \text{ECS}+}=5$. Scale bar: 300 μm.

paminergic neurons (Isaacs et al., 1996), and increase synthesis and release of neurotrophins in cerebellar granule neurons (Marini et al., 1998; Lipsky and Marini, 2007). Moreover, in cultured striatal astrocytes, NMDA increases GDNF mRNA levels (Ho et al., 1995), while glutamate induces mRNA expression and release of GDNF in a concentration-dependent manner (Yamagata et al., 2002). A similar mechanism may be functional *in vivo*. Indeed, NMDA activation differentially regulates GDNF mRNA and GDNF localization in rat striatum (Marco et al., 2002), and GDNF mRNA increases in the striatum and hippocampus of rats after experimental seizures (Schmidt-Kastner et al., 1994; Humpel et al., 1994). These changes could reflect an endogenous trophic response of neurons to an excitotoxic injury. Considering that ECS-induced glutamate release is massive but brief (behaviorally observed by 10–15 s lasting seizures), it may transiently activate glutamate receptors that could be beneficial for neuronal survival through the induction of TFstrophic factors such as GDNF as a neuroprotective attempt. Activation of metabotropic glutamate receptor (mGluR) may also contribute to the protective mechanism of ECS because mGluR agonists

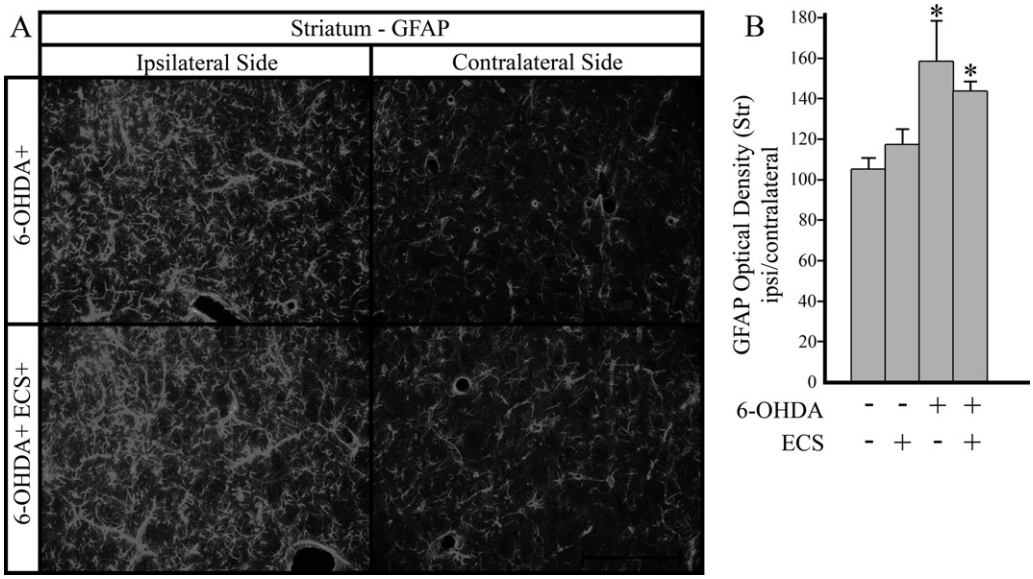

Fig. 6. Striatal astrocytic reaction 7 days after 6-OHDA. (A) Microphotographs of striatal sections stained for GFAP. (B) GFAP optical density quantification. Animals received vehicle (6-OHDA−) or 6-OHDA (6-OHDA+) injection, with sham ECS (ECS−) or with ECS (ECS+) treatment. Str, striatum. Bars represent mean±SEM. * $P<0.05$. $n^{6\text{-OHDA}-\ \text{ECS}-}=3$; $n^{6\text{-OHDA}-\ \text{ECS}+}=5$; $n^{6\text{-OHDA}+\ \text{ECS}-}=5$; $n^{6\text{-OHDA}+\ \text{ECS}+}=5$. Scale bar: 300 μm.

are neuroprotective against excitotoxicity *in vitro* and against 6-OHDA toxicity *in vivo* (Vernon et al., 2005; Murray et al., 2002).

GDNF is expressed in the SN and striatum (Choi-Lundberg and Bohn, 1995; Oo et al., 2005; Altar and DeStefano, 1998; Tomac et al., 1995b; Barroso-Chinea et al., 2005). GDNF+ cells are most commonly (but not exclusively) found in the SNpc, rather than in the SNpr, or other surrounding areas (Kawamoto et al., 2000). ECS may increase GDNF-dependent cellular actions by enhancing GDNF synthesis in glial and/or neuronal cells, increasing GDNF release, and by modifying GDNF transport. Regardless of the mechanism by which ECS increases GDNF, it is clear that such an increase would promote survival of injured dopaminergic neurons more efficiently than other TFs (Krieglstein, 2004), and it has been argued on this basis that GDNF may have a role in the treatment of PD (Gill et al., 2003; Love et al., 2005; Rangasamy et al., 2010).

