Brain Research 1657 (2017) 156–166

Contents lists available at ScienceDirect

Brain Research

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

Research report

Cardiovascular dysfunction associated with neurodegeneration in an experimental model of Parkinson's disease

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ARTICLE INFO

Article history:
Received 29 July 2016
Received in revised form 6 December 2016
Accepted 7 December 2016
Available online 9 December 2016

Keywords:
Parkinson disease
Baroreflex sensitivity
Vascular reactivity
Brainstem

ABSTRACT

Patients with Parkinson's disease (PD) exhibit both motor and non-motor symptoms. Among the non-motor symptoms, cardiovascular autonomic dysfunction is frequently observed. Here, we evaluated baroreflex function, vascular reactivity and neuroanatomical changes in brainstem regions involved in the neural control of circulation in the 6-hydroxydopamine (6-OHDA) model of PD. Male Wistar rats received a bilateral injection of 6-OHDA or vehicle into the striatum. After 61 days, baroreflex function and vascular reactivity were assessed. The 6-OHDA and vehicle groups showed similar increases in mean arterial pressure (MAP) in response to phenylephrine (PE). However, the bradycardia observed in the vehicle group was blunted in the 6-OHDA-treated rats. Injection of sodium nitroprusside (SNP) decreased hypotension, tachycardia and vascular relaxation in 6-OHDA-treated rats. Bilateral intrastriatal 6-OHDA led to massive degeneration of tyrosine hydroxylase (TH)-immunoreactive neurons in the substantia nigra and to reductions in the numbers of A1/C1 and A5 catecholaminergic neurons while sparing A2 neurons within the nucleus of the solitary tract (NTS). 6-OHDA-treated rats also showed decreases in Phox2b-expressing neurons in the NTS and in choline acetyltransferase (ChAT) immunoreactivity in the nucleus ambiguus. Altogether, our data suggest that this model of PD includes neuroanatomical and functional changes that lead to cardiovascular impairment.

1. Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative disorders. Clinically, it is well known by its motor symptoms such as bradykinesia, rigidity, tremor and postural instability. However, while the motor symptoms of PD are considered pathological hallmarks of the disease (Fearnley and Lees, 1991), several debilitating symptoms that substantially impair patients' quality of life are related to the non-motor aspects of PD (Wolters, 2009). Some common non-motor symptoms of PD include sleep disturbances, neuropsychiatric and cognitive deficits, sensory dysfunction, and breathing instability, as well as cardiovascular autonomic dysfunction (Bassetti, 2011; Chaudhuri et al., 2011; Dickson et al., 2009; Truong et al., 2008; Tuppy et al., 2015). There is no doubt that the motor symptoms of PD are associated with the loss of a specific group of dopaminergic neurons located in the substantia nigra (SN) and that this underlies the physiopathology of the disease; however, the specific populations of neurons responsible for various non-motor symptoms remain unclear. Orthostatic hypotension (OH), the most common cardiovascular dysfunction in PD, results from the impairment of baroreflex function and cardiac sympathetic innervations (Cai et al., 2005; Tipre and Goldstein, 2005). Baroreflex dysfunction can also be associated with neurodegeneration in important regions of the brainstem. Previous reports show that the brainstems of patients with PD show considerable loss of an important adrenergic region involved in the neural control of circulation, i.e., the C1 region (Gai et al., 1993; Guyenet et al., 2013). However, according to other studies, patients with PD and OH showed marked individual variations in the numbers of catecholaminergic neurons in the C1 region, obscuring the correlations among OH, PD and catecholaminergic neurons (Benarroch et al., 2000). Despite the controversial observations in patients with PD as described above, no previous studies have reported cardiovascular autonomic dysfunc-

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http://dx.doi.org/10.1016/j.brainres.2016.12.008
0006-8993/

tion in a rat model of PD and its relationship with neurodegeneration in specific areas of the brainstem that are responsible for the neural control of blood pressure (Kuo et al., 2010; Lu et al., 1995; Takatsu et al., 2000). Here, we selected a widely used rat model of PD that is generated by injection of 6-hydroxydopamine (6-OHDA) into the striatum. The neuroanatomical and functional assessment of cardiovascular involvement in the 6-OHDA model of PD may represent an important step for future clarification of the mechanisms underlying the appearance of cardiovascular autonomic dysfunction in PD.

Therefore, it is important to use the 6-OHDA model of PD to evaluate cardiovascular dysfunction, vascular reactivity and neuroanatomical changes in the brainstem regions involved in the neural control of circulation.

2. Results

2.1. Animal model of Parkinson's disease: bilateral intrastriatal injection of 6-OHDA destroyed tyrosine hydroxylase-expressing neurons of the substantia nigra

The 6-OHDA neurotoxic lesion within the nigrostriatal dopaminergic system is one of the most widely used methods to model PD in rodents (McDowell and Chesselet, 2012). In our study, 6-OHDA (24 μg/μL) was injected into the dorsal striatum of rats. The rostral-caudal extent of the lesion was determined by counting the neurons in the SN showing tyrosine hydroxylase immunoreactivity (TH-ir) in every sixth 40-μm brain section from each rat (from 5.32 to 6.04 mm caudal to bregma). Compared to vehicle-injected control rats, 6-OHDA dramatically reduced the number of neurons showing TH-ir in the SN (125 ± 10, vs. vehicle: 907 ± 11 neurons, $p < 0.001$) (Fig. 1A, B and I).

2.2. Intrastriatal injection of 6-OHDA selectively reduces the number of brainstem neurons involved in cardiovascular function

To further assess the effects of bilateral intrastriatal 6-OHDA on neurons involved in blood pressure control, at 61 days after the injections, TH-ir, choline acetyltransferase immunoreactivity (ChAT-ir) and Phox2b immunoreactivity (Phox2b-ir) were examined in the regions A1/C1, A2/C2 and A5, in the dorsal motor nucleus of the vagus nerve (DMV) and in the nucleus ambiguus (NA), as well as in the intermediate and commissural regions of the nucleus of the solitary tract (NTSint and NTSc, respectively). In all regions analyzed, except for the A2/C2 and DMV, significant reductions in immunoreactive neurons were observed. For example, the numbers of neurons with TH-ir that were counted in the A5 and A1/C1 regions were reduced by 60% and 70%, respectively, after 6-OHDA (24 μg) was injected into the striatum (A5: 43 ± 1, vs. vehicle: 107 ± 5 neurons and A1/C1: 123 ± 14, vs. vehicle: 404 ± 2 neurons, $p < 0.001$) (Fig. 1C–F and I). The numbers of cells showing ChAT-ir in the NA were decreased by 30% (191 ± 20, vs. vehicle: 269 ± 13 neurons, $p < 0.05$) (Fig. 2C–E). Nuclei in the NTSint and NTSc with Phox2b-ir were reduced by 48% and 73%, respectively (Fig. 3A–E). Interestingly, the area of the NTS with reduced Phox2b-ir was the site of the first synapse of viscerosensory afferents in the brainstem, including those related to cardiorespiratory afferents (Ergene et al., 1994; Kang et al., 2007; Paton et al., 2001; Takakura et al., 2006). The Phox2b-expressing neurons in the NTS are presumably glutamatergic; they innervate the ventrolateral medulla and are collectively responsible for both the cardiovagal and the sympathetic components of the baroreflex (Chan and Sawchenko, 1994; Kang et al., 2007; Weston et al., 2003) (Fig. 3A–E). We also analyzed TH-ir in A2/C2 and ChAT-ir in the DMV; compared to control rats, intrastriatal 6-OHDA did not change the numbers of neurons showing TH-ir in A2/C2 or showing ChAT-ir in the DMV (Figs. 1G–I, 2A, B and E).

