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The C-terminal domain of the heavy chain of tetanus toxin given by intramuscular injection causes neuroprotection and improves the motor behavior in rats treated with 6-hydroxydopamine

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ABSTRACT

We have previously shown that the intrastriatal injection of the C-terminal domain of tetanus toxin (Hc-TeTx) protects the nigrostriatal-dopaminergic pathways and improves motor behavior in hemiparkinsonism-rat models caused by MPP^+ (1-methyl-4-phenylpyridinium). Here we have investigated the protective effects of the intramuscular application of the Hc-TeTx on motor asymmetry and neurodegeneration in the striatum of 6-hydroxydopamine (6-OHDA)-treated rats. Adult male rats were intramuscularly injected with the recombinant Hc-TeTx protein (0.1–20 μg/kg, daily) 3 days before the stereotaxic injection of 6-OHDA into the left striatum. Our results showed that the motor-improvement functions were extended for 4 weeks in all Hc-TeTx-treated groups, obtaining the maximum performance with the highest dose of Hc-TeTx (20 μg/kg). The improvements found were 97%, 87%, and 70% in the turning behavior, stepping test, and cylinder test, respectively. The striatal levels of dopamine and its metabolites did not vary compared to the control group. Moreover, the peripheral treatment with Hc-TeTx in rats prevents, for 30 days, the neurodegeneration in the striatum caused by the toxicity of the 6-OHDA. Our results lead us to believe that the Hc-TeTx could be a potential therapeutic agent in pathologies caused by impairment of dopaminergic innervations such as Parkinson's disease.

1. Introduction

Dopamine (DA) plays a critical role in the control of locomotion and it is the main neurotransmitter system affected in Parkinson's disease (PD). This neurodegenerative disorder is characterized by the progressive loss of dopaminergic neurons that originate from the substantia nigra and project to the nucleus striatum. The major clinical features of patients with PD are tremor at rest, rigidity, bradykinesia, and postural instability (Obeso et al., 2010).

Several models of selective DA-neurons degeneration have been established, including animals treated with the neurotoxin 6-hydroxydopamine (6-OHDA). The unilateral injection of 6-OHDA into the striatum generates a progressive retrograde degeneration of the nigrostriatal pathway over a period of weeks, which has been used as a successful model to study the progression of PD (Przedborski et al., 1995; Kirik et al., 1998). Furthermore, the 6-OHDA-treated animals show impairments in motor functions (Sauer and Oertel, 1994; Chang et al., 1999).

At the molecular level, studies have shown that 6-OHDA causes oxidative stress that result in cell death (Kumar et al., 1995; Liang et al., 2004; Park et al., 2002; Mazzio et al., 2004). There is an increase of astroglial and microglial activations accompanied by proinflammatory cytokines that can lead to DA-neuronal vulnerability, atrophy, and neuronal death (Cicchetti et al., 2002; Mladenovic et al., 2004). Thus, rodents treated with 6-OHDA are a suitable model system to study the effects of potential new antiparkinsonian drugs that may have survival-promoting activities on the remaining DA neurons (Kirik et al., 1998; Emborg, 2004; Ceravolo et al., 2006).

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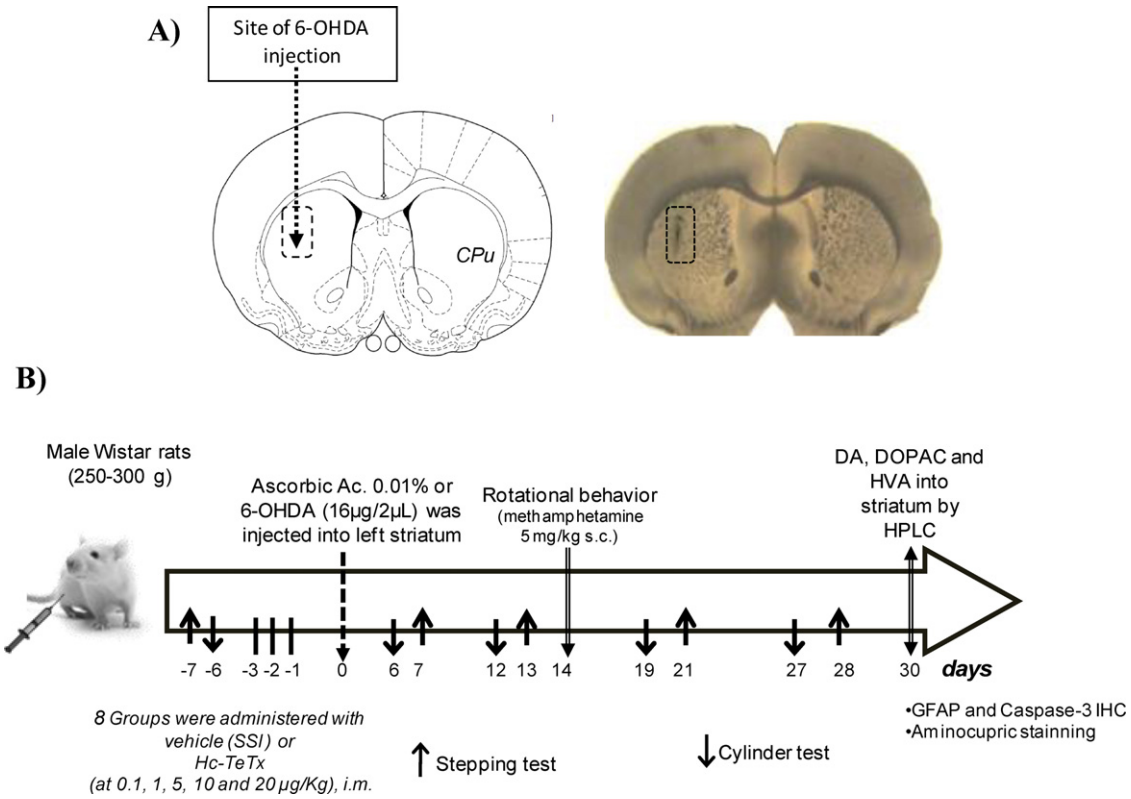

Fig. 1. Location of the striatal injection and diagrammatic representation of design. (A) The figure shows the site of striatal injection from the Paxinos and Watson Atlas compared to a coronal slice obtained to corroborate the coordinates. (B) A diagrammatic representation of the experimental design. Eight groups were injected with vehicle or Hc-TeTx at different doses (0.1, 1, 5, 10, and 20 µg/kg i.m.; $n = 10$) during 3 days before the stereotaxical surgery. At time 0, the 2 µL of 0.1% ascorbic acid vehicle or 6-OHDA was injected into the striatum. After that, all groups were tested for the motor behavior; turning (double line down arrow), cylinder (single up dark arrow), and stepping tests (single down dark arrow) over 28 days. At 30 days post-lesion all animals were killed to obtain the ipsilateral and contralateral striatum to make the immunohistochemical and biochemical analyses (double arrow).

Several trophic factors have been proposed to be effective in protecting dopaminergic neurons in vivo (Kearns and Gash, 1995; Winkler et al., 1996; Rosenblad et al., 2000). These protective effects are observed after intrastriatal or intracerebroventricular applications, but not with systemic administration of these agents. Thus, potential therapeutic agents that do not cross the blood–brain barrier (BBB) will not be effective in treating PD.

The C-terminal domain of tetanus toxin (Hc-TeTx) is used by the clostidial neurotoxin to bind to the neuronal membrane, after it is endocyted and carried through axons to arrive at the inhibitory glycinergic and GABAergic neurons of the spinal cord and brain stem. That is why the Hc-TeTx fragment has been proposed as a carrier to deliver molecules to the CNS (Payne et al., 2006; Larsen et al., 2006; Li et al., 2009; Ciriza et al., 2008). In addition, evidence suggests that the intramuscular administration of Hc-TeTx in rodents results in the delivery of the fragment to the brain. The Hc-TeTx is able to be internalized by motoneurons at the neuromuscular junction followed by a fast axonal retrograde-transport to the brain (Fishman and Carrigan, 1987; Fishman et al., 1990; Roux et al., 2005a,b; Perreault et al., 2006; Deinhardt et al., 2006). However, there are a few studies that indicate that the Hc-TeTx is able to modulate the cellular signaling. Previous reports have demonstrated that this nontoxic fragment has a trophic action highly associated with the stimulation of phosphorylation of tyrosine kinase receptors (Trk receptors) in cultured-cortical neurons and cerebellar-granule neurons (CGN) (Gil et al., 2003; Chaib-Oükadour et al., 2004). In addition, the Hc-TeTx fragment protects against cell death in a cellular model of PD (Chaib-Oükadour et al., 2009). We have previously demonstrated for the first time that the intrastriatal injection of Hc-TeTx protects against DA loss and improves motor behavior in rats treated with MPP^+ (Mendieta et al., 2009). In our present study, we sought to determine if rats treated with 6-OHDA are protected by Hc-TeTx given by intramuscular injection; application that would have strong therapeutic relevance.

2. Materials and methods

2.1. Animals

Adult, male Wistar rats weighing 200–250 g were provided by the animal breeding facility of the Benemérita Universidad Autónoma de Puebla (BUAP). They were housed until the behavioral tests in groups of 3–5 animals in a room maintained at $23 \pm 2\,^{\circ}C$ and a 12 h:12 h light–dark cycle (lights on at 0700) with free access to food and water. All procedures were done in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the institutional animal-care committee of BUAP.

