

Dopaminergic degeneration is enhanced by chronic brain hypoperfusion and inhibited by angiotensin receptor blockage

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Abstract The possible interaction between brain hypoperfusion related to aging and/or vascular disease, vascular parkinsonism and Parkinson's disease, as well as the possible contribution of aging-related chronic brain hypoperfusion in the development or severity of Parkinson's disease are largely unknown. We used a rat model of chronic cerebral hypoperfusion to study the long-term effects of hypoperfusion on dopaminergic neurons and the possible synergistic effects between chronic hypoperfusion and factors that are deleterious to dopaminergic neurons, such as the dopaminergic neurotoxin 6-hydroxydopamine. Chronic hypoperfusion induced significant loss of dopaminergic neurons and striatal dopaminergic terminals and a reduction in striatal dopamine levels. Furthermore,

intrastratial administration of 6-hydroxydopamine in rats subjected to chronic hypoperfusion induced a significantly greater loss of dopaminergic neurons than in sham-operated control rats. The dopaminergic neuron loss was significantly reduced by oral treatment with angiotensin type 1 receptor antagonist candesartan (3 mg/kg/day). The levels of angiotensin type 2 receptors were lower and the levels of angiotensin type 1 receptors, interleukin-1 β and nicotinamide adenine dinucleotide phosphate oxidase activity were higher in the substantia nigra of rats subjected to chronic hypoperfusion than in control rats; this was significantly reduced by treatment with candesartan. The results suggest that early treatment of vascular disease should be considered in the treatment of aged Parkinson's disease patients and Parkinson's disease patients with cerebrovascular risk factors. The findings also suggest that inhibition of brain renin–angiotensin activity may be useful as a neuroprotective strategy.

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Introduction

Vascular parkinsonism (VP) is an aging-related heterogeneous clinical entity caused by cerebrovascular disease, which is generally considered distinct from

idiopathic Parkinson's disease (PD). However, the literature is somewhat conflicting and this simple dichotomy may need revision. It remains largely unknown whether there is an interaction between vascular pathology and PD and whether aging-related vascular pathology contributes to PD development or severity. An increase in the severity of PD may be merely the result of an additive effect of two independent but convergent diseases; PD and cerebrovascular disease increase in prevalence with age, and therefore may occur concomitantly and overlap in their symptomatology (Bohnen and Albin 2011; Jellinger 2006; Papapetropoulos et al. 2004). However, both diseases may also be interdependent; aging-related chronic cerebral hypoperfusion may induce dopaminergic cell death or act synergistically with other factors to induce or exacerbate dopaminergic degeneration. It is known that aging and cerebrovascular disease are accompanied by a reduced cerebral blood flow (Farkas and Luiten 2001), and we have recently shown an age-dependent decrease in density of nigral microvessels (Villar-Cheda et al. 2009). As dopaminergic neurons are highly sensitive to metabolic stress and more vulnerable to damage than those in other brain regions (Mercuri et al. 1994; Singh et al. 2007), modifications of the vascular microenvironment may affect the vulnerability of the dopaminergic neurons, and contribute to development or exacerbation of PD. Consistent with this, recent studies have observed dopaminergic cell loss and parkinsonian signs in elders without PD (Buchman et al. 2012), and reduction in presynaptic dopaminergic function in majority of patients with VP (Zijlmans et al. 2007). However, the contributions of cerebrovascular disease and chronic hypoperfusion to dopaminergic neuron degeneration have not been demonstrated experimentally. Furthermore, identification of potential factors and mechanisms linking hypoperfusion and neurodegeneration may lead to neuroprotective strategies.

The brain renin–angiotensin system (RAS) may play a major role in linking vascular disease and dopaminergic degeneration. Local AII, via angiotensin receptors type 1 (AT1) receptors, is one of the most important known inducers of inflammation and oxidative stress, and the link between arterial wall RAS and the pathophysiology of the vascular disease is well known (Griendling et al. 2000; Münzel and Keany 2001; Ruiz-Ortega et al. 2001). Furthermore, numerous studies in different tissues have shown that local RAS is involved in age-related degenerative changes

and longevity (Basso et al. 2005; Benigni et al. 2009, 2012). Finally, in recent studies, we have shown that brain AII, via AT1 receptors, exacerbates dopaminergic cell death and may play a synergistic role in the pathogenesis and progression of PD (Joglar et al. 2009; Rodriguez-Pallares et al. 2008), and that aging decreases nigral microvascularization (Villar-Cheda et al. 2009) and enhances nigral RAS activity (Rodriguez-Perez et al. 2012; Villar-Cheda et al. 2012). In the present study, we used a well-known rat model of chronic cerebral hypoperfusion (CHP; Farkas et al. 2007) to study the long-term effects of hypoperfusion on dopaminergic neurons and the possible synergistic effects between hypoperfusion and factors that are deleterious to dopaminergic neurons (low doses of the dopaminergic neurotoxin 6-hydroxydopamine), which may lead to increased dopaminergic cell death. Furthermore, we studied the involvement of the nigral RAS, and considered whether the blockage of AT1 receptors may inhibit the hypoperfusion-derived dopaminergic degeneration.