2.3. Intrastriatal injection of 6-OHDA impaired cardiac baroreflex

Analysis of the relationship between mean arterial pressure (MAP) and heart rate (HR) revealed a change in the sensitivity of the cardiac baroreflex in the 6-OHDA model of PD. The baroreflex (elicited by intravenous (IV) injections of either phenylephrine (PE) or sodium nitroprusside (SNP)) was tested 61 days after intrastriatal injection of 6-OHDA (24 μg/μL).

Baseline MAP and HR did not differ between 6-OHDA-lesioned and control rats (113 ± 1.25 vs. vehicle: 110 ± 0.7 mmHg and 328 ± 2.17 vs. vehicle: 333 ± 1.54 bpm, $p > 0.05$). In control animals, injections of PE (0.1–12.8 μg/kg) produced dose-dependent increases and decreases in MAP and HR, respectively, and injections of SNP (6.4 and 51.2 μg/kg) produced dose-dependent decreases and increases in MAP and HR, respectively. For example, PE increased MAP (0.2 μg/kg: Δ = 5.5 ± 1; 1.6 μg/kg: 27.5 ± 1.5; 25.6 μg/kg: 58.8 ± 0.9 mmHg, $p < 0.001$) (Fig. 4A and C) and decreased HR (0.2 μg/kg: Δ = −10.8 ± 0.6; 1.6 μg/kg: −34.5 ± 0.7; 25.6 μg/kg: −148.5 ± 9.9 bpm, $p < 0.001$) (Fig. 4A and D). In contrast, SNP decreased MAP (0.2 μg/kg: Δ = −4.5 ± 0.9; 1.6 μg/kg: −21.8 ± 1; 51.2 μg/kg: −43 ± 4 mmHg, $p < 0.001$) (Fig. 4B and E) and increased HR (0.2 μg/kg: Δ = 14 ± 8; 1.6 μg/kg: 41 ± 14; 51.2 μg/kg: 121.3 ± 15.2 bpm, $p < 0.001$) (Fig. 4B and F). In the 6-OHDA group, dose-dependent effects were observed only for the effects of PE on the MAP. Specifically, PE increased MAP (0.2 μg/kg: Δ = 12 ± 2.7; 6.4 μg/kg: 59.3 ± 8.5; 25.6 μg/kg: 75.5 ± 2.9 mmHg, $p < 0.001$) (Fig. 4A and C). These same doses were not able to decrease HR in a dose-dependent manner (0.2 μg/kg: Δ = −11.5 ± 4.9; 6.4 μg/kg: −45.3 ± 5.1; 25.6 μg/kg: −62 ± 6.1 bpm, $p > 0.05$) (Fig. 4A and D).

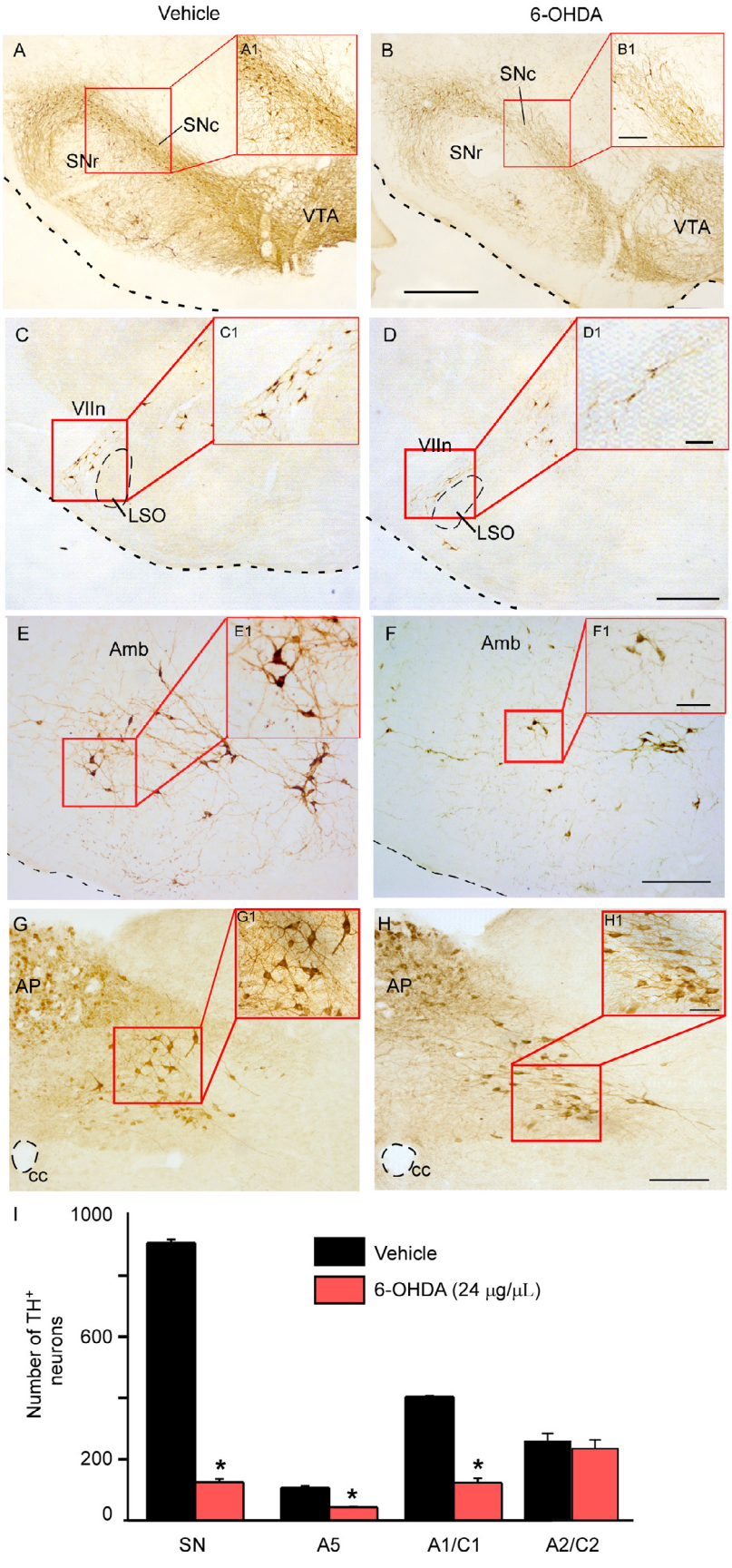
We observed similar increases in MAP in the 6-OHDA and vehicle groups (Δ = 7 ± 2.6 to 66.5 ± 6.1, vs. vehicle: 7.3 ± 0.5 to 60.3 ± 1.6 mmHg, $p > 0.05$; Fig. 4A and C). The highest dose of PE (25.6 μg/kg) produced a higher increase in MAP in 6-OHDA compared to the vehicle group (Δ = 75.5 ± 2.9, vs. vehicle: 58.8 ± 0.9 mmHg, $p < 0.001$). However, bradycardia was blunted in 6-OHDA-lesioned rats with the three highest doses of PE (Δ = −45.5 ± 5 to −62 ± 6.0, vs. vehicle: −119.7 ± 10 to −148.5 ± 9.8 bpm, $p < 0.001$; Fig. 4A and D).

In 6-OHDA-lesioned rats, IV injection of SNP (6.4 and 51.2 μg/kg) produced a smaller decrease in MAP (Δ = −18.5 ± 3 and −24.3 ± 0.8 vs. vehicle: −30.5 ± 1.9 to −43.5 ± 3.9 mmHg, $p < 0.01$; Fig. 4B and E), and a smaller increase in HR was observed in response to doses of 1.6–51.2 μg/kg (Δ = 15.8 ± 5 to 49.5 ± 13.6 vs. vehicle: 41 ± 14 to 121.3 ± 15.2 bpm, $p < 0.05$; Fig. 4B and F).