2.2. Synthesis and purification of Hc-TeTx

The non-toxic C-terminal domain of tetanus toxin that is the binding domain of TeTx and formed by 685–1315 residues (50 kDa) was synthesized in accordance to Chaib-Oükadour et al. (2004) with some modifications. Briefly, *Escherichia coli* BL21 cells were grown in Luria Beltane medium containing 100 µg/mL ampicillin. Protein expression was induced by the addition of 0.4 mM isopropyl-β-D-thiogalactoside (IPTG). After 3 h, cells were pelleted by centrifugation at $4000 \times g$ for 20 min at 4 °C, resuspended in lysis buffer [50 mM NaH_2PO_4, 300 mM NaCl and 1% Triton X-100; pH

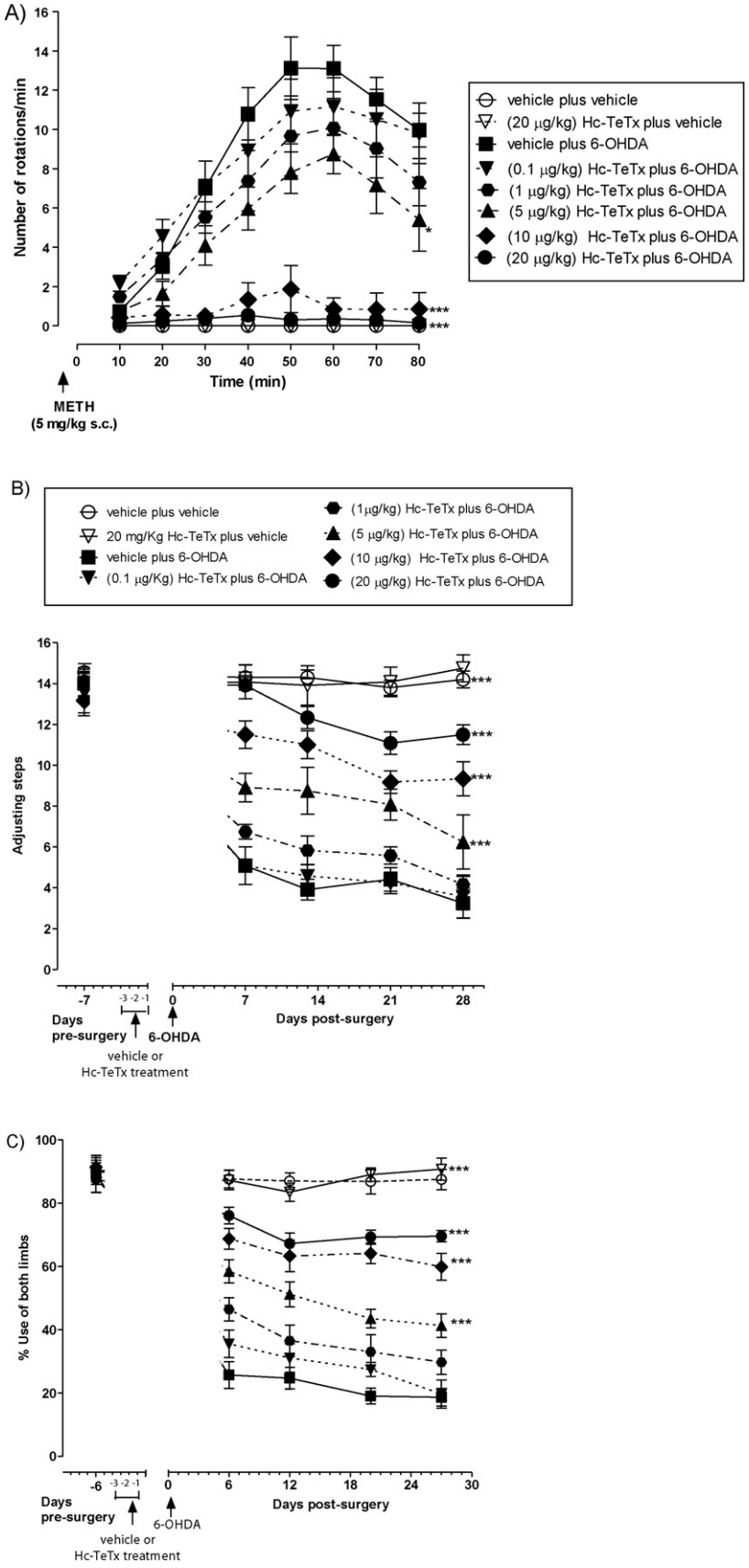

Fig. 2. Effect of Hc-TeTx treatment on motor asymmetry in hemiparkinsonian rats. One group was assigned for each dose of the Hc-TeTx fragment protein (0.1, 1, 5, 10, and 20 μg/kg daily) or vehicle before making the dopaminergic lesion ($n = 7$ in each group). In (A) during the rotational behavior the groups Hc-TeTx plus 6-OHDA at 5, 10, and 20 μg/kg had significantly lower rotations than the 6-OHDA group (***$P < 0.001$). (B) The graph shows the number of contralateral forelimb adjusting steps evaluated with the four trials. The group with a dopaminergic lesion generated by 6-OHDA showed a lower performance during the test compared to the control group. The groups Hc-TeTx

8] and sonicated on ice for six 30 s periods. The suspension was centrifuged at 30,000 × *g* for 30 min at 4 °C. The clear supernatant, which contains the His-tagged protein, was purified by cobalt affinity chromatography. The proteins, without His-Tags, were eluted by washing the resin with elution buffer [50 mM $NaH_2PO_4 \cdot H_2O$ and 300 mM NaCl; pH 7]. Hc-TeTx contains six histidines it is retained in the resin forming a Co-complex. Hc-TeTx was eluted with the elution buffer [50 mM $NaH_2PO_4 \cdot H_2O$, 300 mM NaCl and 150 mM imidazole; pH 7], and those fractions containing purified Hc-TeTx protein were dialyzed [40 mM Na_2HPO_4, 10 mM NaH_2PO_4 and 150 mM NaCl; pH 7.4], overnight at 4 °C, and for 2 h with new buffer. The Hc-TeTx was stored in aliquots at −20 °C. The recombinant Hc-TeTx fragment was obtained with a high grade of purity. It has been patented by the Universidad Autònoma de Barcelona, Spain (Patent No. P200600132) as an invention concerning the treatment of Parkinson's disease.

2.3. Treatments

One group was assigned for each dose of the Hc-TeTx fragment (0.1, 1, 5, 10, and 20 μg/kg daily) or SSI (isotonic saline solution), which were injected intramuscularly (i.m.) into the *gastrocnemius* muscle for 3 days before producing the dopaminergic lesion.

2.4. Surgery

The animals were anesthetized with chloral hydrate (350 mg/kg, i.p.; Sigma–Aldrich Corp, St. Louis, MO, USA). The rats were randomly assigned to receive the vehicle or 6-OHDA injected into the left striatum ($n = 10$ per group). The stereotaxic apparatus (Stoelting Co., Wood Dale, IL, USA) was used to cause dopaminergic denervation by injecting 2 μL of 6-OHDA (8 μg/μL; Sigma–Aldrich Corp, St. Louis, MO, USA) dissolved in 0.01% ascorbic acid solution, pH 7.4 (Coordinates: A: +0.5 mm from Bregma, L: +3.6 mm from midline, DV1: −4.9 and DV2: −5.5 below dura) with reference to the stereotaxic atlas (Paxinos and Watson, 1998). Proper postoperative care was provided until the animals completely recovered.

2.5. Behavioral testing

Three behavioral tests were used to assess the ability of the Hc-TeTx fragment to improve the contralateral motor performance after the unilateral 6-OHDA lesion of the striatum. The time sequences of the behavioral test are shown in Fig. 1.

2.5.1. Rotational behavior

On test day the rats were removed from their home cages and placed in the behavioral room for habituation for 30 min. Then, the rats were placed into cages and the rotations in each group were counted for 80 min after injection of methamphetamine. The rotations (360°, short axis) ipsilateral to the side of the injection were counted by individuals trained in behavioral observation. The animals were tested following the response to methamphetamine (5 mg/kg, sc; Sigma–Aldrich Corp., St. Louis, MO, USA) on the 14th day after stereotaxic injection in all groups, as described by Ungerstedt and Arbuthnott (1970). The number of ipsilateral rotations in each 10-min period was registered as the mean rotations ± SE.

2.5.2. Forelimb-use asymmetry in the cylinder test

Forelimb akinesia was assessed using the cylinder test as reported by Mendieta et al. (2009). This test evaluates the use of the forelimb to support the body against the walls of a cylinder. Six days before of the dopaminergic lesion, the animals were assessed for the asymmetry test in the cylinder to obtain the base values. The animals were placed in the cylinder test for 5 min with one trial every week, and their activity was videotaped. The forelimb-use asymmetry test was made in four assays because the exploratory behavior of rats showed habituation upon daily exposure to the cylinder. The number of wall contacts made with the forelimbs was counted because it represents a more sensitive index of striatal-dopamine depletion than landing movements. The wall contacts were classified as contralateral forelimb (CF), ipsilateral forelimb (IF), or both forelimbs (BF). A minimum of 8 wall contacts per trial must occur to be considered a valid test. The percentage of the use of independent or simultaneous forelimbs, relative to the total number of contacts made by each animal, was calculated.

2.5.3. Adjusting steps test

Seven days before the surgery, the numbers of adjusting steps made with the ipsilateral and contralateral forepaws were recorded to obtain the base values. Rats were held at the rear part of the torso by one hand with their hindlimbs lifted and one forepaw was held steadily with the other hand of the experimenter to cause the rat to bear its weight on the other forepaw. The animals did the stepping test on the surface of the table for 1 m, at a speed of about 14 cm/s. Each stepping test consisted of two trials for each forepaw, alternating between forepaws and videotaped for later analysis. The same procedure was done in the four consecutive weeks after the lesion had been made.