Methods

Experimental design

Male Sprague–Dawley rats (10 weeks old at the beginning of the experiments; 18 weeks old when the brains were analyzed) were used in the present study. All experiments were carried out in accordance with Directive 2010/63/EU and the Directive 86/609/CEE and approved by the corresponding committee at the University of Santiago de Compostela. The first set of experiments (Table 1) was carried out to investigate the effects of chronic cerebral hypoperfusion on dopaminergic neuron degeneration and the possible role of nigral RAS activation in this effect. CHP was induced by permanent bilateral common carotid artery occlusion under ketamine/medetomidine (0.5/75 mg/kg) anesthesia. In some mammal species, carotid occlusion leads to severe forebrain ischemia. In contrast, the complete circle of Willis in the rat affords incessant (but reduced) blood flow from the vertebral arteries after the onset of occlusion, so that cerebral hypoperfusion rather than stroke occurs (see for details on this animal model Farkas et al. 2007). The rats were divided into three groups. Rats in group A1 were sham-operated controls ($n=24$). Rats in group B1 were

Table 1 Experimental design

Group	Day/treatment			Processed for
A1	0 / Sham	→ 20 / Vehicle	→ 56 / Perfusion; decapitation	Histology HPLC RT-PCR Western Blot
B1	0 / Carotid occlusion	→ 20 / Vehicle	→ 56 / Perfusion; decapitation	
C1	0 / Carotid occlusion	→ 20 / Candesartan	→ 56 / Perfusion; decapitation	
A2	0 / Sham	→ 15 / Vehicle	→ 25 / Sham injection	56 / Perfusion
B2	0 / Carotid occlusion	→ 15 / Vehicle	→ 25 / 6-OHDA injection	
C2	0 / Carotid occlusion	→ 15 / Candesartan	→ 25 / 6-OHDA injection	

subjected to carotid artery occlusion ($n=24$). Rats in group C1 ($n=24$) were subjected to carotid artery occlusion and oral treatment with the AT1 receptor antagonist candesartan (Astra-Zeneca; 3 mg/kg/day) from 20 days postsurgery until they were killed (56 days postsurgery). The powdered drug was administered orally to the rats mixed with peanut butter; animals in control groups were given only peanut butter. Some rats from different groups ($n=5$ per group) were perfused and processed for immunohistochemistry (see below). The remaining rats were killed by decapitation and the brains were rapidly removed. The area of the substantia nigra in the ventral mesencephalon and the striatum were carefully dissected on an ice-cold plate, frozen on dry ice, and stored at -80°C until analysis. The samples were processed for high-performance liquid chromatography (HPLC), real-time reverse transcription polymerase chain reaction (RT-PCR), western blot or chemiluminescence to determine the levels of dopamine and metabolites, expression of AT1 and angiotensin receptors type 2 (AT2) receptors, levels of the pro-inflammatory cytokine interleukin-1 β (IL-1 β), expression of the nicotinamide adenine dinucleotide phosphate (NADPH) cytosolic subunit p47 (as an indicator of the level of activation of the NADPH complex) and levels of NADPH oxidase activity. In a second series of experiments, rats in the different groups (control rats, group A2; CHP rats, group B2; rats with CHP and treated with candesartan, group C2) were injected with low doses of the dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA) or vehicle (CHP+vehicle, group B1) in the right striatum (25 days after the bilateral common carotid occlusion; $n=6$ per group). Rats in group C2 were treated with candesartan from 10 days before 6-OHDA lesion until they were killed as above (56 days after

carotid artery occlusion or sham surgery) and processed for immunohistochemistry to investigate the possible synergistic effect of chronic cerebral hypoperfusion and other possible deleterious factors acting on dopaminergic neurons.

Chronic cerebral hypoperfusion surgery and intrastriatal injection of 6-OHDA

A midline ventral cervical incision was made to expose both common carotid arteries. The carotid arteries were then carefully isolated from the carotid sheath and vagus nerve, and each isolated artery was ligated with a 5/0 silk suture. The mortality rate was 15–20 %. Careful isolation of the carotid arteries and in particular very careful handling of the vagus nerve by a researcher with experience in surgical procedures is essential to minimize mortality. Animals in the sham-operated group (group A) were subjected to the same surgical procedure, except that the arteries were not ligated. During the surgical procedure, the rectal temperature of the rats was maintained at $37\pm 0.5^{\circ}\text{C}$ by using a temperature-maintained platform. As postsurgical care, each rat was kept in a separate cage until recovery.

Rats in the second series of experiments were injected intrastriatally with 6-OHDA or vehicle (Przedborski et al. 1995; Sauer and Oertel 1994). Thirty minutes prior to intrastriatal injection with 6-OHDA or vehicle, rats were treated with desipramine (Sigma, 25 mg/kg*i.p.*), a selective inhibitor of the norepinephrine transporter, to prevent uptake of 6-OHDA by noradrenergic terminals. The rats were injected in the right striatum with 7 μg of 6-OHDA (in 3 μl of saline containing 0.2 % ascorbic acid; Sigma, USA). Stereotaxic coordinates were 1 mm anterior to bregma, 3.0 mm right of midline, and 5.5 mm ventral to the dura, tooth bar at -3.3 . Control animals

were injected with 3 μ l of sterile ascorbate saline alone. Finally, rats were killed by an overdose of chloral hydrate (400 mg/kg), and then perfused and processed for histology (see below). The location of the 6-OHDA injection site was confirmed in the immunostained striatal sections.

