Neuroscience Research xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Neuroscience Research

journal homepage: www.elsevier.com/locate/neures

The restorative effect of intramuscular injection of tetanus toxin C-fragment in hemiparkinsonian rats

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ARTICLE INFO

Article history:
Received 22 November 2013
Received in revised form 1 April 2014
Accepted 26 April 2014
Available online xxx

Keywords:
C-terminal domain of the heavy chain of tetanus toxin
Motor asymmetry
Neurodegeneration
Restoration
Tyrosine hydroxylase
Substantia nigra pars compacta
Parkinson's disease

ABSTRACT

The C-terminal domain of the heavy chain of tetanus toxin (Hc-TeTx) is a peptide that has a neuroprotective action against dopaminergic damage by MPP^+, both in vitro and in vivo. The trophic effects of Hc-TeTx have been related to its ability to activate the pathways of the tropomyosin receptor kinase, which are crucial for survival process. Our group had previously shown neuroprotective effect of intramuscular Hc-TeTx treatment on animals with a dopaminergic lesion; however, there is no evidence indicating its restorative effects on advanced dopaminergic neurodegeneration. The aim of our study was to examine the restorative effects of an intramuscular injection of the Hc-TeTx fragment on the nigrostriatal system of hemiparkinsonian rats. The animals were administered with a vehicle or Hc-TeTx (20 μg/kg) in the *gastrocnemius* muscle for three consecutive days post-dopaminergic lesion, which was made using 6-hydroxydopamine. Post-Hc-TeTx treatment, the hemiparkinsonian rats showed constant motor asymmetry. Moreover, the ipsilateral striatum of the post-Hc-TeTx group had a lower number of argyrophilic structures and a major immunorreactivity to Tyrosine Hydroxylase in the striatum and the substantia nigra *pars compacta* compared to the 6-OHDA group. Our results show the restorative effect of the Hc-TeTx fragment during the dopaminergic neurodegeneration caused by 6-OHDA.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder that affects more than five millions people around the world (Liviu and Rüdiger, 2011). PD is characterized by the presence of tremor, akinesia, rigidity, difficulty on trying to walk and loss of postural reflexes (Obeso et al., 2000). These symptoms are caused by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), which is linked to a more than 80% loss of the level of dopamine in the striatum (Kirik et al., 1998). This fact has motivated researchers to reproduce the pathological features of PD in animal models in order to find therapeutic alternatives involving dopaminergic system restoration (Björklund, 2006; Kirik et al., 2006; Enciu et al., 2011).

Unilateral infusion of 6-hydroxydopamine (6-OHDA) into the striatum, where the dopaminergic terminals that module the corticostriatal neuronal activity are placed, promotes a progressive retrograde degeneration of the nigrostriatal pathway over time. This kind of lesion has been used as a major model in the study of the progression of PD (Sauer and Oertel, 1994; Björklund et al., 1997; Deumens et al., 2002) and the evaluation of restorative substances (Sagi et al., 2007; Massa et al., 2010).

In recent years, neurotrophins have been proposed as a possible pharmacological alterative to other conventional PD treatments. By activating the tropomyosin receptor kinase (Trk), the trophic effects of these agents active survival signaling (Kirik et al., 2000). However, neurotrophins have insufficient bioavailability to cross the blood-brain barrier (Poduslo and Curran, 1996). This feature is a major disadvantage of the peripheral administration of neurotrophins. For this reason, it is necessary to search for molecules that can access the Central Nervous System (CNS) and activate cellular survival in the same way as neurotrophins.

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http://dx.doi.org/10.1016/j.neures.2014.04.008
0168-0102/

The C-terminal domain of the heavy chain of tetanus toxin (Hc-TeTx fragment) has emerged as a probable treatment for PD because it has similarities to neurotrophins in its promotion of cellular survival and its neuroprotective action against stress models (Gil et al., 2001, 2003; Chaib-Oükadour et al., 2004) and a dopaminergic lesion model in vitro (Chaib-Oükadour et al., 2009). We have previously shown the neuroprotective effects of the Hc-TeTx fragment on nigrostriatal dopaminergic pathways and motor behavior in a MPP^+ rat model (Mendieta et al., 2009). In recent years we have also shown that the intramuscular injection of the Hc-TeTx fragment (at 20 μg/kg), causes an improvement in motor behavior, acting against the neurodegeneration process of nigrostriatal neurons in animals treated with 6-OHDA (Mendieta et al., 2012). Moreover, the Hc-TeTx fragment has a potential neuroprotective effect on spinal motor neurons through an autophagic signaling pathway that acts against glutamate excitotoxicity (Herrando-Grabulosa et al., 2012). In accordance with these last findings, we proposed the reproduction of a hemiparkinsonian model in the rat in order to explore, for the first time, the restorative effect of Hc-TeTx on motor behavior and the neurodegeneration process.

2. Materials and methods

2.1. Animals

Adult Wistar rats weighing 250–270 g at the commencement of the experiments ($N = 40$) were bred in the vivarium at the Benemérita Universidad Autónoma de Puebla, México. Animals were housed in groups of 5 in a humidity-controlled room and a 12 h:12 h light–dark cycle with ad libitum food and water.

All procedures were carried out in accordance with the Mexican Council for Care and Use of Laboratory Animals and the *Norma Oficial Mexicana* NOM-062-ZOO-1999.