2.6. HPLC-EC determination of catecholamines and metabolites

Dopamine and its metabolites were estimated by HPLC using an electrochemical detector. The striatum of each group of rats ($n = 7$) was used to determine the endogenous levels of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA). The striatum was dissected, weighed, and frozen in isopentane. Then it was homogenized with a Dynatech Sonic Dismembrator Model 300 at 4 °C for 40 s in 500 μL of an antioxidant solution of 0.25 M perchloric acid containing 250 μM EDTA-Na_2, 100 μM potassium metabisulfite, and 1000 ng/mL of 3,4-dihydroxybenzylamine (DHBA) as the internal standard for catecholamine determination. The homogenates were stored at −70 °C. They were centrifuged in an Eppendorf microcentrifuge for 10 min at 8000 rpm, the supernatant was collected, and it was further filtered through 0.25 μm nylon filters before injecting it into the HPLC device. Samples (5 μL) were injected manually to be analyzed by HPLC (LaChrom, Merck-Hitachi). Standards of the amine and its metabolites were made to evaluate the peaks. The analysis was made at room temperature and the elution was run at a flow rate of 1 mL/min. The mobile phase consisted of 0.1 M anhydrous citric acid, 0.05 mM EDTA-$Na_2 \cdot H_2O$, 1 mM sodium octyl sulfate, and 0.3% acetonitrile. The pH was adjusted to 2.3 with triethylamine. The HPLC consists of a BAS LC-4C electrochemical detector with a unijet-electrode analytical cell. The electrode potential was 0.75 V and the analysis software was ChromGraph™.

plus 6-OHDA at 5, 10, and 20 μg/kg had a significantly higher performance compared to the 6-OHDA group (***$P < 0.001$). (C) The graph of the number of wall contacts made simultaneously by the ipsilateral and contralateral forepaws inside the cylinder is a percentage of use of both limbs. The results show that the intramuscular Hc-TeTx injection improves the simultaneous forelimb use in rats with unilateral 6-OHDA lesions (***$P < 0.001$). The vehicle and Hc-TeTx groups showed a high performance during the stepping and cylinder tests. The data are the mean ± SE and they were analyzed using a one-way ANOVA for repeated measures followed by Bonferroni multiple-comparison test.

2.7. Histological examination

After behavioral experiments, the rats ($n = 6$) from the vehicle, Hc-TeTx, 6-OHDA, and 20 μg/kg Hc-TeTx plus 6-OHDA groups were anesthetized and intracardially perfused with 70 mL of PBS and 300 mL of 4% paraformaldehyde-PBS to kill them. All their brains were removed and postfixed in more 4% paraformaldehyde for 48 h. Some of them ($n = 3$ per group) were embedded in paraffin and coronal 5-μm thick sections were taken containing the striatum from each brain at the level of the anterior temporal area, approximately −0.5 to 1.5 from Bregma, to follow the fluorescence immunohistochemistry. Other brains ($n = 3$ per group) were transferred to 30% sucrose–4% paraformaldehyde–PBS, and coronal sections at −5.0 to −6.0 from Bregma were taken containing the substantia nigra (70 μm, 6 per rat) to be collected in PBS–Triton (0.2%). Finally, they were processed for free-floating TH immunohistochemistry ($n = 3$ per group).

2.7.1. Immunohistochemistry to tyrosine hydroxylase

The tissue sections containing the SNpc were first washed in 3% H_2O_2 and PBS–Triton (Tx at 0.2%) after that, they were washed on PBS–Tx (0.2%), both steps using cycles of 10 min. The tissue sections were blocked using 5% BSA–PBS free of IgG for 1 h. They were incubated overnight at 4 °C with a mouse monoclonal anti-TH antibody (1:1000 Millipore) followed by incubation with biotinylated antimouse IgG for 2 h at room temperature. After rinsing in 0.1 M phosphate buffer (pH 7.4), the sections were incubated in phosphate buffer with ABC Complex (Dako, CA, USA) and avidin-biotinylated horseradish peroxidase complexes (Kit Dako) at room temperature for 1 h. The signal was developed by incubation with diaminobenzidine (50 mg/100 mL TRIS; pH 7.6) in the presence of 0.03% H_2O_2. Then, the sections were mounted on gelatin-subbed slides and air-dried. Finally, each tissue from the ipsilateral or contralateral SNpc were observed using the DM750 Leica microscope and captured at the same level of contrast and sharpness using the 4× and 10× objective and Leica software. The digitized images were and transformed into JPEG files for storage and analysis in the ImageJ software.

2.7.2. Immunohistochemistry to GFAP and caspase-3

For the glial-fibrillary-acidic-protein (GFAP) immunohistochemistry, the sections containing the striatum were blocked by incubating in IgG-free 2% bovine serum albumin (BSA, Sigma) for 30 min. Then the slides were incubated overnight at 4 °C with a polyclonal rabbit antibody anti-GFAP (Dako Denmark, Gostrup, Denmark), all at 1:100 dilutions. The antibody labeling was recognized with an isospecific, secondary FITC-conjugated antibody and visualized in the green channel. The same procedure was done for caspase-3 (Santa Cruz Biotechnology Inc., CA, USA) at 1:100 dilutions, and this antibody labeling was recognized with an isospecific secondary rhodamine and visualized in the red channel. Slides were counterstained with VectaShield with DAPI (Vector Labs., Burlingame, CA, USA) for nuclei staining and visualized in the blue channel. The striatum regions were photographed with a Leica DMLS microscope at 40× (Leica Microsystems GmbH, Wetzlar, Germany) using a Leica digital camera DFC-300FX (Leica Microsystems Digital Imaging, Cambridge, UK). The digitized images were captured at the same level of contrast and sharpness using the IM1000 software (Imagic Bildverarbeitung AG, Leica Microsystems; Heerbrugg, Switzerland) and transformed into JPEG files for storage and analysis in the software ImageJ.

2.7.3. Amino-cupric-silver stain

Neurodegeneration caused by the dopaminergic denervation was seen by the precipitation of the ionic silver-staining method (De Olmos et al., 1994). The pre-impregnation solution contained cupric nitrate, silver nitrate, cadmium nitrate, lanthanum nitrate, and pyridine (Sigma–Aldrich, St. Louis, MO, USA), dissolved in deionized water. After the components were well-mixed, the solution was warmed to 45–50 °C. First, the sections containing the striatum were incubated in this solution overnight. The impregnation solution contained silver nitrate, ethanol, acetone, lithium hydroxide, and ammonium hydroxide (Sigma–Aldrich, St. Louis, MO, USA) in deionized water. Second, the sections were rinsed in deionized water, next in acetone, and then placed into the impregnation solution to be incubated in this solution for 50 min. The reducing solution contained ethanol, Formalin, and citric acid (Sigma–Aldrich, St. Louis, MO, USA) in deionized water. Third, the sections were transferred from the impregnation solution into the reducing solution and placed in a water bath with a temperature between 32 °C and 35 °C. After that, the sections were incubated for 25 min in the reducing solution, then were transferred into deionized water and rinsed twice. Next, the sections were counterstained with neutral red (Sigma–Aldrich, St. Louis, MO, USA). Finally, all sections were transferred into alcohol and xylene and then mounted permanently with Entellan (Merck, Darmstadt, Germany). The slices stained in the striatum were examined through a DM750 Leica microscope at 40× (Leica Microsystems GmbH, Wetzlar) and photographed using a Leica digital camera ICC50 (Leica Microsystems Digital Imaging, Coldhams Lane, Cambridge, UK). The digitized images were captured at the same level of contrast and sharpness using the Leica software and transformed into JPEG files for storage and analysis in the ImageJ software for the measurement of the neurodegenerative cells.

2.8. Data analysis

All values are the mean ± SE. A one-way analysis of variance (ANOVA) for repeated measures followed by a Bonferroni multiple-comparison test was used to analyze the differences in the behavioral test. A one-way analysis of variance (ANOVA) followed by a Bonferroni multiple-comparison test was used to analyze the cell numbers with an argyrophilic stain, the number of positive cells for caspase-3 or GFAP, and the catecholamine levels between groups. The differences were considered significant when $P < 0.05$. The statistical analysis was made using Prism 5 Software (Prism5, San Diego, CA, USA).

3. Results

3.1. Rotational behavior

Fig. 2A shows the mean number of ipsilateral turns for every 10 min and the SE for all groups. We found that the Hc-TeTx plus 6-OHDA groups decreases the number of ipsilateral turns per minute; at 20 and 10 μg/kg of Hc-TeTx (0.3 ± 0.2 and 0.9 ± 0.2 turns/min; respectively; $P < 0.001$), at 5 μg/kg of Hc-TeTx (5.2 ± 0.9 turns/min; $P < 0.05$), with no significant changes in the turning behavior at 1.0 and 0.1 μg/kg of Hc-TeTx (6.7 ± 1.0, 8.1 ± 1.0 turns/min; n.s.), all compared with compared with the 6-OHDA group (8.7 ± 1.2 turns/min). The vehicle and Hc-TeTx groups did not show a change in turning behavior caused by methamphetamine during the test. The vehicle and Hc-TeTx groups did not show rotational behavior caused by methamphetamine during the test. These data were analyzed using one-way ANOVA for repeated measures [$F_{(7,63)} = 32.67$], followed by a Bonferroni multiple comparison test.

3.2. Adjusting steps

Fig. 2B shows the time-course of the changes in the forelimb adjusting steps. All animals were, before making the dopaminergic