Immunohistochemistry cresyl violet staining and dopaminergic neuron quantification

The animals used for immunohistochemistry were perfused, firstly with 0.9 % saline, and then with cold 4 % paraformaldehyde in 0.1 M phosphate buffer, pH7.4. The brains were removed, washed, and cryoprotected in the same buffer containing 20 % sucrose, and finally cut on a freezing microtome (30 μ m thick). Sections were processed for tyrosine hydroxylase (TH) immunohistochemistry as follows. The sections were incubated for 1 h in 10 % normal swine serum with 0.25 % Triton X-100 in 20 mM potassium phosphate-buffered saline containing 1 % bovine serum albumin (KPBS-BSA) and then incubated overnight at 4 °C with a mouse monoclonal anti-serum to TH (1:10,000; Sigma). The sections were subsequently incubated; first, with the corresponding biotinylated secondary antibody (horse antimouse, 1:200), and then with avidin–biotin–peroxidase complex (ABC, 1:100, Vector). Finally, the labeling was revealed by treatment with 0.04 % hydrogen peroxide and 0.05 % 3,3'-diaminobenzidine (Sigma).

The total number of TH-immunoreactive (TH-ir) neurons in the substantia nigra compacta (SNc) was estimated by an unbiased stereological method (i.e., the optical fractionator). The stereological analysis was carried out with the Olympus CAST-Grid system (Computer-Assisted Stereological Toolbox; Olympus, Denmark). Uniform randomly chosen sections through the substantia nigra (i.e., every fourth section) were analyzed for the total number of TH-ir cells by means of a stereological grid (fractionator), and the nigral volume was estimated according to Cavalieri's method (Gundersen et al. 1988). To confirm that 6-OHDA induces neuron death (i.e., the loss of TH-ir is not due to phenotypic downregulation of TH activity) series of sections through the entire substantia nigra of control rats and rats treated with 6-OHDA were counterstained with cresyl violet, and the total number of neurons in the SNc was estimated by the unbiased stereology method described above for TH-ir cells (see Rey et al. 2007 for details).

The density of striatal dopaminergic terminals was estimated as the optical density of the striatal TH-ir with the aid of NIH-Image 1.55 image analysis software (Wayne Rasband, MIMH) in a personal computer coupled to a video camera (CCD-72, MTI) and a constant illumination light table (Northern Light, St. Catharines, Canada). At least four sections, through the central striatum of each animal, were measured (both the right and left striatum) and for each section, the optical densities were corrected by subtraction of background, as observed in the corpus callosum.

RNA extraction and real-time quantitative RT-PCR

Total RNA from the nigral region was extracted with Trizol (Invitrogen), according to the manufacturer's instructions. Total RNA (2.5 μ g) was reverse-transcribed to cDNA with dNTPs, random primers, and Moloney murine leukemia virus reverse transcriptase (M-MLV; 200U; Invitrogen). Real-time PCR was used to examine the relative levels of IL-1 β , p47, angiotensin receptors type 1 (AT1a) and type 2 (AT2) mRNA. Experiments were performed with a real-time iCyclerTM PCR platform (BioRad). β -Actin was used as a housekeeping gene and was amplified in parallel with the genes of interest. The comparative Ct method was used to examine the relative mRNA expression. The expression of each gene was obtained as relative to the housekeeping transcripts. The data were then normalized to the values of the control group (group A) of the same batch (100 %), to counteract any possible variability among batches. The specificity of the RT-PCR data used to identify RAS components has been confirmed by negative controls, omitting reverse transcriptase (Fig. 1). Finally, the results were expressed as means \pm SEM. Primer sequences were as follows: for IL-1 β forward 5'-ATCTCACAGCAGCATCTC, reverse 5'-TAGCAGGTCGTCATCATC; for AT1a, forward 5'-TTCAACCTCTACGCCAGTGTG-3', reverse 5'-GCCAAGCCAGCCATCAGC-3'; for AT2, forward 5'-AACATCTGCTGAAGACCAATAG-3', reverse 5'-AGAAGGTCAGAACATGGAAGG-3; for p47^{phox}, forward 5'-CCACACCTCTTGAACCTTCTTC-3', reverse 5'-CTCGTAGTCAGCGATGGC-3'; for β -actin, forward 5'-TCGTGCGTGACATTAAGAG-3', reverse 5'-TGCCACAGGATTCCATACC-3'.

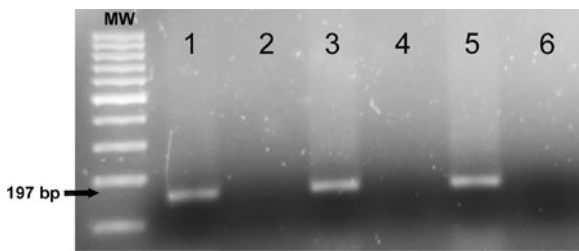


Fig. 1 AT1 receptor gene amplification in 2 % agarose gel stained with Sybr[®]Safe (Invitrogen). 1, 3, and 5 Positive amplification of cDNA from control (sham operated) rats, and rats subjected to CHP and chronic cerebral hypoperfusion+candesartan (CHP+cand), respectively. 2, 4, and 6 The corresponding negative controls without reverse transcription, which show negative amplification

Western blot analysis and NADPH oxidase activity

Tissue was homogenized in RIPA buffer containing protease inhibitor cocktail (P8340, Sigma) and PMSF (P7626, Sigma). Homogenates were centrifuged and protein concentrations were determined with the Bradford protein assay. Equal amounts of protein were separated by 5–10 % Bis-Tris polyacrylamide gel, and transferred to nitrocellulose membranes. The membranes were incubated overnight with primary antibodies (1:200) against AT1 receptor (sc-31181), AT2 receptor (sc-9040), p47-phox (sc-7660), and IL-1 β (sc-1252; all obtained from Santa Cruz Biotechnology). The specificity of the antibodies used to identify RAS components has been established in our previous studies (Rodriguez-Perez et al. 2010; Valenzuela et al. 2010). The horseradish peroxidase (HRP)-conjugated secondary antibodies used were Protein A (NA9120V, GE Healthcare) and Protein G (18–161, Upstate-Millipore). Immunoreactivity was detected with an Immun-Star HRP Chemiluminescent Kit (170–5044, BioRad) and imaged with a chemiluminescence detection system (Molecular Imager ChemiDoc XRS System, BioRad). Blots were stripped and reprobed for anti-GAPDH (G9545, Sigma; 1:25,000) as loading control. In each animal, protein expression was measured by densitometry of the corresponding band and expressed as relative to the GAPDH band value. The data were then normalized to the values of the control group of the same batch (i.e., expressed relative to the value obtained for the control rats; 100 %) to counteract possible variability among batches. Finally, the results were expressed as means \pm SEM.