Fig. 5A-B show the baroreflex sensitivity in response to PE and SNP injections based on analysis of the slope of the curves. Reflex bradycardia in response to the PE-induced increase in MAP was reduced in the 6-OHDA group (−0.794 ± 0.159, vs. vehicle: −2.053 ± 0.307 bpm/mmHg, $p < 0.05$), whereas the reflex tachycardia in response to the SNP-induced fall in MAP was similar between the 6-OHDA and control groups (−2.37 ± 0.099, vs. vehicle: −3.034 ± 0.655 bpm/mmHg, $p > 0.05$) (Fig. 5A-B).

2.4. Intrastriatal injection of 6-OHDA does not change the vasoconstrictor effect of PE in denuded mesenteric arteries of rats

PE (10 nmol/L to 0.1 mmol/L) induced a concentration-dependent constrictor effect in resistance mesenteric arteries in both the control and 6-OHDA-injected groups (Fig. 6A). The efficacy of PE was similar between denuded (Fig. 6A) mesenteric arteries (E−) of the 6-OHDA and control groups (Maximum effect (ME): 21.63 ± 3.97 vs. vehicle: 20.30 ± 3.18%, N = 5 artery rings/5 ani-


Vehicle
6-OHDA
A
A1
SNc
SNr
VTA
B
B1
SNc
SNr
VTA
C
C1
VIIn
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D
D1
VIIn
LSO
E
E1
Amb
F
F1
Amb
G
G1
AP
cc
H
H1
AP
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I
1000
600
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SN
A5
A1/C1
A2/C2

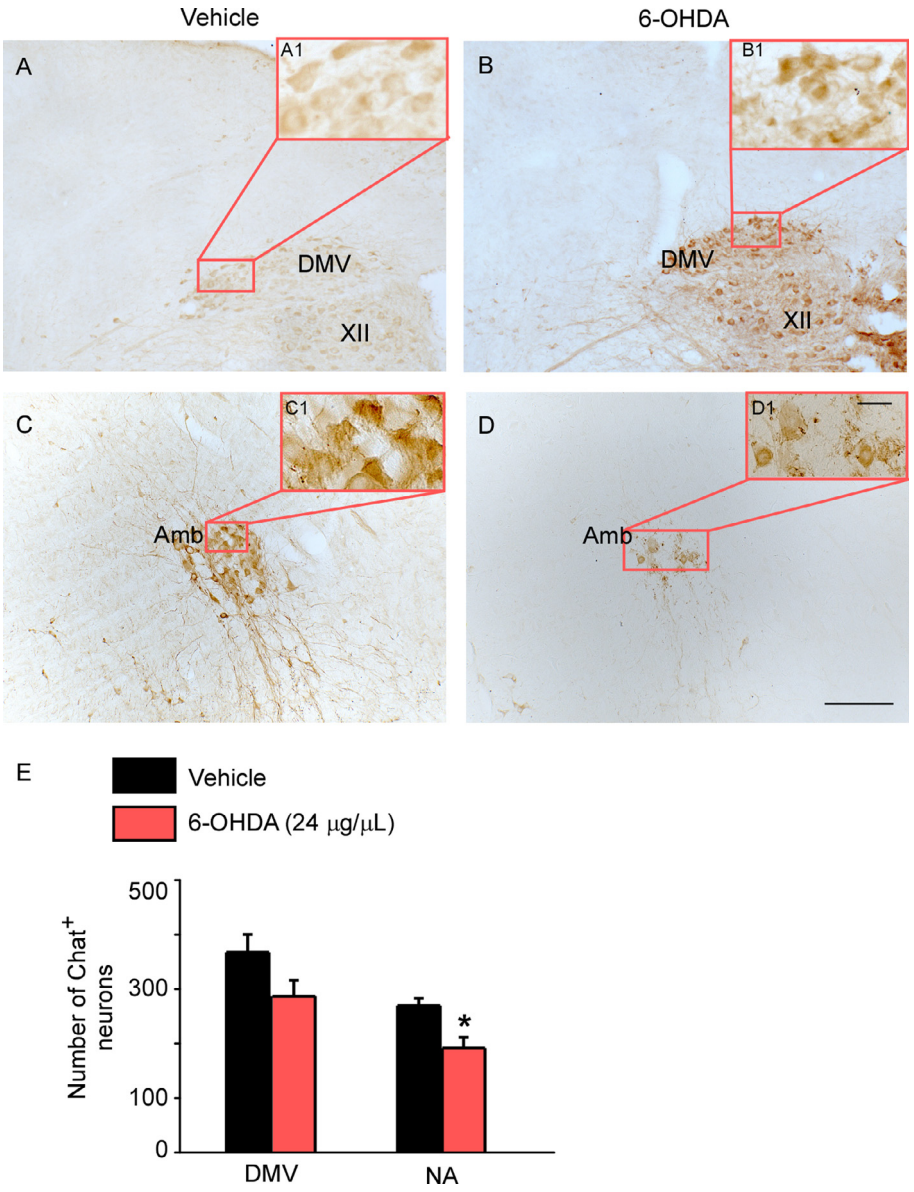

Fig. 2. Bilateral intrastriatal injections of 6-OHDA reduce ChAT-ir neurons within the nucleus ambiguus. Photomicrographs from a control animal that received bilateral intrastriatal injections of vehicle (left column, panels A, C) and from an animal that received 24 μg of 6-OHDA (right column, panels B, D). Compared to vehicle, 6-OHDA reduced the numbers of ChAT-immunoreactive neurons at the level of the NA (C, D) but not at DMV (A, B). E) Group data. Each column of NA represents the total number of neurons of a given type in six sequential 40-μm-thick coronal sections, separated by 240 μm. Each column of DMV represents the total number of neurons of a given type in three sequential 40-μm-thick coronal sections, each separated by 240 μm. Scale bar in D = 200 μm for panels A-D and D1 = 50 μm for panels A1-D1. Abbreviations: XII: hypoglossal motor nucleus, DMV: dorsal motor vagus nucleus, Amb: nucleus ambiguus. $^{*}p < 0.05$ relative to vehicle. N = 5 per group.

mals/group; $p > 0.05$). The potency of PE in denuded mesenteric arteries was similar between the 6-OHDA and control groups (pD2: 5.69 ± 0.21 vs. vehicle: 5.75 ± 0.13, N = 5 artery rings/5 animals/group, $p > 0.05$; Fig. 6A).

2.5. Intrastriatal injection of 6-OHDA impaired the relaxation to SNP and acetylcholine (ACh) in resistance mesenteric arteries

SNP (0.1 nmol/L to 0.1 mmol/L) induced a concentration-dependent dilator effect in denuded resistance mesenteric rings

Fig. 1. Bilateral intrastriatal injections of 6-OHDA destroy TH-ir neurons within the substantia nigra (SN) and catecholaminergic neurons in the ventrolateral pons (A5) and ventrolateral medulla (A1/C1). Photomicrographs from a control animal that received bilateral intrastriatal injections of vehicle (left column, panels A, C, E, G) and from an animal that received 24 μg of 6-OHDA (right column, panels B, D, F, H). Compared to vehicle, 6-OHDA caused an almost complete loss of TH-ir at the level of the SN (A, B), A5 (C, D) and A1/C1 (E, F), but there were no changes at the level of the A2/C2 (G, H). I) Group data. Each column of the SN represents the total number of neurons of a given type in five sequential 40-μm-thick coronal sections, each separated by 240 μm. Each column of A5 represents the total number of neurons of a given type in three sequential 40-μm-thick coronal sections, separated by 240 μm. Each column of A1/C1 represents the total number of neurons of a given type in eight sequential 40-μm-thick coronal sections, each separated by 240 μm. Each column of A2/C2 represents the total number of neurons of a given type in five sequential 40 μm-thick coronal sections, separated by 240 μm. Scale bar in B = 500 μm for panels A-B; B1 = 100 μm for panels A1-B1; D, F and H = 200 μm for panels C-D, E-F and G-H, respectively; D1 and F1 = 50 μm for panels C1-D1 and E1-F1, respectively; H1 = 25 μm for panels G1-H1. Abbreviations: SNr: substantia nigra pars reticulata, SNc: substantia nigra pars compacta, VTA: ventral tegmental area, VIIn: facial nerve, LSO: lateral superior olive, Amb: nucleus ambiguus, AP: area postrema, cc: central canal. $^{*}p < 0.05$ relative to vehicle. N = 11 per group.