2.2. Unilateral 6-hydroxydopamine intrastriatal lesion

The rats were divided in two groups, one with a dopaminergic lesion induced by 6-hydroxydopamine and the other with ascorbic acid as a vehicle. Each animal was anesthetized with ketamine (55 mg/kg i.p.) and xylazine (5 mg/kg i.p.). The rats were placed in a stereotaxical apparatus (Stoelting Co., Wood Dale, IL, USA) for the administration of a solution of either 2 μL of vehicle or 2 μL of 6-hydroxydopamine (6-OHDA 8 μg/μL, Sigma Aldrich, St. Louis, MO, USA, dissolved in 0.01% ascorbic acid (a.a.)) to the left striatum.

The 6-hydroxydopamine was injected using a 10 μL Hamilton syringe and each μL was infused for 300 seconds. The stereotaxic coordinates were AP: +0.7 mm from Bregma, L: +3.7 mm from Midline, DV1: −4.9 mm and DV2: −5.5 mm below Dura (according to Paxinos and Watson's Stereotaxic Atlas, 1998). Proper post-operative attention was given until the animal made a full recovery. Eight days after the surgery, the lesion was evaluated by measuring the methamphetamine induced ipsilateral turning behavior (5 mg/kg s.c.). Animals lesioned with 6-OHDA, which performed more than fifteen turns per minute were considered to be demostrating hemiparkinsonian behavior and considered for posterior experiments. Animals administered with the vehicle did not demonstrate turning behavior.

2.3. Intramuscular injection of Hc-TeTx

One day subsequent to the recording of the turning behavior, one half of both groups of animals were administered with either the vehicle (SSI) or the Hc-TeTx fragment (20 μg/kg) in the *gastroctemius* muscle for three consecutive days. The experimental groups were as follows: sham rats, 6-OHDA lesioned rats, sham rats administered with the vehicle via intramuscular injection and 6-OHDA lesioned rats administered with an intramuscular Hc-TeTx fragment injection. The Hc-TeTx fragment was synthetized in accordance with Herrando-Grabulosa et al. (2012).

2.4. Motor activity evaluation

Forelimb asymmetry was evaluated using the cylinder test, proposed by Schallert and Tilleron (2000). In this test, rats use their forelimbs in the vertical exploration along the wall of the cylinder (30 cm to height and 20 cm to diameter), and to land after they had reared up.

A day before the dopaminergic lesion, the animals were assessed in the cylinder test to obtain the base values. Then, the test was made every seventh day over 4 weeks in order to evaluate the dopaminergic lesion and the effect of the Hc-TeTx treatment. Rats were placed in a transparent acrylic cylinder and their activity was videotaped for five minutes. A minimum of eight wall contacts was considered as a valid data. The measurement of the number of forelimb wall contacts produced scores that represent overall asymmetry, which is correlated closely with the degree of striatal DA depletion (Schallert and Tilleron, 2000; Cenci and Lundblad, 2005). The individual use of each forelimb was calculated according to Schallert (2006), and using the following calculation: % ipsilateral forelimb = [(ipsilateral + (1/2) both)/(ipsilateral + contralateral + both)] × 100 and % contralateral forelimb = [(contralateral + (1/2) both)/(ipsilateral + contralateral + both)] × 100.

2.5. Histological analysis

Once the behavior evaluation had been performed, the rats were anesthetized and perfused with 200 mL of 4% paraformaldehyde. The brains were then removed and stored in the same fixative solution for 48 h, following which the brains obtained from each group were divided into two different histological protocols. Each tissue was fixed and embedded in paraffin to obtain 5 μm thick coronal sections from the striatum approximately +1.2 mm to −0.3 mm from the bregma. The other protocol required that 40 μm thick sections of the striatum and the SNpc were obtained by microtome apparatus and then stored in a 15% sucrose solution until immunostaining procedures were carried out.

2.5.1. Amino-cupric-silver stain

The neurodegeneration caused by dopaminergic terminal lesions to the striatum was observed using the precipitation that takes place during the ionic-silver staining method (Mendieta et al., 2012).

Neurodegenerative structures in the striatum were examined through a Leica DM750 microscopy at 40× and photographed using a Leica digital camera ICC50. Photographs were captured using the Leica Application Suite LAS EZ software, version 1.8.0.

2.5.2. Immunohistochemistry

Immunostaining for 40 μm sections of striatum and SNpc were carried out using the free-floating method with standard biotin–strepavidin immunohistochemistry protocols (Granado et al., 2008; Grealish et al., 2008). Briefly, following the suppressing of endogenous peroxidase activity (3% hydrogen peroxide/100% methanol solution) and the blocking of non-specific secondary antibody binding (3% bovine serum albumin in phosphates buffered saline, Sigma), sections were incubated overnight in the primary TH antibody (mouse monoclonal 1:1000 dilution, Millipore). Then, the sections were incubated in a Dako LSAB + System-HRP. Immunolabeling was revealed by incubating the sections in a 0.5% solution of diaminobenzidine tetrahydrocloride in a mixture of phosphate buffered saline and 0.3 μL/mL of hydrogen peroxide. Sections were

Please cite this article in press as: Sánchez-González, A., et al., The restorative effect of intramuscular injection of tetanus toxin C-fragment in hemiparkinsonian rats. Neurosci. Res. (2014), http://dx.doi.org/10.1016/j.neures.2014.04.008