NADPH oxidase activity in ventral mesencephalic tissue was measured by lucigenin-enhanced chemiluminescence with an Infinite M200 multiwell plate

reader (TECAN), as described by Griendling et al. (2000); Griendling et al. (1994) and Hong et al. (2006). Chemiluminescence was expressed as relative light units (RLU; RLU per minute per milligram protein). The level of activation of the NADPH oxidase complex was confirmed by determination of the p47^{phox} expression (see above). NADPH oxidase complex is composed of membrane-bound subunits and cytosolic subunits such as p47^{phox}, which is considered a key subunit for NADPH activation (Li and Shah 2003). The translocation of cytosolic subunits to the membrane is necessary for NADPH activation, which leads to ROS generation. The level of the NADPH oxidase subunit p47^{phox} is correlated with NADPH activity and NADPH-derived superoxide formation. (Rueckschloss et al. 2002; Touyz et al. 2002).

High-performance liquid chromatography

The striata were dissected on an ice-cold plate and the striatal tissue frozen on dry ice and stored at -80°C until analysis. Striatal tissue was homogenized and then centrifuged at 14,000 \times g for 20 min at 4°C . The supernatant fractions were decanted, filtered (0.22 μm), and injected (20 μl /injection) into the HPLC system (Shimadzu LC prominence). Dopamine and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid were separated with a reverse phase analytical column (Waters Symmetry300 C18; 150×3.9 mm, 5 μm particle size; Waters). The mobile phase (70 mM KH_2PO_4 , 1 mM octanesulfonic acid, 1 mM EDTA, 10 % MeOH, pH4) was delivered at a rate of 1 ml/min. Detection was performed with a coulometric electrochemical detector (ESA Coulochem III). The first and second electrodes of the analytical cell were set at +50 and +350 mV, respectively; the guard cell was set at -100 mV. Data were acquired and processed with Shimadzu LCsolution software. Results were expressed in nanograms per microgram of tissue (wet weight) and presented as means \pm SEM ($n=5$ per group).

Statistical analysis

All data were obtained from at least three independent experiments and were expressed as means \pm SEM. Two-group comparisons were analyzed by a Student's *t* test and multiple comparisons were analyzed by one-way analysis of variance (ANOVA) followed by a post hoc Bonferroni test. The normality of populations and

homogeneity of variances were tested before each ANOVA. Differences were considered significant at $p < 0.05$. Statistical analyses were carried out with SigmaStat 3.0 from Jandel Scientific (San Rafael, CA, USA).

Results

Effect of chronic cerebral hypoperfusion on dopaminergic degeneration and dopamine levels. Neuroprotective effect of the AT1 antagonist candesartan

In control rats (group A1), the dopaminergic neurons in the substantia nigra compacta were intensely immunoreactive to TH, and dense, evenly distributed TH-ir was observed throughout the striatum, indicating the presence of a dense network of nigrostriatal dopaminergic

terminals. A loss of about 33 % of nigral TH-ir neurons was observed in the SNc of rats subjected to chronic cerebral hypoperfusion (group B1) in comparison with the SNc of control rats (group A1; Fig. 2). In control rats (group A1), the number of neurons counted in cresyl violet-stained sections ($22,792 \pm 335$) was slightly higher than the number of TH-ir (Fig. 2a) neurons since some nondopaminergic neurons located in the SNc were also included. However, sections from group B1 rats showed a significant reduction in the number of cresyl violet-stained neurons in the SNc ($16,087 \pm 988$), which confirms that chronic cerebral hypoperfusion induces cell death and not just phenotypic downregulation in TH activity. Rats subjected to chronic cerebral hypoperfusion and treated with the AT1 antagonist candesartan (group C1) showed a significant loss of dopaminergic neurons (about 18 % decrease). However, the loss was