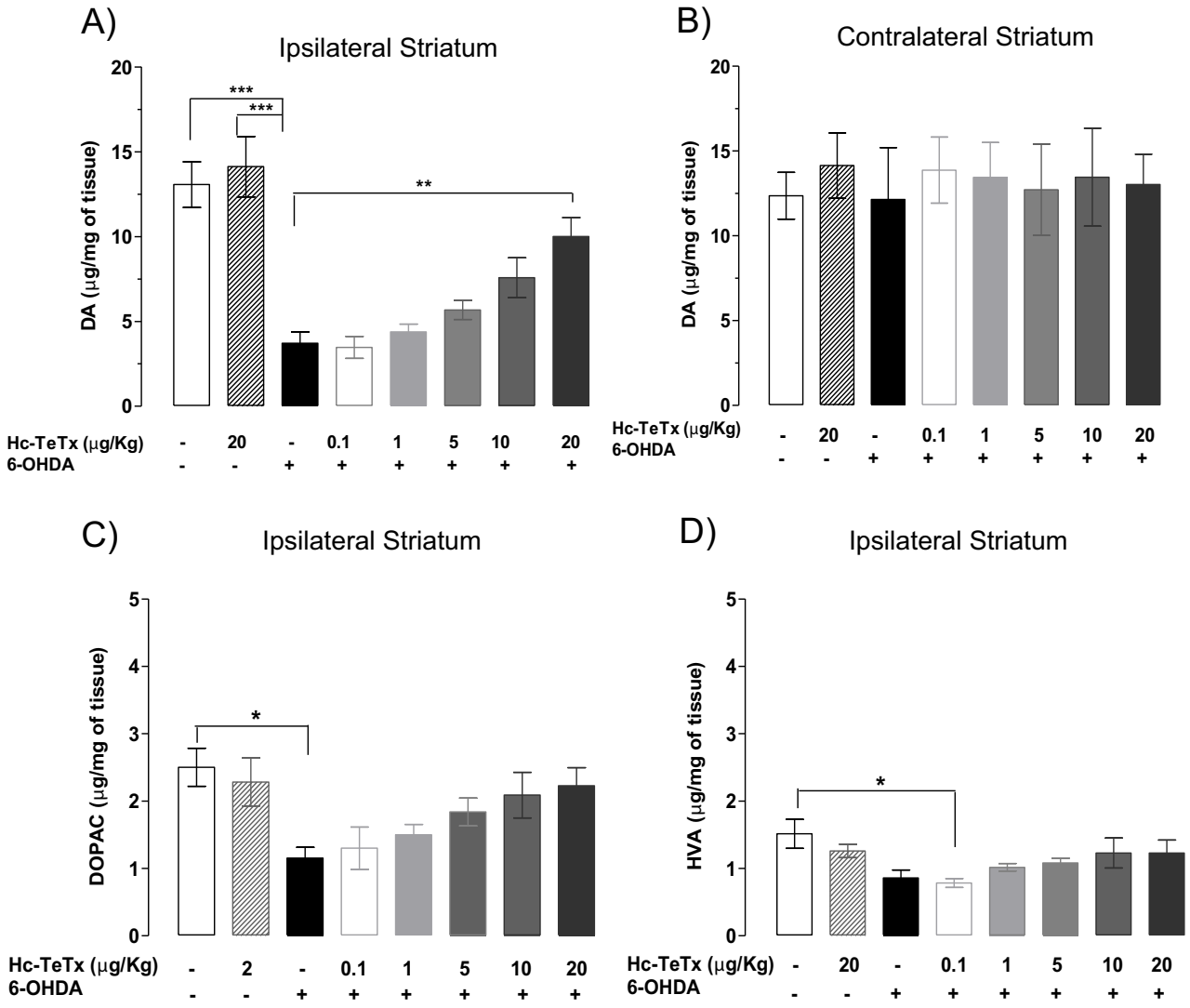

Fig. 3. The effect of intramuscular Hc-TeTx injection at different doses on the dopamine and metabolite levels in the striatum of rats with 6-OHDA lesion. (A) The ipsilateral striatums have a reduction of catecholamines (dopamine, DOPAC, and HVA) levels compared to the contralateral striatum in rats treated with 6-OHDA. (B) The reduction is partially restored with the pretreatment of Hc-TeTx at the dose of 20 μg/kg. (C and D) Figures showed the concentrations of DOPAC and HVA that show that the Hc-TeTx did not produce changes in the metabolism of DA in both metabolites in the striatum. The data are the mean (μg/mg of the tissue wet weight) ± SE (bars) with each group ($n = 7$ in each group).*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ were considered significant in the statistical analyses using a one-way ANOVA followed by Bonferroni multiple-comparison test.

lesion, assessed for the stepping test using one trial, 14 ± 1 mean of ipsilateral or contralateral steps (mean ± SE). After the surgery, all the groups were assessed in four trials on different days. The 6-OHDA group showed the contralateral forelimb adjusting steps significantly decreased 4 weeks after the dopaminergic lesion (at last trial 4.1 ± 0.4 steps) and did not show any change in ipsilateral forelimb steps (13.5 ± 0.1 steps; mean ± SE). Similar levels of steps were registered, before and after the surgery for all groups of rats (graph not shown).

The other groups of rats with Hc-TeTx plus 6-OHDA showed the contralateral forelimb adjusting steps were significantly increased during the 4 weeks post-lesion; principally the groups with 20, 10 and 5 μg/kg of Hc-TeTx (12.2 ± 0.6 and 10.3 ± 0.6, and 8.0 ± 0.6, respectively; $P < 0.001$), all compared to 6-OHDA group (4.1 ± 0.4 steps) (Fig. 2B). The lower doses of Hc-TeTx (1.0 and 0.1 μg/kg) had no significant improvement of the contralateral forelimb adjusting steps (4.4 ± 0.3 and 5.6 ± 0.5 steps, respectively). The results clearly indicate that the better performances were caused by the Hc-TeTx (20 and 10 μg/kg) plus 6-OHDA groups at 87% and 73% compared to the 6-OHDA group that had a low performance of 29%. The vehicle and Hc-TeTx groups had a high performance during the test (14.1 ± 0.1 and 14.2 ± 0.1 steps, respectively). These data were analyzed using a one-way ANOVA for repeated measures [$F_{(7,31)} = 159.7$], followed by a Bonferroni multiple comparison test.

3.3. Cylinder test

Animals were tested for symmetric forelimb use in the cylinder at day 6 before the dopaminergic lesion was made. We have found that 6 days before treatment all animals showed symmetrical use of both forelimbs; about 90% in the cylinder test for vertical exploration (Fig. 2C). The animals treated with 6-OHDA group were severely impaired in the use of the contralateral paw in the cylinder test yielding a low performance of 22 ± 2% (mean ± SE) in the use of both forelimbs during the 4 weeks postlesion (Fig. 2C). The recovery was seen for all groups with the Hc-TeTx, particularly

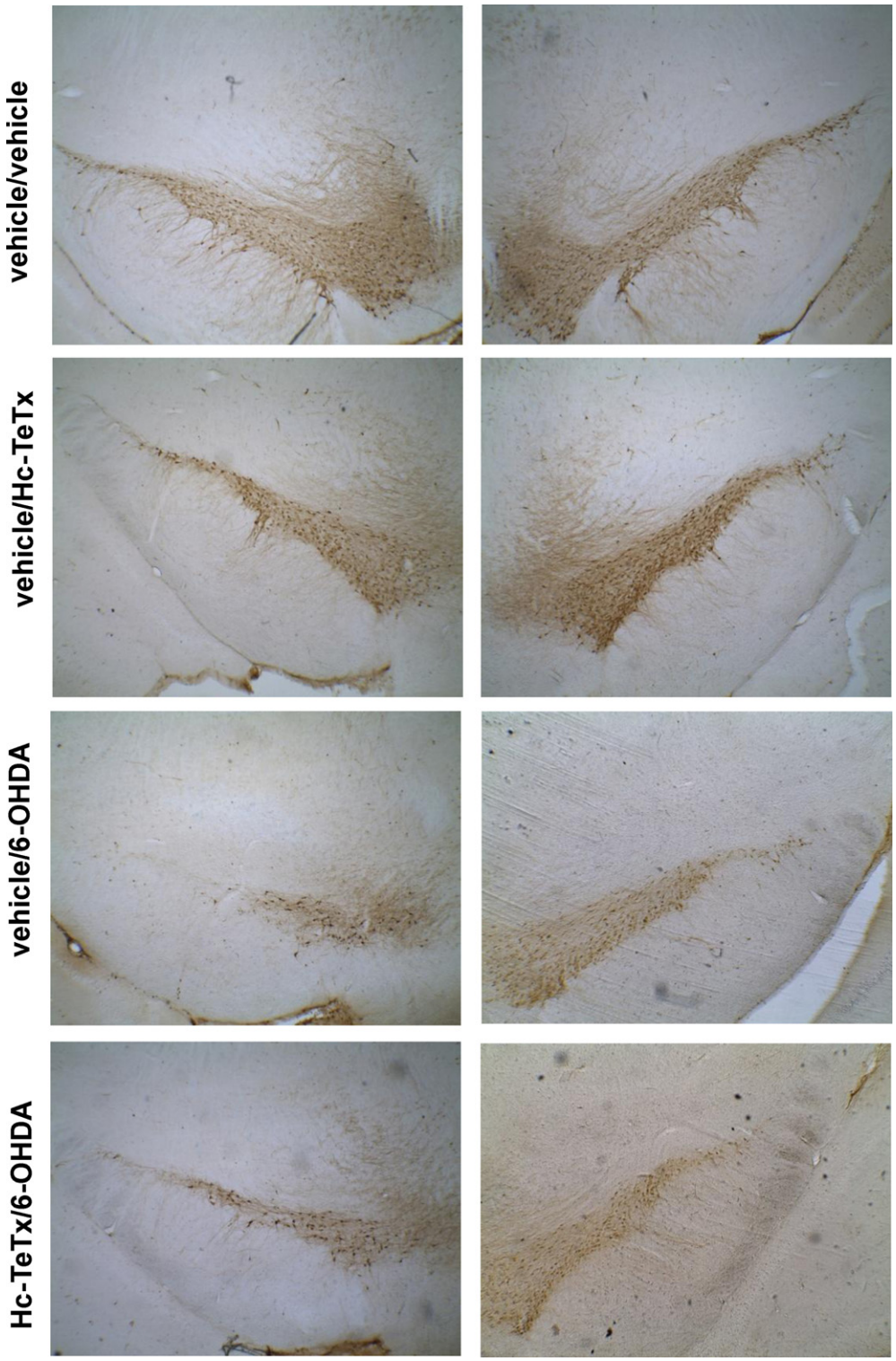

Fig. 4. Tyrosine hydroxylase immunohistochemistry following treatment with Hc-TeTx and 6-hydroxydopamine. The Hc-TeTx treatment, at the dose of 20 μg/kg i.m., causes neuroprotective effects on the side ipsilateral side to the 6-OHDA lesion. More TH-IR neurons were observed in Hc-TeTx/6-OHDA group than in the vehicle/6-OHDA group, where the number of neurons TH-IR was markedly reduced in the latter. The control (vehicle) and Hc-TeTx groups, showed a similar TH-IR in the side ipsilateral side of the SNpc. Photomicrography of TH immunoreactive neurons on the ipsilateral or contralateral SNpc of animals ($n = 3$ in each group) with vehicle, Hc-TeTx plus vehicle, vehicle plus 6-OHDA, and Hc-TeTx plus 6-OHDA. All were observed and captured at 4×.

the higher performance in the use of both limbs during the four trials measured for 20 and 10 μg/kg of Hc-TeTx plus 6-OHDA-treated groups (70 ± 2% and 64 ± 2%, respectively; $P < 0.001$). The groups of 5 and 1 μg/kg of Hc-TeTx decreased use of both forelimbs (48 ± 4% and 36 ± 4%, respectively; $P < 0.001$). However, we found an exception for the low dose of 0.1 μg/kg of the Hc-TeTx (28 ± 3%, n.s.), all groups compared with the 6-OHDA group. These data were analyzed using one-way ANOVA for repeated measures [$F_{(7,31)} = 182.6$], followed by a Bonferroni multiple comparison test.