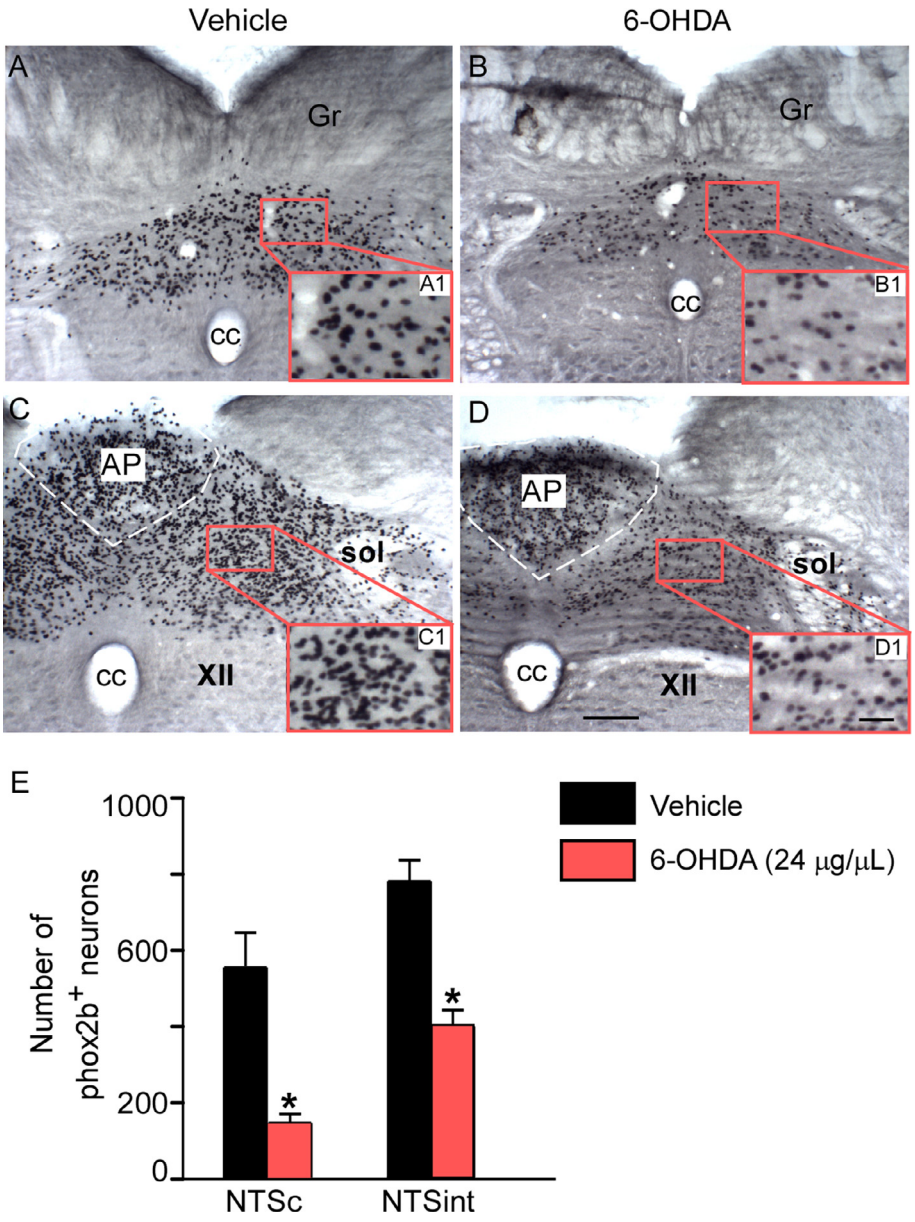

Fig. 3. Bilateral intrastriatal injections of 6-OHDA reduce Phox2b-ir neurons within commissural and intermediate NTS. Photomicrographs from a control animal that received bilateral intrastriatal injections of vehicle (left column, panels A, C) or 24 μg of 6-OHDA (right column, panels B, D). Compared to vehicle, 6-OHDA reduced Phox2b-immunoreactive neurons at the level of the commissural NTS (A, B) and intermediate NTS (C, D). E) Group data. Each column of NTS commissural represents the total number of neurons of a given type in two sequential 40-μm-thick coronal sections, separated by 240 μm. Each column of NTS intermediate represents the total number of neurons of a given type in four sequential 40-μm-thick coronal sections, separated by 240 μm. Scale bar in D = 200 μm for panels A-D and D1 = 50 μm for panels A1-D1. Abbreviations: cc: central canal, Gr: nucleus gracilis, AP: area postrema, XII: hypoglossal motor nucleus, sol: solitary tract. *p < 0.05 relative to vehicle. N = 6 per group.

in both groups (Fig. 6B). The efficacy of SNP on the E-mesenteric arteries was similar between the 6-OHDA and control groups (ME: 89.04 ± 3.26 vs. vehicle: 94.41 ± 1.81%, p > 0.05) (Fig. 6B). In denuded arteries, the potency of SNP was decreased in 6-OHDA rats compared to the control group (pD2: 6.13 ± 0.10, vs. vehicle: 7.22 ± 0.11, p < 0.001; Fig. 6B).

The maximum effect of ACh (0.1 nmol/L to 0.1 mmol/L) as seen in the concentration-effect curves (Fig. 6C) was similar in arteries of the two groups (ME: 95.42 ± 0.94 vs. vehicle: 93.93 ± 0.91%, p > 0.05). ACh potency in intact mesenteric resistance arteries (E +) was lower in 6-OHDA rats than in vehicle-injected rats (pD2: 5.82 ± 0.10 vs. vehicle: 7.44 ± 0.09, p < 0.001; Fig. 6C).

3. Discussion

The current study reveals cardiovascular autonomic deficits after degeneration of dopaminergic neurons in the SN. We suggest that the 6-OHDA model of PD produces a dysfunction in baroreflex sensitivity and considerable changes in neuronal cytoarchitecture in the brainstem regions involved in the regulation of circulation.

Approximately 30–40% of PD patients have orthostatic hypotension (OH) (Goldstein, 2003; Velseboer et al., 2011), a key manifestation of cardiovascular dysautonomia. One of the pathophysiological mechanisms underlying the cardiovascular autonomic abnormalities of PD is arterial baroreflex failure due to