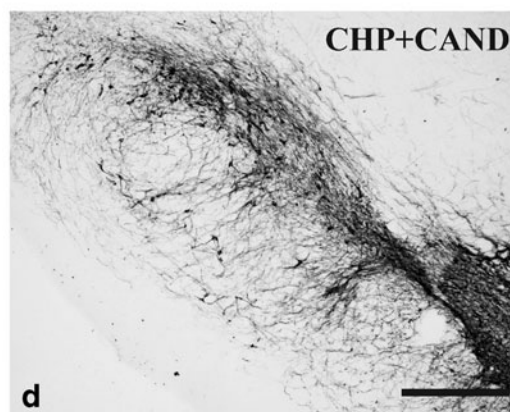
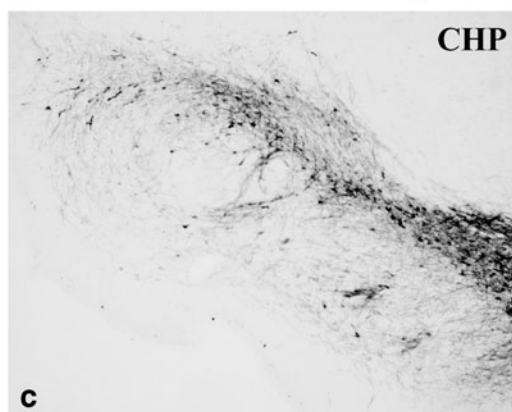
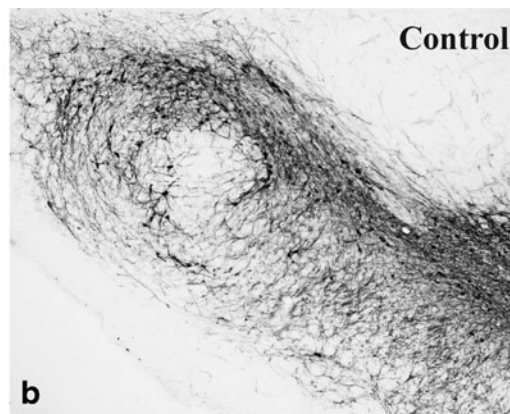
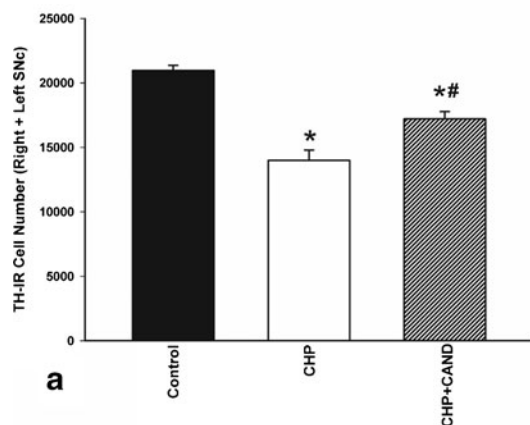


Fig. 2 Dopaminergic (TH-ir) neurons (**a–d**) in the SNc of controls (sham-operated rats), rats subjected to CHP or chronic cerebral hypoperfusion and treatment with candesartan (CHP+cand). Representative photomicrographs of the SNc of different groups of rats are shown in **b–d**. The dopaminergic neurons

were quantified as the total number of TH-ir neurons in the right +left SNc (**a**). Data are means \pm SEM. * $p < 0.05$ relative to the control group, # $p < 0.05$ relative to the group subjected to chronic hypoperfusion (one-way ANOVA and Bonferroni post hoc test). Scale bar 500 μ m

significantly lower than in untreated rats. Similarly, the density of striatal TH-ir terminals, relative to control rats, was significantly lower in rats subjected to chronic hypoperfusion than in control rats, as a consequence of the loss of striatal dopaminergic terminals (Fig. 3). However, the reduction in the density of striatal TH-ir terminals was significantly lower in group C1 rats treated with candesartan. No striatal infarcts or ischemic lesions, which could induce significant degeneration of dopaminergic neurons, were observed (Fig. 3c–d).

The functional effects of chronic cerebral hypoperfusion were confirmed by determination of the striatal levels of dopamine and its metabolites by HPLC (Fig. 4). Levels (nanogram per milligram wet weight of tissue) of dopamine and DOPAC in control rats (group A1) were significantly higher than those observed in rats

with chronic cerebral hypoperfusion (group B1), but not significantly different to those observed in rats with chronic cerebral hypoperfusion treated with candesartan (group C1). However, the DOPAC/dopamine ratio values were not significantly different between control rats (0.094 ± 0.021), rats with chronic hypoperfusion (0.089 ± 0.024) and rats subjected to chronic hypoperfusion and treated with candesartan (0.092 ± 0.019).

Effect of chronic cerebral hypoperfusion on angiotensin AT1 and AT2 receptors in the substantia nigra. Effect of treatment with candesartan

Real-time RT-PCR analysis revealed significantly higher expression of AT1 receptor mRNA and lower levels of AT2 mRNA in rats with chronic

Fig. 3 Striatal TH-ir in controls (sham-operated rats) and rats subjected to CHP or chronic cerebral hypoperfusion and treatment with candesartan (CHP+cand). TH-ir (i.e., spared dopaminergic terminals) is higher in the treated group than in the rats that did not receive candesartan (a). Representative photomicrographs of the striatum of different groups of rats are shown in b–d. Density of striatal dopaminergic terminals was estimated as optical density and expressed as a percentage of the value obtained in the control group. Data are means \pm SEM. * $p < 0.05$ relative to the control group, # $p < 0.05$ relative to the group subjected to chronic hypoperfusion (one-way ANOVA and Bonferroni post hoc test). Scale bar 250 μ m