3.4. Determination of catecholamine and metabolites

To determine if a unilateral change in dopaminergic transmission was associated with the improvement in motor behavior after the Hc-TeTx i.m. application, the tissue levels of DA, DOPAC, and HVA for the ipsilateral or contralateral striatum were measured and expressed as μg/mg of tissue. The data obtained for three determinations of dopamine and its metabolites for each experimental group are reported as the mean ± SE. In Fig. 3A the DA levels in the ipsilateral striatum are shown 30 days after of the Hc-TeTx injection (20 μg/kg) and dopaminergic lesion with 6-OHDA (10.0 ± 1.0; $P < 0.01$), which were found to be higher compared to the 6-OHDA-treated group (3.7 ± 0.6); without important changes at the lower doses of the Hc-TeTx (one-way ANOVA [$F_{(7,47)} = 15.84$]). Moreover, the vehicle group showed similar DA levels in the ipsilateral and contralateral striatum (13.0 ± 1.3 and 12.4 ± 1.3). These results were contrasted with the lower DA levels in the ipsilateral striatum of the 6-OHDA-treated group (∼28%). However, the deterioration of the catecholamine system in the 6-OHDA group was partially prevented by the Hc-TeTx injection at 20 μg/kg by ∼49% ($P < 0.01$). In Fig. 3B the data show that there are no changes in the DA levels in the contralateral striatum for the different groups with or without the Hc-TeTx, which means that the contralateral area is not affected [$F_{(7,47)} = 0.0964$].

Fig. 3C and D shows the reduction of the main metabolites DOPAC and HVA (1.1 ± 0.1 and 0.8 ± 0.1, respectively) for the 6-OHDA-treated group, compared to vehicle group (2.5 ± 0.3 and 1.5 ± 0.2, respectively). The Hc-TeTx plus the 6-OHDA-treated group showed levels similar to vehicle group of these metabolites (2.2 ± 0.3 and 1.2 ± 0.2). In the ipsilateral striatum the DOPAC was found decreased in the 6-OHDA-treated group compared to the control group (one-way ANOVA [$F_{(7,54)} = 3.128$; $P < 0.05$]) and there were no significant differences in the other groups treated with Hc-TeTx plus 6-OHDA or the group using only the Hc-TeTx. Moreover, the data indicated that all groups had no significant changes in the HVA levels. Our findings of the deterioration of the catecholamine system in the 6-OHDA group compared to the Hc-TeTx plus 6-OHDA group show an effective protection by the Hc-TeTx fragment on the DA levels and its metabolites at 20 μg/kg (one-way ANOVA [$F_{(7,47)} = 0.037$]).

3.5. Tyrosine hydroxylase immunoreactivity in the SNpc

Our findings of the Hc-TeTx effects on motor asymmetry and the DA system in the striatum suggest that the trophic effects are most evident when we use the intramuscular injection dose of 20 μg/kg of Hc-TeTx, therefore we decided to use this dose in the other group of animals for examination of the dopaminergic neurodegeneration of the nigrostriatal system.

Animals of the following groups: vehicle-vehicle, Hc-TeTx-vehicle, vehicle-6-OHDA, and Hc-TeTx-6-OHDA were examined to tyrosine hydroxylase (TH) immunoreactivity (IR) in the ipsilateral and contralateral SNpc 30 days after the dopaminergic lesion. The principal findings were the major positive-TH-IR (151.0 ± 5.0) in the neurons from ipsilateral SNpc to the Hc-TeTx plus 6-OHDA-treated group, compared to 6-OHDA group (67.0 ± 14) (Fig. 4). The number of positive to TH-IR in the contralateral SNpc of both groups was similar (140.0 ± 7.0). Moreover, the control groups, Hc-TeTx or vehicle, did not show a different number of positive TH-IR cells from the ipsilateral and contralateral SNpc. The photographs captured at 10× were analyzed using ImageJ software.

3.6. Caspase-3 and GFAP immunoreactivity (IR) in the striatum

The caspase-3-IR was assessed in the ipsilateral and contralateral striatum as a marker of apoptosis caused 30 days after the dopaminergic lesion (Fig. 5A–H). In Fig. 5E the caspase-3-positive cells were found located close to the site of injection in the ipsilateral striatum for the 6-OHDA-treated group and the quantitative analysis showed a large number of caspase-3-positive cells. Contrarily, the Hc-TeTx plus 6-OHDA-treated group in the ipsilateral striatum showed fewer caspase-3 positive cells in the caspase-3-IR compared to the 6-OHDA-treated group (Fig. 5F and E). These findings indicate that the neuronal damage was prevented by the intramuscular injection of the Hc-TeTx. Consistent with the findings from the argyrophilic staining, we did not find caspase-3-positive cells in the ipsilateral striatum of the vehicle and Hc-TeTx groups. Therefore, there were no caspase-3-positive cells in the contralateral striatum of each group when they were assessed (Fig. 5C, D, G, and H).

The immunohistological examination 30 days after the dopaminergic lesion showed a strong expression of GFAP, a marker for astrocytosis. The aim of immunofluorescence analysis was to estimate the effect of the Hc-TeTx injection plus the dopaminergic lesion on astrocytosis over the long term (Fig. 5I–O). In the 6-OHDA-treated group the GFAP-IR increased compared to the vehicle group in the ipsilateral striatum, as the photomicrographs show (Fig. 5M and I). The most interesting finding was the lower GFAP-IR of the Hc-TeTx and 6-OHDA-treated group compared to the 6-OHDA-treated group (Fig. 5N and M). In addition, we found that Hc-TeTx by itself showed a low immunoreactivity to GFAP in the same area of the ipsilateral striatum (Fig. 5J; $P < 0.05$). The quantitative analysis of positive GFAP cells confirms the result that the Hc-TeTx treatment causes a small number of positive cells to GFAP compared to the control 6-OHDA in the ipsilateral striatum (Fig. 5R). The GFAP-IR for the contralateral striatum was found to be small for each group after 30 days (Fig. 5K, L, O, and P). Fig. 5Q and R shows the quantitative data of the caspase-3- and the GFAP-immunopositive cells in the ipsilateral striatum. The statistical analysis was made using a one-way ANOVA [$F_{(3,11)} = 44.95$ and $F_{(3,12)} = 24.17$] followed by a Bonferroni multiple comparison test and indicates that there is a significant difference between the Hc-TeTx plus 6-OHDA and 6-OHDA groups for caspase-3 ($P < 0.001$) and GFAP immunoreactivity ($^{**}P < 0.01$, $^{***}P < 0.001$).

3.7. Neurodegeneration

The effects of the neuronal damage caused by the intrastriatal injection of 6-OHDA and the protection by the previous intramuscular injection of Hc-TeTx were studied with the aminocupric-silver stain. As shown in Fig. 6B, C, F, and G, the regions close to the site of the injection were studied and analyzed in the ipsilateral striatum and in the same region of the contralateral striatum (Fig. 6D, E, H and I).

We found an evident argyrophilic reaction that characterizes the neuronal damage in the ipsilateral striatum of animals with a dopaminergic lesion (Fig. 6F), which was more evident close to the site of the injection. This was shown by the notably dark cells from the 6-OHDA-treated group compared to the fewer number of dark cells in the same areas of the Hc-TeTx plus 6-OHDA group (Fig. 6G).

On another hand, control groups that were only treated with Hc-TeTx or the vehicle groups did not show a significant argyrophilic stain in the ipsilateral striatum (Fig. 6B and C). The contralateral striatum did not show argyrophilic cells for any group analyzed (Fig. 6D, E and H–I). The number of positive argyrophilic cells was measured using the ImageJ software, by using the mean of five photomicrographs captured at 4×. The quantitative data of the positive argyrophilic cells in the ipsilateral striatum for the 6-OHDA-treated group was 34 ± 4 (mean ± SE), although the Hc-TeTx plus 6-OHDA-treated group that had a low number of positive argyrophilic cells,