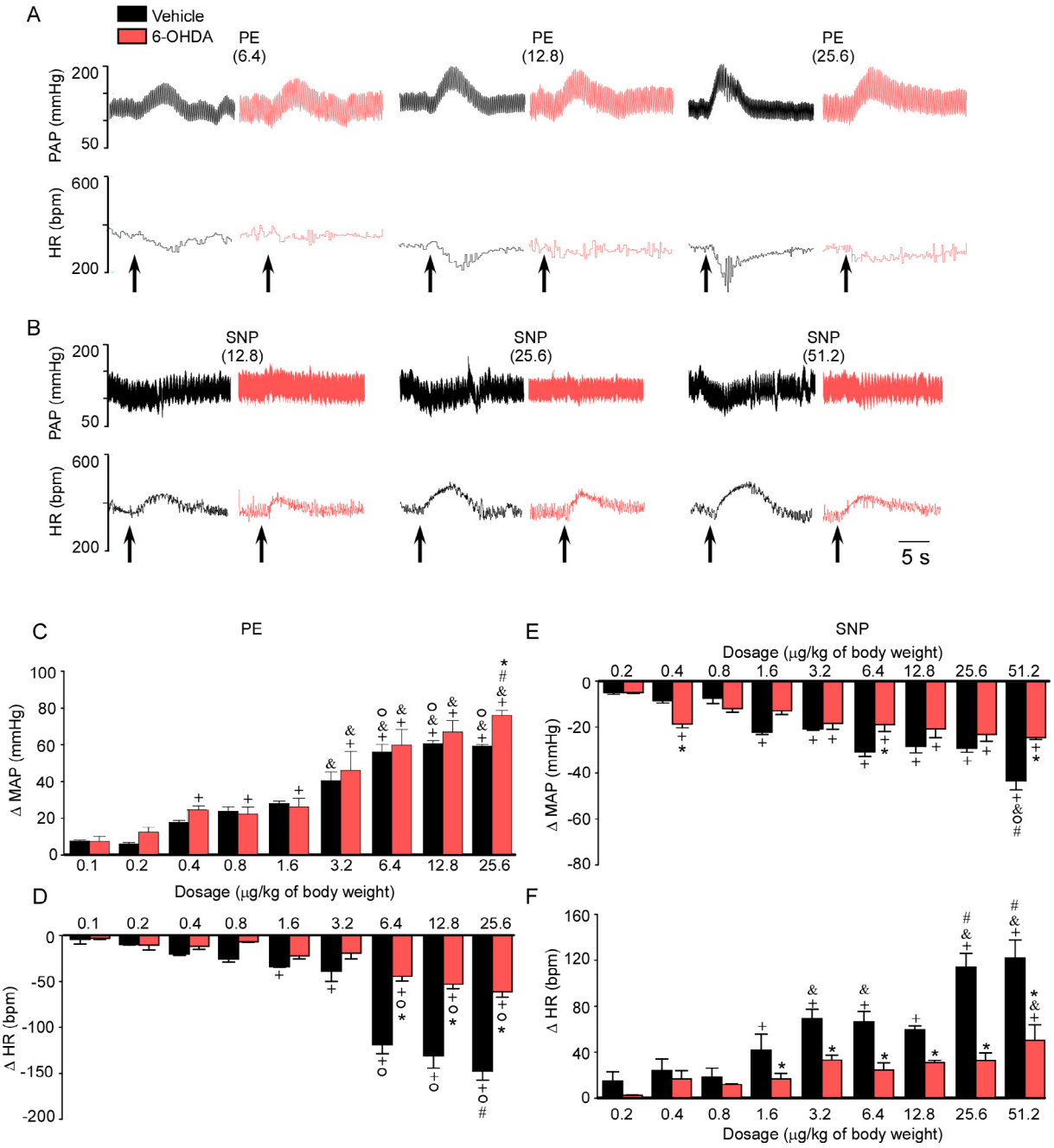

Fig. 4. 6-OHDA affected the cardiac baroreflex. Typical recordings showing changes in pulsatile arterial pressure (PAP) and heart rate (HR) produced by IV injection of the three highest doses tested of A) phenylephrine (PE: 6.4, 12.8 and 25.6 μg/kg of body weight) and B) sodium nitroprusside (SNP: 12.8, 25.6 and 51.2 μg/kg of body weight) in vehicle and 6-OHDA-lesioned rats. Arrows indicate the moment of the injection. Changes in mean arterial pressure (Δ MAP) and HR (Δ HR) produced by C-D) PE (0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 μg/kg of body weight, IV) and E-F) SNP (0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, and 51.2 μg/kg of body weight, IV) at 61 days after bilateral intrastriatal 6-OHDA (24 μg) or vehicle. Significant differences between doses in the same group: + $p < 0.05$ relative to 0.1 μg/kg (PE) and 0.2 μg/kg (SNP); & $p < 0.05$ relative to 1.6 μg/kg (PE and SNP); o $p < 0.05$ relative to 3.2 μg/kg (PE and SNP); # $p < 0.05$ relative to 6.4 μg/kg (PE and SNP). Significant differences between groups in the same dose: *$p < 0.05$ relative to vehicle into the striatum (group effects). N = 6/group.

decreased function of both the parasympathetic and sympathetic components. Conflicting reports propose the possibility that medullary neuronal loss may underlie these autonomic dysfunctions in patients with PD (Benarroch et al., 2000; Gai et al., 1993; Saper et al., 1991). However, those studies analyzed only the catecholaminergic neurons of the C1 region.

Our study is the first to show that functional deficits in this model can be associated with specific neuroanatomical changes in key regions involved in neural control of circulation, i.e., A1/C1, NTS, NA and A5.

4. Cardiovascular deficits and Parkinsonism

Our results show that the bilateral intrastriatal 6-OHDA model of PD has a significant reduction in the number of neurons in regions involved in baroreflex control, such as the NTS, NA and the catecholaminergic neurons within the A1/C1 and A5 regions. For several reasons, the decreases in the catecholaminergic neurons of the A1/C1 and A5 regions are probably not due to catecholaminergic or non-catecholaminergic neurons in different brain areas coming into contact with 6-OHDA by the diffusion of

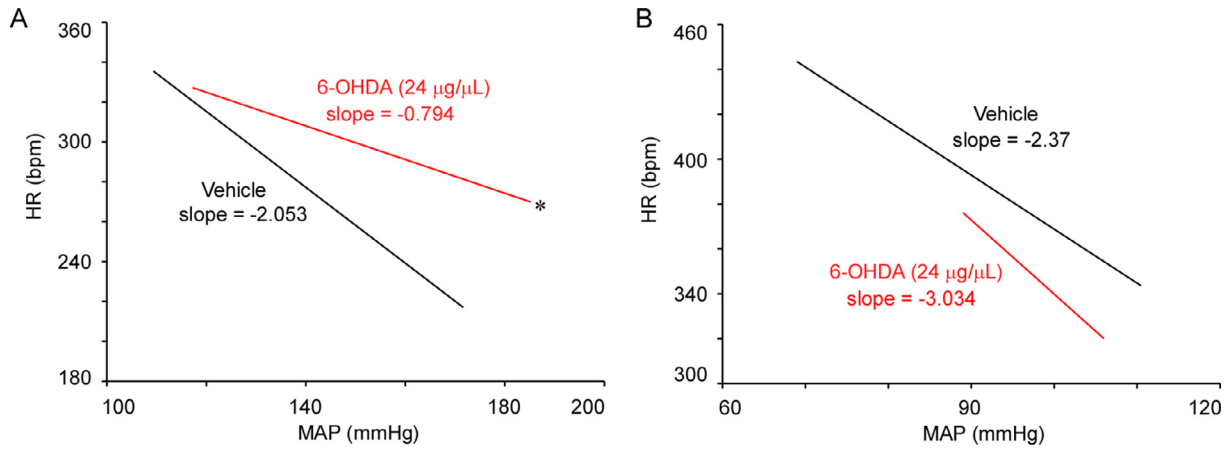

Fig. 5. Baroreceptor reflex sensitivity for heart rate. Linear regression graphs showing A) the bradycardic reflex response to increase mean arterial pressure (MAP) produced by IV injection of phenylephrine and B) the tachycardic reflex response to decrease the MAP produced by IV injection of sodium nitroprusside in the control (vehicle) and 6-OHDA-lesioned rats. $^{*}p < 0.05$ relative to vehicle into the striatum (Student's *t*-test).

the toxin through the ventricular system. i) Phenylethanolamine-N-methyltransferase neurons are resistant to 6-OHDA (Fety et al., 1984); ii) using the same concentration and methodology as in the present study, a previous study from our laboratory showed that there is no reduction in the number of neurons showing TH-ir in the A6 region (Tuppy et al., 2015); iii) in a previous study, we did not observe a reduction in the density of neurokinin-1 receptors in the Bötzinger complex and the caudal ventral respiratory group (an important cluster of neurons involved in breathing activity) (Tuppy et al., 2015); iv) here, we did not observe a change in the number of neurons showing ChAT-ir in the DMV or TH-ir in the A2/C2.