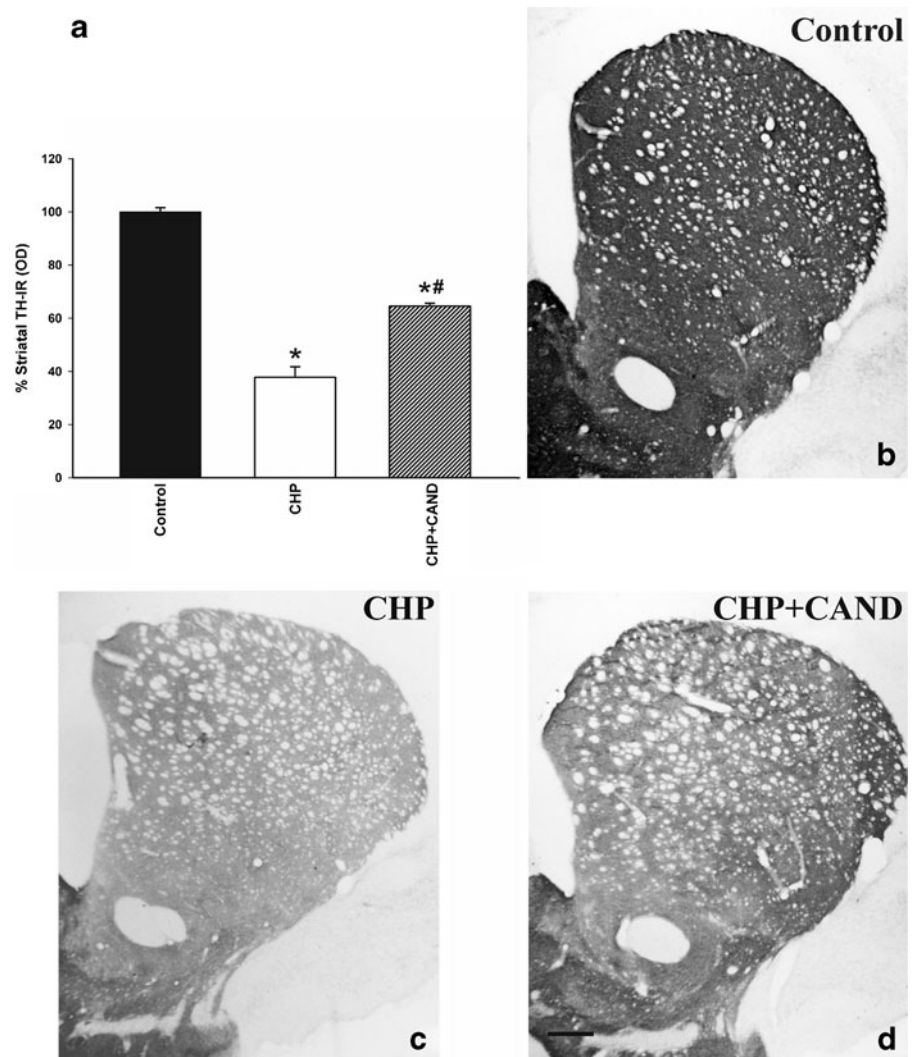
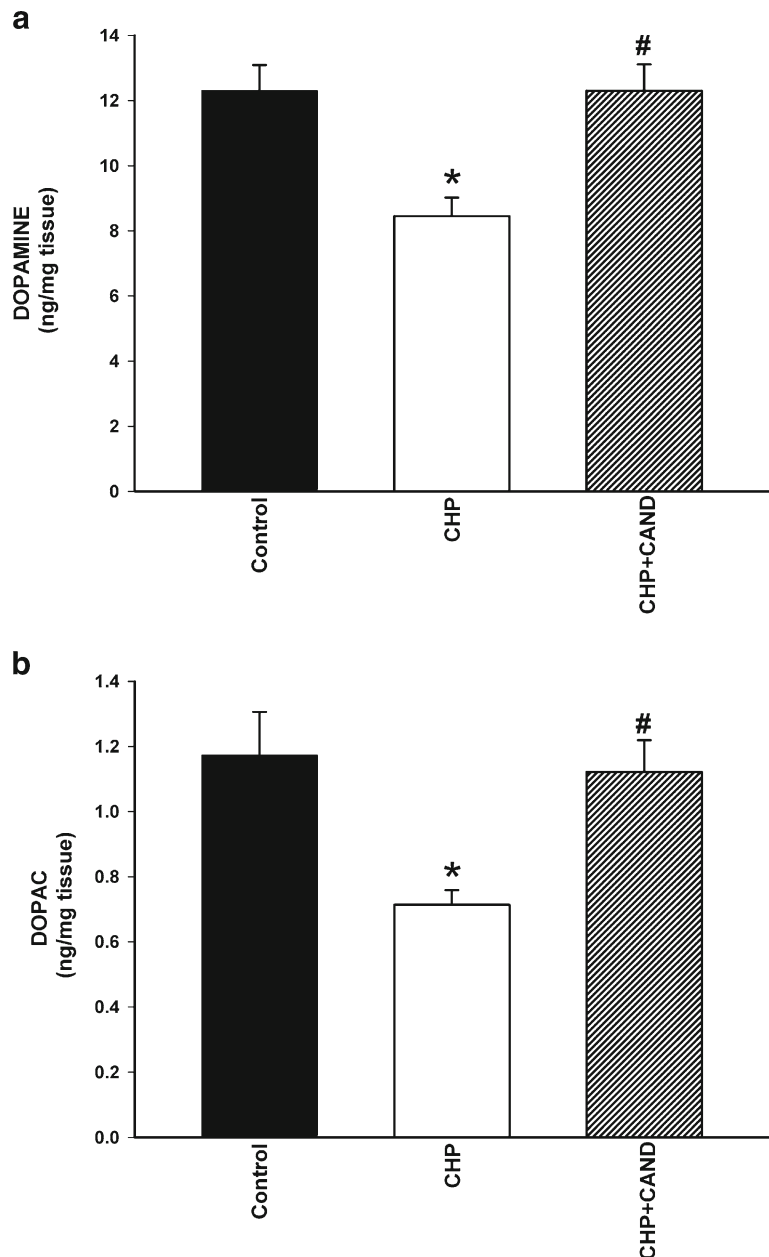