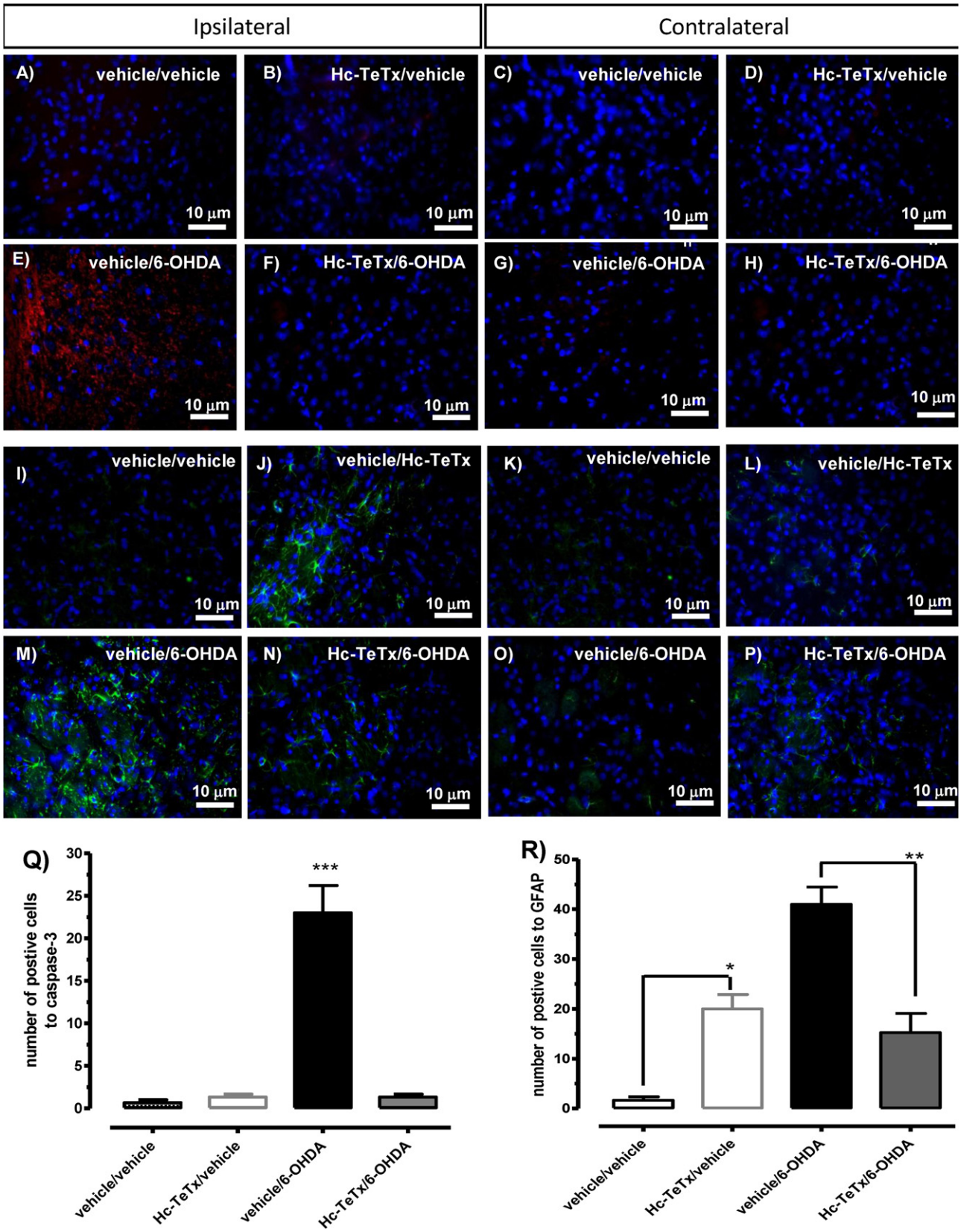

Fig. 5. The injection of Hc-TeTx into hemiparkinsonian rats caused changes in the immunoreactivity to caspase-3 and GFAP in the striatum. The photomicrographs show the caspase-3 (red color) in the ipsilateral and contralateral striatum (A–H). The immunohistochemical assessments were done 30 days after the injection of the 6-OHDA ($n = 3$ in each group). The photomicrographs show that the Hc-TeTx/6-OHDA-treated group had a decrease in the caspase-3 immunoreactivity compared to the 6-OHDA group (F and E). The photomicrographs show the GFAP (green color) in the ipsilateral and contralateral striatum (I–P). The photomicrographs show that the astrocytosis of Hc-TeTx/6-OHDA-treated group was lower than the 6-OHDA group (M and N). The nucleus was observed with DAPI staining (blue stain). All the slices were observed at 40×. In (Q) the graph shows the quantification for positive caspase-3 cells for the ipsilateral striatum. Our results show a few positive caspase-3 cells compared to

10 ± 1 (mean ± SE; $P < 0.001$) (Fig. 6A). The statistical analysis (one-way ANOVA followed by a Bonferroni multiple comparison test) indicates that there is a significant difference between the groups (one-way ANOVA [$F_{(3,18)} = 34.20$]). The control groups of vehicle and Hc-TeTx show a small number of positive argyrophilic cells, 9 ± 1 and 4 ± 1 (mean ± SE) for the ipsilateral striatum.

4. Discussion

In recent years the investigation of the neuroprotective effects of the C-terminal domain of tetanus toxin has increased (reviewed in Toivonen et al., 2010) and subsequently revealed that its action on the antiapoptotic and survival pathways is similar to neurotrophins in the animal models of neurodegenerative diseases (Moreno-Igoa et al., 2010; Chaib-Oükadour et al., 2004). In our study, the effects of the intramuscular Hc-TeTx injection to arrive at the brain in a rat model of PD were explored. The principal findings are the improvements of several motor behaviors and protection against the loss of DA associated with amelioration of the neurodegenerative process in the striatum.

During these behavioral tests, we found that the motor asymmetry was a minimum for the Hc-TeTx using the doses of 10 and 20 μg/kg compared with the 6-OHDA group. There was a severe depletion of striatal dopamine resulting in a lower ability of the animals to make adjusting steps or use both forelimbs during the exploration in the cylinder test, and these results were corroborated with other reports (Johnson et al., 1999; Kirik et al., 2004; Dowd et al., 2005; Grealish et al., 2008).

Furthermore, the effects on rats were shown on the motor asymmetry over the long term. The lower doses of Hc-TeTx (0.1, 1 or 5 μg/kg) did not cause improvement in the motor asymmetry. These findings suggest that the protective action of Hc-TeTx is related to the dose, because the higher doses of the Hc-fragment produced better effects on the motor asymmetry than the lower doses, as we had shown in the model of a striatal lesion with MPP^+ (Mendieta et al., 2009).

The lower doses of Hc-TeTx did not cause changes in the striatal dopamine levels of the rats compared to the 6-OHDA-treated animals. However, only the 20 μg/kg dose of Hc-TeTx in rats showed a high significant increase in the amounts of dopamine in the ipsilateral striatum compared with the 6-OHDA-treated group. Moreover, these results suggest that Hc-TeTx exerts its neuroprotective effects in an dose-dependent way in animals with a dopaminergic lesion made by 6-OHDA. The main finding is that the 20 μg/kg dose of the Hc-TeTx in the animals causes improvement in their motor behavior. The improvement in the DA levels is related to the size of the Hc-TeTx dose because there are no important changes in the DA levels using a dose of <10 μg/kg Hc-TeTx. The results obtained at 20 μg/kg of Hc-TeTx also showed that it is efficient in preventing the degeneration of the nigral-dopamine cell bodies over the long-term. It is probable that the Hc-TeTx treatment can preserve the functional dopamine innervations if it were administered early in the degenerative process.

The dopaminergic neurotransmission is the key to the regulation of the basal ganglia for the movement performance; consequently the principal therapy for PD has been focused on the replacement of this neurotransmitter by its precursor levodopa which results in a useful drug that leads to a remarkable amelioration of the symptoms. However, the levodopa loses its efficacy after several years of treatment because the patients develop motor complications, such as dyskinesia, and the neurodegeneration is not stopped, as might be expected with the use of trophic agents or using the Hc-TeTx fragment that promotes neuronal survival over the long term.

The molecular mechanisms underlying the neuroprotective action of the Hc-TeTx has been shown through the Trk pathways (Chaib-Oükadour et al., 2004), which suggests that Hc-TeTx might have a direct neuroprotective role. In cultured neurons, the Hc-TeTx acts similarly to growth factors causing the activation of several prosurvival pathways, including the phosphatidylinositol-3-kinase (PI3K)-protein kinase B (Akt), Ras-extracellular-signal-regulated kinase (ERK), and the phospholipase C-γ (PLC-γ) (Gil et al., 2000, 2001). The first evidence of the neuroprotective action of Hc-TeTx was assessed in a model for the study of PD, using the neurotoxin MPP^+ in cerebellar-granule neurons (CGN). These experiments showed that the Hc-TeTx fragment protects against MPP^+ by inhibiting apoptosis (Chaib-Oükadour et al., 2004).

In addition, during the study of neuroprotection by the glial-derived-growth factor (GDNF), the fusion of Hc-TeTx with this growth factor showed the maintenance of the neurotrophic effects in a model of neurodegeneration (Ciriza et al., 2008). The Hc-TeTx fragment was later proved to be a neuroprotective agent in an animal model of amyotrophic lateral sclerosis, and it caused the same antiapoptotic effects and had a high efficiency to cause cellular survival (Moreno-Igoa et al., 2010).

There is another possibility for the action of Hc-TeTx that is directly associated with the increase in the dopamine levels, such as we found in the striatum. The activation of dopamine receptors, such as the D1 type, has been associated with a neuroprotective response because these kinds of receptors are G-protein-coupled receptors (GPCRs) causing the transactivation of Trk receptors after the activation of the classical cascade of signaling, PI3-K/Akt, and p21 ras/ERK1–2, independent of the neurotrophins' action or along with them (Iwakura et al., 2008). In an attempt to understand the molecular mechanisms that involved the trophic action of Hc-TeTx in rats with a striatal dopaminergic lesion, we are assessing the TrkB pathways because it is plausible that the Akt and mitogen-activated protein kinase (MAPK) pathways are crucial in promoting the survival of dopaminergic neurons, as other studies have suggested (Ugarte et al., 2003).

The quantitative analysis of argyrophilic-positive cells confirmed this idea that the Hc-TeTx decreases neuronal death in the ipsilateral striatum. The 6-OHDA group had a large amount of caspase-3-IRin the ipsilateral striatum, and we found the Hc-TeTx-treated group showed low caspase-3-IR in the same area of the striatum. These data suggest the protective action of the Hc-TeTx against the apoptotic process caused by the 6-OHDA. Interestingly, there was almost complete rescue of the nigral neurons (TH-IR cells) at the 20 μg/kg dose of Hc-TeTx. We report for first time that the intramuscular application of the Hc-TeTx is also effective in decreasing the striatal neurodegeneration of hemiparkinsonian rats even after 30 days. Its effects are associated with the protection at the site of the lesion or in the terminal neurons remaining in the nigrostriatal system caused by the sprouting generated in the damaged neurons, similar to the action of GDNF in the striatum (Kirik et al., 2000, 2004).