As expected, we observed that phenylephrine elicited a dose-dependent pressor response, and the magnitude of the increase in blood pressure was similar between the two groups (control and 6-OHDA) except with the highest dose used, suggesting that there was no change in the vascular function mediated by adrenergic receptors. The bradycardic reflex to baroreceptor activation was milder in the 6-OHDA group, even with the highest dose, which produced a higher increase in blood pressure. This suggests a bradycardic impairment that is potentially associated with the reduction in parasympathetic activity (Goldstein, 2003). The reduction in parasympathetic activity observed in the 6-OHDA group may be associated with the observed reduction in the numbers of neurons showing Phox2b-ir in the NTS and ChAT-ir in the NA. The first central relay of the baroreflex consists of glutamatergic neurons located in the NTS, which receives monosynaptic inputs from baroreceptor afferents and projects to parasympathetic nuclei and to inhibitory neurons of the caudal ventrolateral medulla (CVLM) that in turn modulate the activity of presympathetic neurons in the C1 region (Guyenet, 2006). Additionally, previous studies have reported that neurons showing Phox2b-ir in the NTS are exclusively glutamatergic (Kang et al., 2007), that in Phox2b-null mutants, parasympathetic ganglia never form (Pattyn et al., 1999), and that neurons with Phox2b-ir in the NTS are involved in the baroreflex (Kang et al., 2007). Previous experiments have shown that 70% of NTS neurons activated by phenylephrine also contain Phox2b-ir. However, those experiments were not able to demonstrate whether Phox2b-expressing neurons of the NTS project to parasympathetic nuclei or to CVLM inhibitory neurons (Kang et al., 2007).

Considering that small-diameter blood vessels are associated with vascular resistance and the maintenance of blood pressure, we calculated the concentration-effect curve to phenylephrine in denuded rings of the second and third branches of mesenteric arteries, and we observed that the potency and maximum effect of phenylephrine were similar between groups, suggesting that 6-OHDA treatment did not change the function of alpha-adrenergic receptors in the vascular smooth muscle.

SNP is a potent vasodilator of arteries and veins. Its metabolization by red blood cells results in the release of nitric oxide (NO) (Al-Sa'doni and Ferro, 2000; Bogusz et al., 1979; Robin and McCauley, 1992). Here, we showed that the hypotension, tachycardia and vascular reactivity in response to SNP were reduced in the 6-OHDA group. However, no changes in the baroreflex sensitivity were observed. Similarly, a previous study demonstrated that the integrity of C1 cells is not essential for the maintenance of resting blood pressure but is crucial for the tachycardia response evoked by baroreflex deactivation (Schreihofer and Guyenet, 2000).

We also observed a reduction in the number of catecholaminergic neurons in the A5 region. Located in the ventrolateral pons, the A5 region has spinal projections exclusively targeting the intermediolateral cell group (Loewy et al., 1979), and these projections are proposed to regulate sympathetic nerve activity and blood pressure (Koshiya and Guyenet, 1994). Evidence from the literature suggests that A5 neurons are under baroreceptor control (Huangfu et al., 1991). Additionally, A5 neurons receive inputs from C1 neurons, suggesting that the C1 region may represent a potential source of excitatory drive to the A5 region. In our model, both regions contain a reduced number of neurons, which may explain an impairment of the baroreflex control.

We cannot exclude the contribution of the inhibition of sympathetic and parasympathetic tone to the changes in heart rate during baroreflex activation and deactivation, respectively. However, our results show that in the 6-OHDA group, although there is also a reduction in C1 neurons, this reduction seems to be insufficient to produce bradycardia during activation of the baroreflex. Similarly, evaluating the deactivation of the baroreflex, we observed a reduction in the number of NA neurons that could produce a tachycardic response. In the former case, we must also consider that SNP in 6-OHDA animals was not able to produce a hypotensive response similar to that in control animals; that lack of response might also be a reason for the observed reduction in the tachycardic response.

A previous study reported an impairment in autonomic modulation in the same model used in the present study (Ariza et al., 2015). However, properly interpreting these results requires the consideration of several important issues. Here, we performed

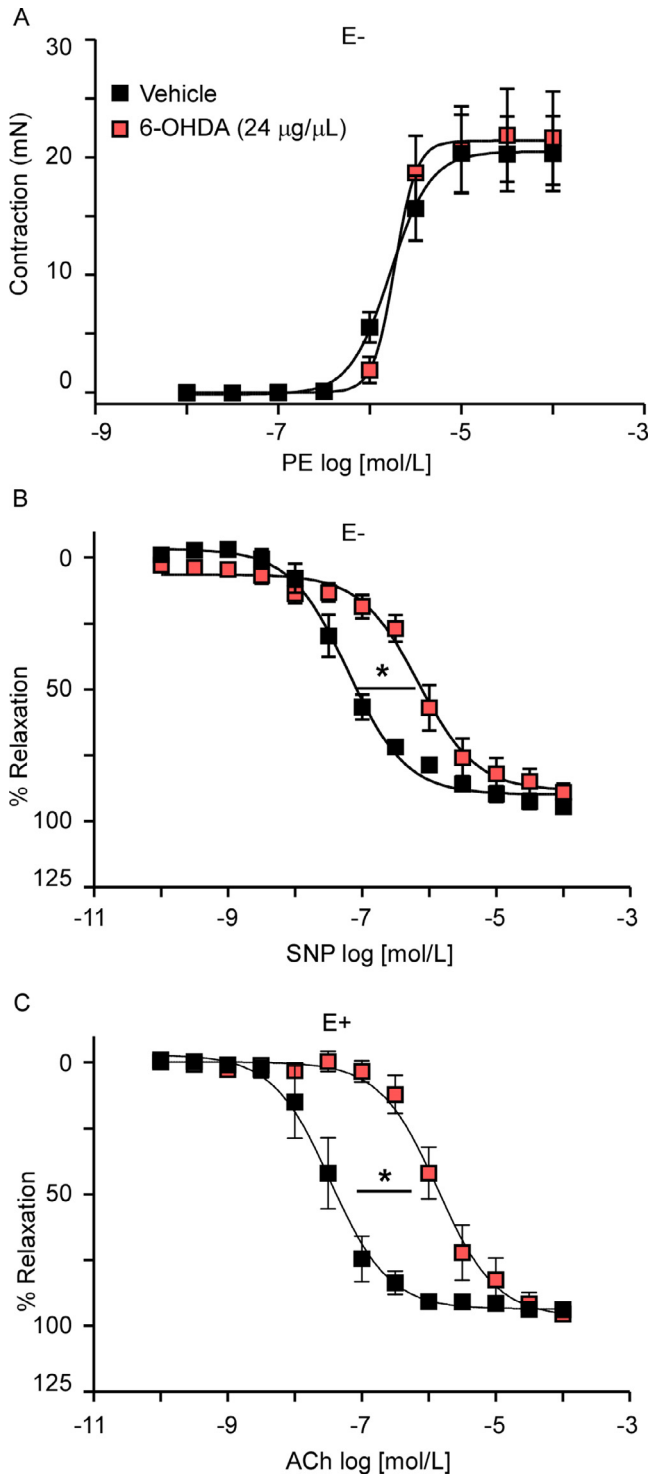

Fig. 6. 6-OHDA impaired the reactivity of the resistance arteries to PE, ACh and SNP. Concentration-response curves to A) phenylephrine (PE, 10 nM – 100 μM) in denuded (E−), B) to sodium nitroprusside (SNP, 0.1 nM – 100 μM) in E− and C) to acetylcholine (ACh, 0.1 nM – 100 μM) in intact (E+) mesenteric resistance arteries of rats injected with 6-OHDA (24 μg) or vehicle. Data are reported as the mean ± SEM. *$p < 0.05$ relative to vehicle into striatum. N = 5 artery rings for each treatment from each of 5 animals per group.

experiments 61 days after 6-OHDA injection into the striatum, while the experiments in the previous study were performed only 7 days after the lesion. An ideal model of PD should exhibit pathological and clinical features that mimic the human disease, including motor and non-motor symptoms, as well as the progressive degenerative nature of PD. The former study used the model in which 6-OHDA was injected directly into the SN and performed the functional analysis only 7 days post-injection, which is insufficient time to create a reliable lesion of the nigrostriatal pathway, a key characteristic of PD (Jeon et al., 1995). We used a progressive degeneration of dopaminergic neurons of the striatum, i.e., the experiments were performed 61 days after the lesion. In our model, we did not observe classic motor symptoms, but we observed a reduction in the baseline breathing (Tuppy et al., 2015) and a cardiovascular imbalance (present results). Additionally, we did not observe changes in baseline blood pressure or heart rate, while the previous study reported considerable reductions in these cardiovascular parameters. This effect may be attributable the fact that the dopaminergic terminals in the striatum are still releasing dopamine at 7 days post-lesion. Dopamine can have an inhibitory effect on the cardiovascular system, leading to a depression of cardiovascular parameters (Hassan et al., 2015; Niewinski et al., 2014; Oliva et al., 2010). Importantly, the present results are the first to address a correlation between functional and specific neuroanatomical changes that can contribute to the dysautonomia observed in this model of PD.