Fig. 4 Striatal levels (nanogram per milligram wet weight of tissue) of dopamine (a) and its metabolite DOPAC (b) in control rats as compared with rats subjected to CHP or chronic cerebral hypoperfusion and treatment with the AT1 blocker candesartan (CHP+cand). Data are means \pm SEM. * p <0.05 relative to the control group, # p <0.05 relative to the group subjected to chronic hypoperfusion (one-way ANOVA and Bonferroni post hoc test)



cerebral hypoperfusion than in control rats (Fig. 5a, c). Similarly, Western blot (WB) studies revealed significantly higher expression of AT1 receptors in rats with chronic cerebral hypoperfusion than in control rats (Fig. 5b), and the expression of AT2 receptors was significantly lower in rats with chronic cerebral hypoperfusion (Fig. 5d). However, treatment of rats with chronic cerebral hypoperfusion with the AT1 receptor blocker candesartan induced a significant increase in the

levels of AT2 receptor mRNA and protein (Fig. 5c, d).

Effect of chronic cerebral hypoperfusion on NADPH complex activation and IL-1 β expression in the substantia nigra. Effect of treatment with candesartan

Rats with chronic cerebral hypoperfusion showed a significantly greater activation of the NADPH

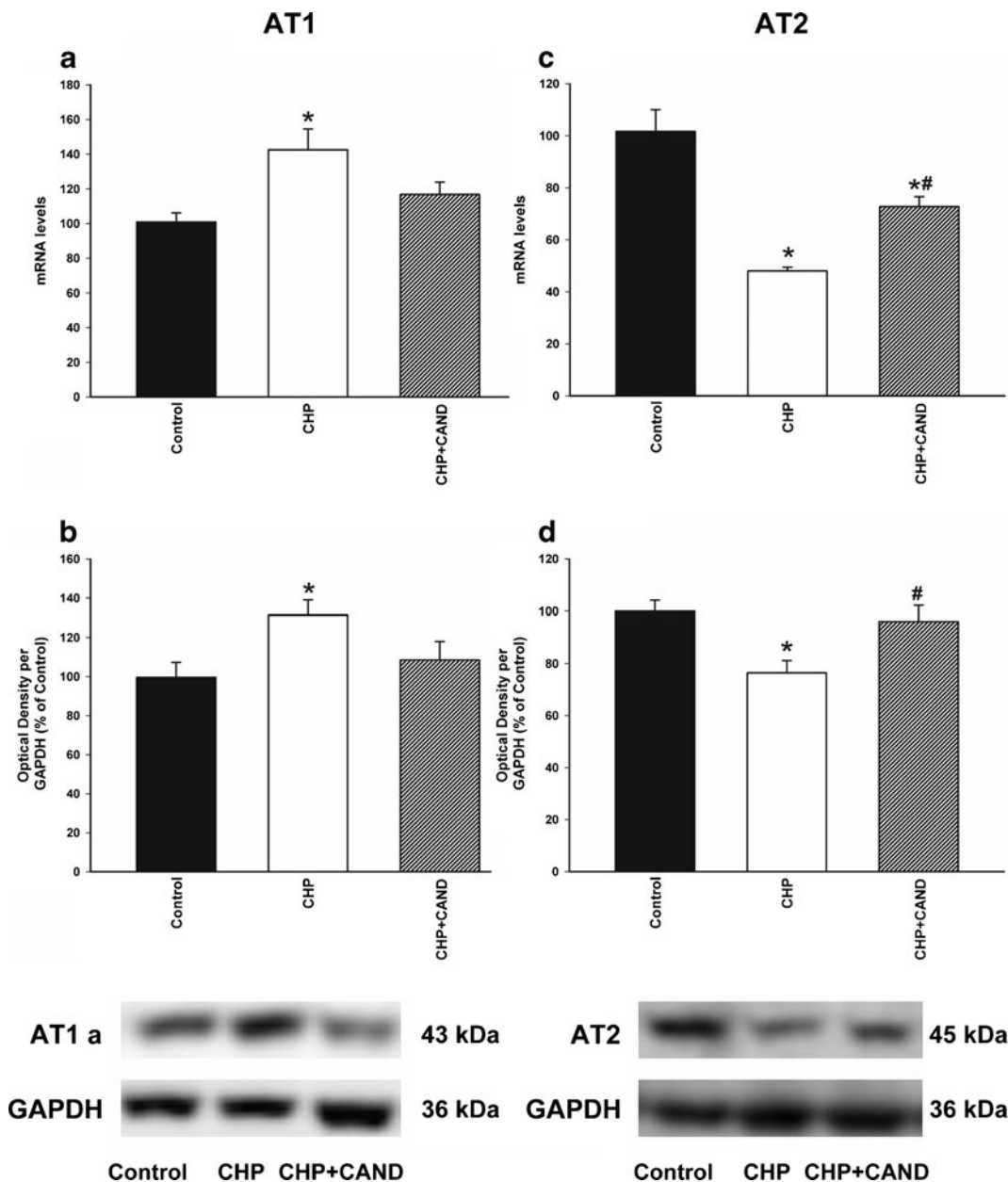


Fig. 5 Real-time quantitative RT-PCR (**a**, **c**) and Western blot (WB; **b**, **d**) analysis of AT1, AT2 receptor expression in control rats as compared with rats subjected to CHP or chronic cerebral hypoperfusion and treatment with candesartan (CHP+cand). Protein expression was obtained relative to the GAPDH band value and the expression of each gene was obtained relative to the housekeeping transcripts

(β -Actin). Note that AT1 receptors are blocked in the CHP+cand group. The results were then normalized to values for control rats (100 %). Data are mean values \pm SEM. * p <0.05 relative to the control group, # p <0.05 relative to the group subjected to chronic hypoperfusion (one-way ANOVA and Bonferroni post hoc test)

complex than control rats, as determined by p47 subunit expression and NADPH oxidase activity. Real-time RT-PCR analysis revealed higher mRNA levels of p47^{phox} in rats with chronic cerebral hypoperfusion than in

control rats (Fig. 6a), and WB also revealed a significant increase in the expression of p47^{phox} in rats with chronic cerebral hypoperfusion (Fig. 6b). Treatment with candesartan induced a significant

decrease in levels of NADPH activity and expression of p47 (Fig. 6).