Previous studies have shown that when a lesion of the nigrostriatal pathway is caused by 6-OHDA, it leads to the astroglial and microglial activation that modulates the response to the injury that triggers neuronal survival or death (Rodrigues et al., 2003; Chadi and Gomide, 2004; Maeda et al., 2008). We found that a high astrocytosis in the ipsilateral striatum of rats with a 6-OHDA

the 6-OHDA group (***$P < 0.001$). The graph (R) shows the quantification for positive GFAP cells for the ipsilateral striatum. Our results showed that astrocytosis (GFAP) is lower for animals with Hc-TeTx plus 6-OHDA group than animals with only the 6-OHDA group. The data are the mean ± SE (*$P < 0.05$; **$P < 0.01$) were considered significant in the statistical analyses using a one-way ANOVA followed by Bonferroni multiple-comparisons test.

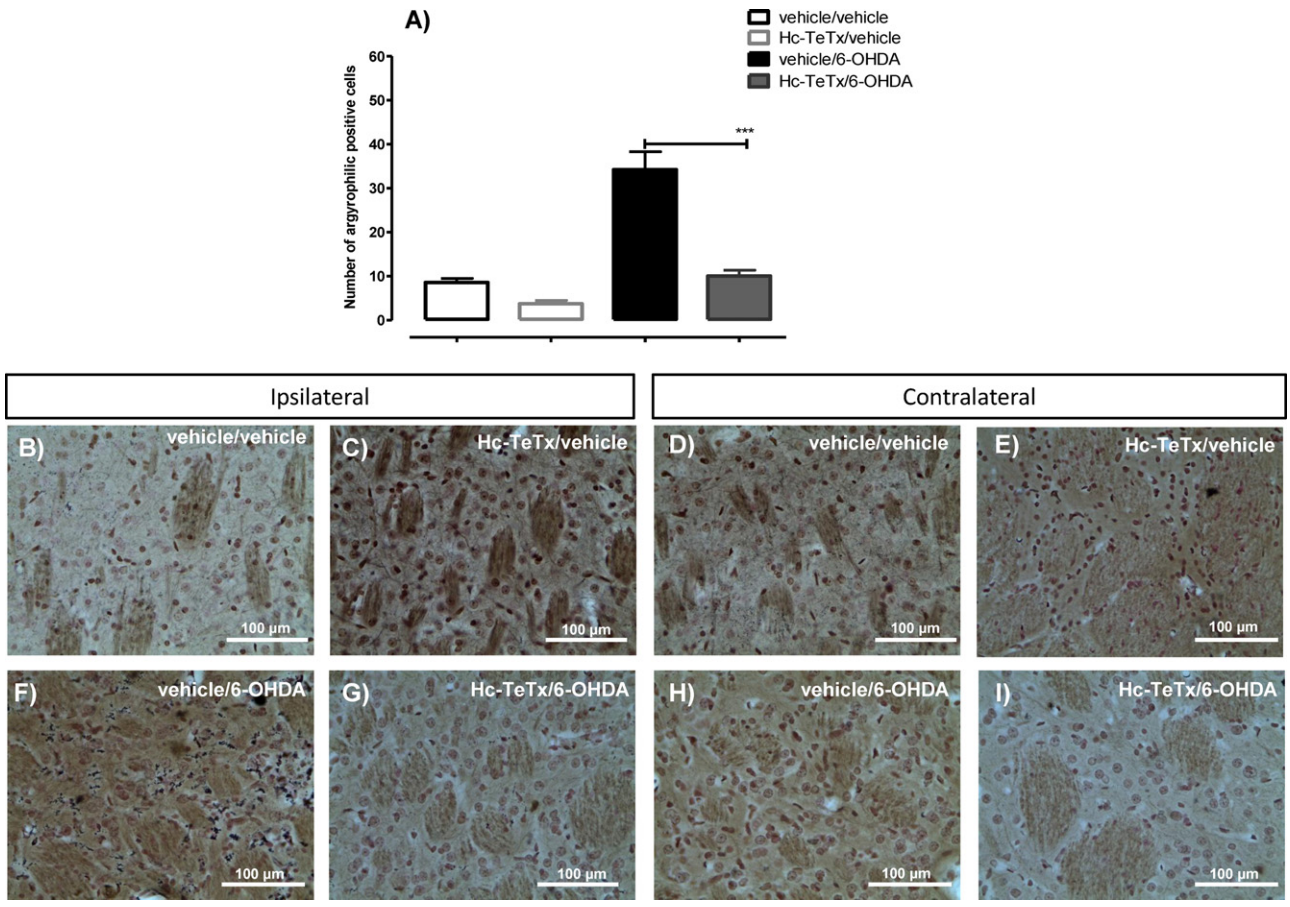

Fig. 6. Effects of Hc-TeTx treatment on the striatal neurodegeneration of rats injured with the specific catecholamine neurotoxin 6-OHDA. (A) Quantification of positive argyrophilic cells in the ipsilateral striatum from the vehicle, Hc-TeTx, 6-OHDA, and Hc-TeTx/6-OHDA groups. The statistical analysis (one-way ANOVA followed by Bonferroni multiple-comparisons test) indicates that there is a significant decrease in the number of positive argyrophilic cells in the Hc-TeTx/6-OHDA-treated group compared with the 6-OHDA group (***$P < 0.001$). The photomicrographs (B–I) show the argyrophilic cells. Photomicrography of the amino-cupric staining of the ipsilateral and contralateral striatum of animals ($n = 3$ in each group) with vehicle/vehicle (B and D), Hc-TeTx/vehicle (C and E), vehicle/6-OHDA (F and H), and Hc-TeTx/6-OHDA (G and I). The slices were observed at 4×.

lesion may be related to the high number of argyrophilic and caspase-3-positive cells, accompanied by low dopamine levels in the striatum. This phenomenon appears be attenuated by the previous treatment with Hc-TeTx, so 30 days after dopaminergic lesion there is a low astrocytosis. Moreover, we found a moderate astrocytosis in the group treated with the Hc-TeTx plus ascorbic acid, but it probably means this is a trophic effect because there was no neuronal damage in the same area of the striatum as shown by the low argyrophilic stain.

However, the trophic effect of astrocytes remains in controversy because it is present in the beginning of a toxic event, so astrocytes cause the release of neurotrophins and increase the antioxidant molecules that give protection to the neurons. If the astrocytosis is chronic and high, it causes neuronal death. Some studies showed that the dopaminergic cells became dramatically resistant to the 6-OHDA neurotoxicity when the cells are in the presence of astrocytes, as reported by Yu and Zuo (1997). These observations suggest that the astroglial activity by the Hc-TeTx can result in beneficial effects for the striatum cells, when this cellular response occurs to a middling degree. Consequently, all evidence and our findings lead to suggest that the Hc-fragment can have a trophic action in the brain. The discovery of Hc-TeTx as a trophic fragment to mimic growth factors represents a promising avenue for exploring the TrkB (and others neurotrophin receptors) pathways in models of PD. This may lead to the understanding of the neurodegenerative disorder. At the same time the Hc-TeTx may have important implications in the future of the neurodegenerative process where there is a decrease in the production of growth factors, and consequently, several deficits in behavior.

5. Conclusion

Altogether, our findings indicate that (i) 20 μg/kg i.m. is the most effective dose of the Hc-TeTx in the animals to cause improvement of their motor behavior, (ii) this dose of Hc-TeTx protects against loss of DA, and (iii) it is able to prevent the nigrostriatal neurons of neurodegeneration by 6-OHDA over the long term. Thus the intramuscular injection of Hc-TeTx may have the therapeutic potential to prevent the motor-behavior deficits and the apoptotic neuronal death in PD. Moreover, several attempts must still be made to establish that the chronic treatment of Hc-TeTx is able to delay the motor-behavioral impairments and the dopaminergic neurodegeneration in parkinsonian patients.

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References

Ceravolo, R., Sgado, P., Frosini, D., Corsini, G.U., 2006. Assessing neuroprotection in Parkinson's disease: from the animal models to molecular neuroimaging in vivo. J. Neural Transm. 71, 133–141.

Chadi, G., Gomide, V.C., 2004. FGF-2 and S100beta immunoreactivities increase in reactive astrocytes, but not in microglia, in ascending dopamine pathways following a striatal 6-OHDA-induced partial lesion of the nigrostriatal system. Cell Biol. Int. 28, 849–861.

Chaib-Oükadour, I., Gil, C., Aguilera, J., 2004. The C-terminal domain of the heavy chain of tetanus toxin rescues cerebellar granule neurons from apoptotic death: involvement of phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways. J. Neurochem. 90, 1227–1236.

Chaib-Oükadour, I., Gil, C., Rodríguez-Alvarez, J., Ortega, A., Aguilera, J., 2009. Tetanus toxin HC fragment reduces neuronal MPP^+ toxicity. Mol. Cell. Neurosci. 41 (3), 297–303.

Chang, J.W., Wachtel, S.R., Young, D., Kang, U.J., 1999. Biochemical and anatomical characterization of forepaw adjusting steps in rat models of Parkinson's disease: studies on medial forebrain bundle and striatal lesions. Neuroscience 88 (2), 617–628.

Cicchetti, F., Brownell, A.L., Williams, K., Chen, Y.I., Livni, E., Isacson, O., 2002. Neuroinflammation of the nigrostriatal pathway during progressive 6-OHDA dopamine degeneration in rats monitored by immunohistochemistry and PET imaging. Eur. J. Neurosci. 15, 991–998.

Ciriza, J., Moreno-Igoa, M., Calvo, A.C., Yague, G., Palacio, J., Miana-Mena, F.J., Muñoz, M.J., Zaragoza, P., Brûlet, P., Osta, R., 2008. A genetic fusion GDNF-C fragment of tetanus toxin prolongs survival in a symptomatic mouse ALS model. Restor. Neurol. Neurosci. 26 (6), 459–465.

De Olmos, J.S., Beltramino, C.A., de Olmos de Lorenzo, S., 1994. Use of an amino-cupric-silver technique for the detection of early and semiacute neuronal degeneration caused by neurotoxicants, hypoxia, and physical trauma. Neurotoxicol. Teratol. 16 (6), 545–561.

Deinhardt, K., Salinas, S., Verastegui, C., Watson, R., Worth, D., Hanrahan, S., Bucci, C., Schiavo, G., 2006. Rab5 and Rab7 control endocytic sorting along the axonal retrograde transport pathway. Neuron 52, 293–305.