The former study suggested that in the 6-OHDA animal model of PD, there is lower sympathetic modulation of the vasculature and no changes in the parasympathetic activity. Our results are in agreement only because we observed a massive reduction in the number of presympathetic neurons in the A1/C1 region. However, the bradycardic response to baroreceptor activation is mediated by the parasympathetic system, and our data showed significant reductions in the number of NA neurons and in the bradycardic response to phenylephrine. These differences may explain some of the discrepancies observed between the current work and the previous study (Ariza et al., 2015).

5. Conclusion

Our results showed that the bilateral intrastriatal 6-OHDA model of PD led to a massive degeneration of neurons with TH-ir in the SN, and this was associated with significant decreases in Phox2b-ir in the NTS, ChAT-ir in the NA and TH-expressing neurons in the A1/C1 and A5 regions. We also observed that other brainstem regions involved in neural control of blood pressure, such as ChAT-ir in the DMV and TH-ir in A2/C2, were not affected in this model. Our conclusion is that those specific neuroanatomical changes may be associated with a deficit in the bradycardic response to baroreflex activation, a non-motor symptom of PD. Moreover, our data suggest that the 6-OHDA PD model may also blunt the response of mesenteric artery resistance. Those results may contribute to future studies trying to elucidate the mechanisms involved in cardiovascular dysfunction in this model.

6. Methods and materials

6.1. Animals

Experiments were performed in 22 adult male Wistar rats (250–350 g), and the entire protocol lasted two months. Animals were used in accordance with the guidelines of the Animal Experimentation Ethics Committee of the Institute of Biomedical Sciences at the University of São Paulo (ICB/USP) and the NIH.

6.2. 6-OHDA injection

The bilateral injection of 6-OHDA hydrochloride (6-OHDA hydrochloride, H4381, Sigma, Saint Louis, MO, USA) into the striatum was performed acutely as previously described (Tuppy et al.,

2015). Briefly, the animals were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (7 mg/kg) intraperitoneally (IP) and placed in a stereotaxic frame (model 900; David Kopf Instruments) to receive two injections of 6-OHDA (24 μg/μL in 0.3% ascorbic acid saline solution) or vehicle (0.3% ascorbic acid) in the dorsal striatum using a 10 μL Hamilton syringe and the following coordinates: 1) 0.0 mm caudal to bregma, ±2.7 mm lateral to the midline, 4.5 mm ventral to the dorsal surface of the brain and 2) 0.5 mm caudal to bregma, ±3.2 mm lateral to midline, 4.5 mm ventral to the dorsal surface of the brain. The volume of the injection was 0.5 μL/site. After the 6-OHDA injection, the animals were allowed to survive for 60–61 days until vascular cannulation or vascular reactivity experiments.

6.3. In vivo experiments

6.3.1. Baroreflex analysis

Pulsatile arterial pressure (PAP), mean arterial pressure (MAP) and heart rate (HR) were recorded in unanesthetized, freely moving rats as previously described (Favero et al., 2011; Takakura et al., 2014; Barna et al., 2016). Briefly, one day before the experiments, under IP injection of ketamine combined with xylazine anesthesia, polyethylene tubing (PE-10 connected to PE-50; Clay Adams, Parsippany, NJ, USA) was inserted into the femoral vein and abdominal aorta through the femoral artery. The cannulas were tunneled subcutaneously to the animal's back to allow for measurement while they were freely moving. Water and food intake and motor activity were unchanged 24 h after cannulation. Although motor activity was not quantified, visual observation in their home cages and during handling revealed no apparent differences in reactivity or locomotion before and after cannulation.

The arterial catheter was connected to a pressure transducer (MLT844, ADInstruments, Sydney, NSW, Australia) coupled to a preamplifier (Bridge Amp, ML221, ADInstruments, Sydney, NSW, Australia) that was connected to a Powerlab computer data acquisition system (PowerLab 16/30, ML880, ADInstruments). A variable period of time (30–50 min) was allowed for the stabilization of cardiovascular parameters before beginning simultaneous measurement of PAP and HR. For baroreceptor stimulation, 100-μL bolus injections of graded doses of phenylephrine (PE: 0.1; 0.2; 0.4; 0.8; 1.6; 3.2; 6.4; 12.8 and 25.6 μg/kg) and sodium nitroprusside (SNP: 0.2; 0.4; 0.8; 1.6; 3.2; 6.4; 12.8; 25.6 and 51.2 μg/kg) were administered into the femoral vein. PE and SNP injections were made in a random order, and subsequent injections were not made until the recorded parameters had returned to baseline levels. Values of matching MAP variations with reflex HR response were separately plotted for each vasoactive drug to create linear regression curves of baroreceptor function for each group, and their slopes (beats/min/mmHg) were compared to test changes in the baroreceptor reflex sensitivity.

6.4. In vitro experiments

6.4.1. Vessel preparation

The animals were anesthetized with halothane (Tanohalo®, Cristália, Itapira, Brazil), euthanized by decapitation and exsanguinated. The mesenteric bed was removed and placed in a Petri plate containing cold Krebs-Henseleit buffer (4 °C) with the following composition (mmol/L): NaCl 130.00; $NaHCO_3$ 14.9; $C_6H_{12}O_6$ 5.5; KCl 4.7; KH_2PO_4 1.18; $MgSO_4$ 1.17; $CaCl_2$ 1.6; pH 7.4. The mesenteric artery was cleaned of connective tissue under a dissection stereomicroscope (Luxeo 2S, Labomed, Los Angeles, USA).

6.4.2. Vascular reactivity

The 2nd or 3rd segments from the mesenteric artery were cut in rings 2-mm in length and mounted in a small vessel dual-chamber myograph for isometric tension measurement. Two tungsten wires (40-μm diameter) were introduced through the lumen of the rings and mounted according to the method described by Mulvany and Halpern (Halpern and Mulvany, 1977). In some rings, the endothelium was removed by gently rubbing the intimal surface with a human hair. After a 30-min equilibration period in oxygenated Krebs solution at 37 °C and pH 7.4, rings were stretched to their optimal lumen diameter for active tension development. The diameter was determined based on the internal circumference-wall tension ratio of the rings by setting their internal circumference (L0) to 90% of the circumference that the vessels would have if they were exposed to a passive tension that was equivalent to that produced by a transmural pressure of 100 mmHg (Halpern and Mulvany, 1977). The effective lumen diameter was calculated as $L0/\pi$. Next, rings were washed three times with Krebs-Henseleit buffer and left to equilibrate for 30 min. To test the viability of the preparations, rings were stimulated with a high-concentration solution of potassium (120 mmol/L, 15 min). Functionality of endothelial cells was verified by relaxation to acetylcholine (ACh: 10 μmol/L), and efficient removal of endothelial cells was verified by the lack of relaxation to ACh (10 μmol/L) in rings pre-contracted with PE (10 μmol/L). Rings with more than 70% relaxation to acetylcholine were classified as rings with intact endothelium, and rings with less than 10% relaxation to acetylcholine were used in the experiments without endothelium. Endothelium releases vasodilator and vasoconstrictor factors. Then, to analyze the maximum response to PE or SNP, we used mesenteric artery rings without endothelium, since endothelium can modulate the responses to PE or SNP. Cumulative concentration-response curves to ACh (0.1 nmol/L to 0.1 mmol/L) were performed in intact resistance mesenteric arteries; cumulative concentration-response curves to SNP (0.1 nmol/L to 0.1 mmol/L) and to PE (10 nmol/L to 0.1 mmol/L) were performed in denuded resistance mesenteric arteries.