We found that 6-OHDA significantly decreased GDNF+ cell number in SNpc, but not GDNF optical density in the striatum (Figs. 1C, D and 2A, B), in agreement with our previously published Western blot analysis (Anastasía et al., 2007), but at odds with unchanged levels found in the SN by Yurek and Fletcher-Turner (2001). Likewise, the literature on the effect of 6-OHDA on striatal GDNF expression is not uniform. One publication found (as we did in this report and previously in Anastasía et al., 2007) no changes in striatal GDNF (Smith et al., 2003), while other reported an upregulation 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 assessed by immunohistochemical analysis (and previously using SDS–polyacrylamide gel electrophoresis and western blot analysis) other studies utilized either RT-PCR (Zhou et al., 2000) or ELISA (Yurek and Fletcher-Turner, 2001).

Decreased GDNF expression in the SNpc following 6-OHDA injection may be due to loss of cells, decreased synthesis, excess degradation, or interruption of striatonigral retrograde transport. Whatever the case, the neuroprotective effect of ECS is associated with increased levels of GDNF in the SNpc of 6-OHDA-injected animals (Fig. 1). Unchanged striatal GDNF levels following ECS, in the face of strong protection of dopaminergic neurons, suggest that GDNF neuroprotection is mediated primarily by a local regulation and/or release on the SN. Rapid transport of newly synthesized striatal GDNF to the SN is also a theoretical possibility. Two weeks after 6-OHDA administration, the number of GDNF+ cells in the SNpc is reduced by ~10%, even though dopaminergic neurons are reduced by 80% at the same time (Anastasía et al., 2007), indicating that other cell populations must be expressing and providing GDNF. Surviving dopaminergic neurons in the SNpc of 6-OHDA-injected animals treated with ECS also retained normal expression of the GDNF receptor complex (Ret and GFRalpha1) (Anastasía et al., 2007), a condition necessary to mediate GDNF participation in ECS-protective mechanism.

We found that anti-GDNF blocking function antibodies (Oo et al., 2003; Burke, 2006; Lu et al., 2009) prevent ECS-induced neuroprotection (Figs. 3 and 4), confirming the role of GDNF in its mediation. Antibodies infused in brain parenchyma are degraded resulting in transitory blocking effects. We found anti-GDNF antibodies in the parenchyma for at least 12 h after the infusion, but by 24 h are no longer detectable (data not shown); these results are consistent with published reports (Bekinschtein et al., 2007; Unsain et al., in press). We tested different infusion protocols and decided to administer three injections of 2 μg of anti-GDNF antibodies every 48 h (Fig. 3A) to partially and transiently inhibit GDNF effects in the SNpc; this protocol aimed at minimizing secondary effects, such as neuronal death, described with higher anti-GDNF doses (Oo et al., 2003). Likewise, to minimize time of exposure to the antibodies, we stopped the experiments after one week, when ECS neuroprotection is already significantly detectable (Figs. 3C and 4A and Anastasía et al., 2007). We found GDNF activity to be necessary for ECS neuroprotection of dopaminergic neurons (Figs. 3C and 4A); however, this result does not exclude the participation of other molecules. For instance, GDNF action requires TGF-β (transforming growth factor-β) *in vitro* as well as *in vivo* (Sariola and Saarma, 2003; Peterziel et al., 2002), suggesting that this growth factor may be also required for ECS effect.

In sum, we present evidence supporting the notion that ECS-induced increase of GDNF occurs locally in the SNpc. First, we have been unable to show an increase in GDNF protein in the striatum (current data and Anastasía et al., 2007). Second, stereotaxic injection of function-blocking anti-GDNF antibodies in the SNpc prevents the ECS-induced neuroprotection, suggesting neutralization of GDNF in the intercellular space.

Loss of dopaminergic phenotype (TH expression) and neuronal death not always coincide (Hagg and Varon, 1993; Bowenkamp et al., 1996; Anastasía et al., 2009); therefore, we evaluated neuronal survival with Cresyl Violet and found that one week after 6-OHDA infusion both markers coincide in the anterior SNpc, albeit not in the posterior segment where loss of phenotype occurs before neuronal death (Figs. 3C and 4A).