Dowd, E., Monville, C., Torres, E.M., Wong, L.F., Azzouz, M., Mazarakis, N.D., Dunnet, S.B., 2005. Lentivector-mediated delivery of GDNF protects complex motor functions relevant to human Parkinsonism in a rat lesion model. Eur. J. Neurosci. 22, 2587–2595.

Emborg, M.E., 2004. Evaluation of animal models of Parkinson's disease for neuroprotective strategies. J. Neurosci. Methods 139 (2), 121–143.

Fishman, P.S., Carrigan, D.R., 1987. Retrograde transneuronal transfer of the C-fragment of tetanus toxin. Brain Res. 406 (1–2), 275–279.

Fishman, P.S., Savitt, J.M., Farrand, D.A., 1990. Enhanced CNS uptake of systemically administered proteins through conjugation with tetanus C-fragment. J. Neurol. Sci. 98, 311–325.

Gil, C., Chaib-Oükadour, I., Pelliccioni, P., Aguilera, J., 2000. Activation of signal transduction pathways involving TrkA, PLCγ-1, PKC isoforms and ERK-1/2 by tetanus toxin. FEBS Lett. 481, 177–182.

Gil, C., Chaib-Oükadour, I., Aguilera, J., 2003. C-terminal fragment of tetanus toxin heavy chain activates Akt and MEK/ERK signalling pathways in a Trk receptor-dependent manner in cultured cortical neurons. Biochem. J. 373, 613–620.

Gil, C., Chaib-Oükadour, I., Blasi, J., Aguilera, J., 2001. Hc-fragment (C-terminal portion of the heavy chain) of tetanus toxin activates protein kinase C isoforms and phosphoproteins involved in signal transduction. Biochem. J. 356, 97–103.

Grealish, S., Xie, L., Kelly, M., Dowd, E., 2008. Unilateral axonal or terminal injection of 6-hydroxydopamine causes rapid-onset nigrostriatal degeneration and contralateral motor impairments in the rat. Brain Res. Bull. 77, 312–319.

Iwakura, Y., Nawa, H., Sora, I., Chao, M.V., 2008. Dopamine D1 receptor-induced signaling through TrkB receptors in striatal neurons. J. Biol. Chem. 283 (23), 15799–15806.

Johnson, R.E., Schallert, T., Becker, J.B., 1999. Akinesia and postural abnormality after unilateral dopamine depletion. Behav. Brain Res. 104 (1–2), 189–196.

Kearns, C.M., Gash, D.M., 1995. GDNF protects nigral dopamine neurons against 6-hydroxydopamine in vivo. Brain Res. 672, 104–111.

Kirik, D., Georgievska, B., Björklund, A., 2004. Localized striatal delivery of GDNF as a treatment for Parkinson disease. Nat. Neurosci. 7 (2), 105–110.

Kirik, D., Rosenblad, C., Björklund, A., 1998. Characterization of behavioral and neurodegenerative changes following partial lesions of the nigrostriatal dopamine system induced by intrastriatal 6-hydroxydopamine in the rat. Exp. Neurol. 152 (2), 259–277.

Kirik, D., Rosenbland, C., Björklund, A., 2000. Preservation of a functional nigrostriatal dopamine pathway by GDNF in the striatal 6-OHDA lesion model depends on the site of administration of the trophic factor. Eur. J. Neurosci. 12, 3871–3882.

Kumar, R., Agarwal, A.K., Seth, P.K., 1995. Free radical-generated neurotoxicity of 6-hydroxydopamine. J. Neurochem. 64, 1703–1707.

Larsen, K.E., Benn, S.C., Ay, I., Chian, R.J., Celia, S.A., Remington, M.P., Bejarano, M., Liu, M., Ross, J., Carmillo, P., Sah, D., Phillips, K.A., Sulzer, D., Pepinsky, R.B., Fishman, P.S., Brown, R.H., Francis Jr., J.W., 2006. A glial cell line-derived neurotrophic factor (GDNF): tetanus toxin fragment C protein conjugate improves delivery of GDNF to spinal cord motor neurons in mice. Brain Res. 1120, 1–12.

Li, J., Chian, R.J., Ay, I., Kashi, B.B., Celia, S.A., Tamrazian, E., Pepinsky, R.B., Fishman, P.S., Brown, R.H., Francis Jr., J.W., 2009. Insect GDNF-TTC fusion protein improves delivery of GDNF to mouse CNS. Biochem. Biophys. Res. Commun. 390, 947–951.

Liang, Q., Liou, A.K., Ding, Y., Cao, G., Xiao, X., Perez, R.G., Chen, J., 2004. 6-Hydroxydopamine induces dopaminergic cell degeneration via a caspase-9-mediated apoptotic pathway that is attenuated by caspase-9dn expression. J. Neurosci. Res. 77, 747–761.

Maeda, T., Nagata, K., Yoshida, Y., 2008. Exogenous L-DOPA induces no dopamine immuno-reactivity in striatal astroglias and microglias of adult rats with extensive nigro-striatal dopaminergic denervation. Neurosci. Lett. 433, 255–258.

Mazzio, E.A., Reams, R.R., Soliman, K.F., 2004. The role of oxidative stress, impaired glycolysis and mitochondrial respiratory redox failure in the cytotoxic effects of 6-hydroxydopamine in vitro. Brain Res. 1004, 29–44.

Mendieta, L., Venegas, B., Moreno, N., Patricio, A., Martínez, I., Aguilera, J., Limón, I.D., 2009. The carboxyl-terminal domain of the heavy chain of tetanus toxin prevents dopaminergic degeneration and improves motor behavior in rats with striatal MPP^+-lesions. Neurosci. Res. 65 (1), 98–106.

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

Moreno-Igoa, M., Calvo, A.C., Penas, C., Manzano, R., Oliván, S., Muñoz, M.J., Mancuso, R., Zaragoza, P., Aguilera, J., Navarro, X., Osta Pinzolas, R., 2010. Fragment C of tetanus toxin, more than a carrier. Novel perspectives in non-viral ALS gene therapy. J. Mol. Med. 88 (3), 297–308.

Obeso, J.A., Rodriguez-Oroz, M.C., Goetz, C.G., Marin, C., Kordower, J.H., Rodriguez, M., Hirsch, E.C., Farrer, M., Schapira, A.H., Halliday, G., 2010. Missing pieces in the Parkinson's disease puzzle. Nat. Med. 16 (6), 653–661.

Park, J.W., Youn, Y.C., Kwon, O.S., Jang, Y.Y., Han, E.S., Lee, C.S., 2002. Protective effect of serotonin on 6-hydroxydopamine- and dopamine-induced oxidative damage of brain mitochondria and synaptosomes and PC12 cells. Neurochem. Int. 40, 223–233.

Paxinos, G., Watson, C., 1998. The Rat Brain in Stereotaxic Coordinates, 4th ed. Academic Press, USA.

Payne, A.M., Zheng, Z., Messi, M.L., Milligan, C.E., Gonzalez, E., Delbono, O., 2006. Motor neurone targeting of IGF-1 prevents specific force decline in ageing mouse muscle. J. Physiol. (Lond.) 570, 283–294.

Perreault, M.C., Bernier, A.P., Renaud, J.S., Roux, S., Glover, J.C., 2006. C fragment of tetanus toxin hybrid proteins evaluated for muscle-specific transsynaptic mapping of spinal motor circuitry in the newborn mouse. Neuroscience 141, 803–816.

Przedborski, S., Levivier, M., Jiang, H., Ferreira, M., Jackson-Lewis, V., Donalson, D., Togasaki, D.M., 1995. Dose-dependent lesions of the dopaminergic nigrostriatal pathway induced by intrastriatal injection of 6-hydroxydopamine. Neuroscience 67, 631–647.

Rodrigues, R.W.P., Gomide, V.C., Chadi, G., 2003. Striatal injection of 6-hydroxydopamine induces retrograde degeneration and glial activation in the nigrostriatal pathway. Acta Cir. Bras. 18 (4), 272–282.

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

Roux, S., Colasante, C., Cloment, C., Barbier, J., Curie, T., Girard, E., Molgo, J., Philippe, B., 2005a. Internalization of GTP-tetanus toxin C-terminal fragment fusion protein at mature mouse neuromuscular junctions. Mol. Cell. Neurosci. 59, 401–415.

Roux, S., Colasante, C., Cloment, C., Barbier, J., Curie, T., Girard, E., Molgo, J., Philippe, B., 2005b. Internalisation of GTP-tetanus toxin C-terminal fragment fusion protein at mature mouse neuromuscular junctions. Mol. Cell. Neurosci. 59, 401–415.

Sauer, H., Oertel, W.H., 1994. Progression degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesion with 6-hydroxydopamin: A combined retrograde tracing and inmunocitochemical study in the rat. Neuroscience 59, 401–415.

Toivonen, J.M., Oliván, S., Osta, R., 2010. Tetanus toxin C-fragment: the courier and the cure? Toxins 2 (11), 2622–2644.

Ugarte, S.D., Lin, E., Klann, E., Zigmond, M.J., Perez, R.G., 2003. Effects of GDNF on 6-OHDA-induced death in a dopaminergic cell line: modulation by inhibitors of PI3 kinase and MEK. J. Neurosci. Res. 73, 105–112.

Ungerstedt, U., Arbuthnott, G.W., 1970. Quantitative recording of rotational behavior in rats after 6-hydroxydopamine lesions of the nigrostriatal dopamine system. Brain Res. 24, 485–493.

Winkler, C., Sauer, H., Lee, C.S., Björklund, A., 1996. Short-term GDNF treatment provides long-term rescue of lesioned nigral dopaminergic neurons in a rat model of Parkinson's disease. J. Neurosci. 16, 7206–7215.

Yu, P.H., Zuo, D.M., 1997. Enhanced tolerance of neuroblastoma cells towards the neurotoxin 6-hydroxydopamine following specific cell–cell interaction with primary astrocytes. Neuroscience 78 (3), 903–912.