6.4.3. Histology

Rats were anesthetized (pentobarbital overdose, 60 mg/kg, IP) and perfused transcardially, first with heparinized saline (300 mL) and then with 4% phosphate-buffered paraformaldehyde (PFA, 500 mL). Brains were removed and postfixed in PFA for 1–2 days at 4 °C. Coronal sections (40 μm) were cut using a vibrating microtome (Vibratome 1000 s) and stored in cryoprotectant solution at −20 °C for up to two weeks until histological processing (Schreihofer and Guyenet, 1997).

All histological procedures were conducted with free-floating sections. No labeling was observed when the primary antibodies were omitted. Sections were rinsed, blocked and subjected to the immunoperoxidase method with specific antibodies. Tyrosine hydroxylase (TH) is the rate-limiting enzyme responsible for the synthesis of dopamine, noradrenaline and adrenaline. Therefore, it was selected for labeling neurons of the SN (dopaminergic neurons), C1 and C2 (adrenergic neurons), and A1, A2 and A5 (noradrenergic neurons). Choline acetyltransferase (ChAT) is a transferase enzyme responsible for the synthesis of the neurotransmitter acetylcholine and was therefore used as a marker for preganglionic parasympathetic neurons in the NA and DMV. The paired-like homeobox gene Phox2b is expressed by central excitatory relays of the sympathetic baroreflex (NTS) and was used in this study as a marker for that nucleus (Stornetta et al., 2006; Barna et al., 2012; Tuppy et al., 2015). For TH and ChAT, color development was carried out using imidazole-diaminobenzidine (DAB), and for Phox2b, the staining used was Nickel-Cobalt-DAB. All of the antibodies used in the present study were experimentally characterized as previously demonstrated (Barna et al., 2012; Pattyn et al., 1997; Stornetta et al., 2006).

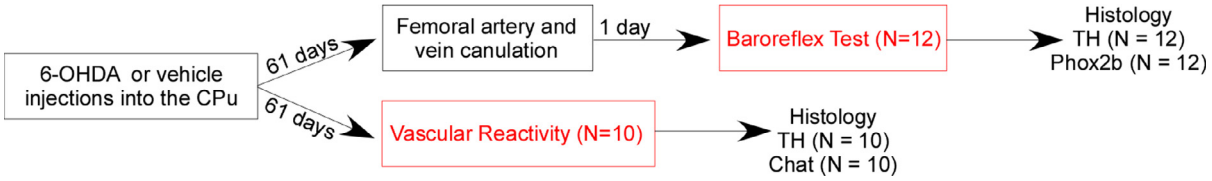

Fig. 7. Experimental design. Indicates the fraction of rats in which each of the anatomical and physiological experiments was performed.

6.4.4. Cell mapping, cell counting and imaging

A conventional multifunction microscope (brightfield, darkfield and epifluorescence; Zeiss Axioskop 2 (Oberkochen, Germany)) was used for all observations unless otherwise indicated. ImageJ software (public domain program available from the NIH; http//rsb.info.nih.gov/ij/) was used to count the various types of neuronal profiles within a defined area.

For each rat, every sixth 40-μm brain section was used, which means that the sections analyzed were 240 μm apart. The sections were counted bilaterally, and the numbers reported in the results section correspond exactly to the counts of every sixth section in the indicated series. Section alignment between brains was performed relative to a reference section, as previously described (Tuppy et al., 2015). Briefly, to align sections around the substantia nigra (SN) level, the most caudal section containing the medial geniculate nucleus was identified in each brain and assigned the level 6.04 mm caudal to Bregma (Bregma = −6.04 mm). To align sections around the nucleus of the solitary tract (NTS) level, the section containing the mid-area postrema was identified in each brain and assigned the level 13.8 mm caudal to Bregma (Bregma = −13.8 mm). Levels rostral or caudal to the reference section were determined by adding a distance corresponding to the interval between sections multiplied by the number of intervening sections. The same method was also used to identify the Bregma levels of the C1, A5, nucleus ambiguus (NA) and dorsal motor nucleus of vagus (DMV) areas. Analyses were performed using 6 sections of the NTS, 5 sections of the SN, 8 sections of the A1/C1, 3 sections of the A5, 5 sections of the A2/C2, 6 sections of the NA and 3 sections of the DMV.

All files were exported to the Canvas 9 drawing program for final modifications. Photographs were taken with a 12-bit color CCD camera (CoolSnap, Roper Scientific, Tucson, AZ; resolution 1392 × 1042 pixels).

The neuroanatomical nomenclature is in accordance with that described in Paxinos and Watson (1998).

6.5. Statistical analysis

Data were analyzed using unpaired Student's *t*-tests or two-way repeated measures ANOVA followed by Tukey or Newman-Keuls post hoc tests. All results are presented as the means ± SEM. Differences were considered significant when $p < 0.05$. The EC50 (concentration of the agent that produced half-maximal relaxation amplitude) was determined after logarithmic transformation of the normalized concentration-response curves and is reported as the negative logarithm (pD2). The maximum effect (ME) was considered the maximal amplitude of response reached in the concentration-response curves for PE, SNP or ACh.

6.6. Experimental protocol

Two injections of 0.5 μL/site of 6-OHDA (24 μg/μL) or saline + 0.3% ascorbic acid (N = 5 animals/group for *in vitro* experiments and N = 6 animals/group for *in vivo* experiments) were performed bilaterally in the striatum (caudate-putamen) to induce a retrograde neuronal nigrostriatal injury (Blum et al., 2001). For *in vivo* experiments, sixty days after 6-OHDA, the femoral artery and vein were cannulated to evaluate the baroreflex, and the experiments were performed the following day. For *in vitro* experiments, sixty-one days after 6-OHDA, the animals were anesthetized to remove segments from the mesenteric artery for experiments. Three artery rings per animal were used to perform 3 different experiments (PE, SNP and ACh). At the end of the *in vivo* experiments, all animals were sacrificed and perfused. The brains of all animals were removed for histological analysis. The animal and experimental protocols used are shown in schematic form in Fig. 7.

Grants

This research was supported by public funding from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (grants: 14/22406-1 to ACT; 12/20398-6 to CA) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (grants: 471263/2013-3 to ACT; 471283/2012-6 to TSM) and by funds from a FAPESP fellowship (2011/21841-8 to MT; 2015/11268-0 to BF) and a CNPq fellowship (305533/2012-6 to TSM and 301651/2013-2 to ACT).

Author contributions

TSM, CA and ACT designed the experiments; BF, MT, SRP and ACT collected and analyzed data; BF, MT, TSM, CA and ACT wrote the paper. All authors approved the final version of the manuscript.

Conflict of interest statement

We declare no conflict of interest.

Acknowledgments

We gratefully acknowledge J.F. Brunet (Departement de Biologie, EcoleNormaleSuperieure, Paris, France) for providing the Phox2b antibody, J.C. Callera (Department of Basic Sciences, School of Dentistry of Araçatuba, Araçatuba, Brazil) for the stereotaxic apparatus and Fabiane C. Fernandes for expert technical assistance. We would like to also acknowledge Dr. Lisete C. Michelini for helping with the baroreflex analysis.

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