Most studies have examined the behavioral and neurochemical effects of 6-OHDA administration after behavioral deficits first occur, typically longer than 2 weeks after injection, even though toxicity to dopaminergic neurons certainly begins almost immediately after the toxin administration. We studied the effects of 6-OHDA 7 days after its injection to assess early changes in dopaminergic neurons and their modifications by ECS treatment. Cresyl Violet staining of the SNpc clearly showed that 6-OHDA-induced neuronal death was restricted to the anterior SNpc (Fig. 4A). Virtually, no Cresyl Violet cell number decrease was observed in the posterior SNpc. This is consistent with the known pattern of degeneration of dopaminergic neurons in the MPTP primate model of PD (Freundlieb et al., 2006),

and it has recently been described 4 days after 6-OHDA administration in rats (Anastasía et al., 2009). Reported differences between anterior and posterior SN (Gibb and Lees, 1991; Damier et al., 1999) may explain the dissimilar vulnerability observed. Nonetheless, to exclude the possibility of parenchymal diffusion of 6-OHDA, we injected 4 μl of India Ink in two normal animals using the same coordinates as 6-OHDA and sacrificed them 4 or 24 h after the administration. We found no ink in the anterior SNpc (data not shown). More importantly, the fact that 6-OHDA induced a similar reduction in TH immunoreactivity in anterior and posterior SNpc 7 days after injection (Fig. 3C) suggests that differences in Cresyl Violet staining are due to differential neuronal vulnerability, rather than to local 6-OHDA parenchymal diffusion. Notably, ECS prevented both neuronal death and loss of the TH+ phenotype in the anterior SNpc, and preserved the dopaminergic phenotype expression in the posterior SNpc.

Astrocytes are key elements in the brain response to injury (Teissmann et al., 2003; McGeer and McGeer, 2008). Astrocytic reaction upregulates antioxidant molecules, membrane transporters, and TFs that support neuronal and glial survival and tissue repair (Liberto et al., 2004). Following 6-OHDA injection, no astrocytic response was detectable at 4 days (Anastasía et al., 2009), but it does become evident weeks later (Gordon et al., 1997; Gomide et al., 2005a,b; Henning et al., 2008). We found a marked increase in astrocytic reaction in the anterior SNpc (Fig. 5B) and a present but less intense gliosis in the posterior SNpc and striatum after one week (Figs. 5 and 6). Most remarkably, astrocytic reaction was greatest in the anterior SNpc in animals treated with ECS one week after 6-OHDA (Fig. 5B), indicating that ECS promoted response to injury where the most susceptible neuronal population is located. Recently we published a similar synergistic GFAP increase 4 days after 6-OHDA in anterior SNpc of animals maintained in an enriched environment (Anastasía et al., 2009). Schallert et al. (2000) described a similar effect in animals with ischemic hemispheric brain lesions that were forced to use the impaired limb.

Astroglial reaction consists of hypertrophy of cellular processes and upregulation of GFAP, but the GFAP increase could be accounted for migration from surrounding areas (McGeer and McGeer, 2008) (such as SNpr) to the SNpc, and/or astrogliogenesis (Aponso et al., 2008); the latter possibility has been contradicted (Lie et al., 2002). GFAP-positive astrocytes have been shown to release GDNF in the SNpc of rodents (Abe et al., 2010). Reactive astrocytes participate in neurorestoration mechanisms triggered at very early times following toxin exposure, possibly involving release of BDNF, GDNF, and nerve growth factor among many others (Nithianantharajah and Hannan, 2006). Upregulation of GDNF by astrocytes or microglial cells occur in several injury models (Batchelor et al., 1999; Bresjanac and Antauer, 2000; Satake et al., 2000; Wei et al., 2000; Miyazaki et al., 2001). Furthermore, astrocytic GDNF production has been proposed to explain the neuroprotective/therapeutic effects of several compounds, including apomorphine, selegiline, riluzole, anti-depressants, melatonin, or vitamin D3. However, we do not exclude the possibility that reactive astrocytes may regulate other neuroprotective molecules, as well as participate in TFs–independent neuroprotective mechanisms.

A sustained motor improvement in human patients with idopathic PD after electroconvulsive therapy has been extensively described (Shulman, 2003; Fregni et al., 2005; Gallinek, 1947; Andersen et al., 1987; Balldin et al., 1981; Rasmussen and Abrams, 1991; Fall et al., 2000; Popeo and Kellner, 2009); however, the mechanism of this benefit is unknown. We previously reported that ECS prevents 6-OHDA-induced neurodegeneration of the nigrostriatal dopaminergic system. More specifically, this treatment reduced 6-OHDA toxicity to dopaminergic neurons, preserved axonal integrity, and preserves unilateral nigrostriatal lesioning behavioral impairment (Anastasía et al., 2007). Here, we report that an increase of GDNF endogenous levels is necessary for ECS neuroprotection. Moreover, we show that an astrocytic reaction targeted to the most susceptible neurons, may contribute to the well-known favorable effect of electroconvulsive therapy on motor impairment in sporadic PD. This work sheds light on the mechanisms of electroconvulsive treatment effect in PD (Fregni et al., 2005; Andersen et al., 1987), but is not intended to suggest this treatment as a therapeutic alternative for PD, even though this case has been made on clinical grounds (Popeo and Kellner, 2009). Instead, this study aims to demonstrate how neuromodulation and modification of endogenous TF levels can induce a neuroprotective effect and be of potential therapeutic interest.

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