In addition, levels of the pro-inflammatory cytokine IL-1 β were determined by real-time RT-PCR analysis and WB, and were significantly higher in rats with chronic cerebral hypoperfusion than in control rats. Treatment with candesartan induced a significant decrease in the expression of IL-1 β mRNA and protein (Fig. 7a, b).

Synergistic effect of chronic cerebral hypoperfusion and 6-OHDA on dopaminergic degeneration. Neuroprotective effect of the AT1 antagonist candesartan

In order to investigate the possible synergistic effect of chronic cerebral hypoperfusion and other possible deleterious factors causing dopaminergic neuron degeneration, rats in the different groups (A2, B2, C2) were injected with low doses of the dopaminergic neurotoxin 6-OHDA (Fig. 8). As described above, rats subjected to chronic hypoperfusion (without 6-OHDA injection) showed a significant loss of TH-ir neurons. Intrastratial administration of 6-OHDA in control rats also induced a significant loss of TH-ir neurons in the ipsilateral SNc (group A2). However, intrastratial administration of 6-OHDA in rats subjected to chronic hypoperfusion (group B2) induced a significantly higher loss of TH-ir neurons than in control rats (group B1). The difference in 6-OHDA-induced loss of TH-ir neurons between control rats and rats subjected to chronic hypoperfusion was significantly reduced by treatment of the latter rats with the AT1 receptor antagonist candesartan (group C2; Fig. 8).

Fig. 6 Real-time quantitative RT-PCR (a), western blot (b), and Lucigenin enhanced chemiluminescence (c) analysis of the NADPH complex activation in the ventral mesencephalon of control rats as compared with rats subjected to CHP or chronic cerebral hypoperfusion and treatment with the AT1 blocker candesartan (CHP+cand). Protein expression was obtained relative to the GAPDH band value and the expression of each gene was obtained relative to the house-keeping transcripts (β -actin). The results were then normalized to values for control rats (100 %). NADPH oxidase activity (c) was expressed as relative light units (RLU/min/mg protein). Data are means \pm SEM. * p <0.05 relative to the control group, # p <0.05 relative to the group subjected to chronic hypoperfusion (one-way ANOVA and Bonferroni post hoc test)

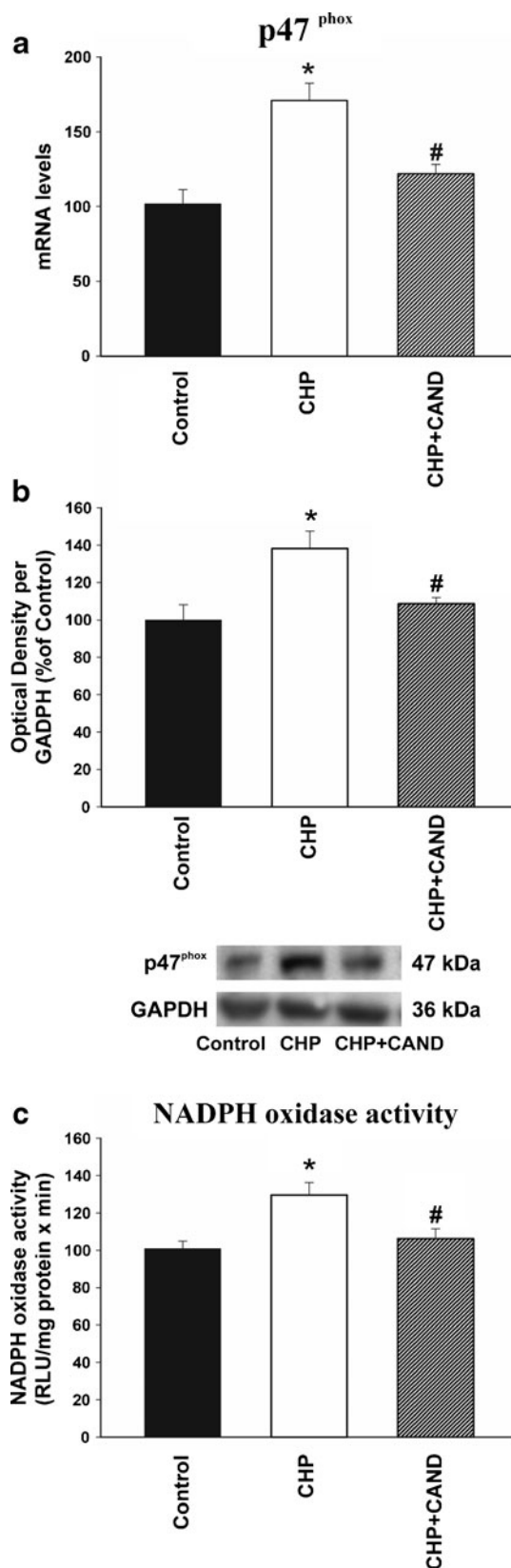
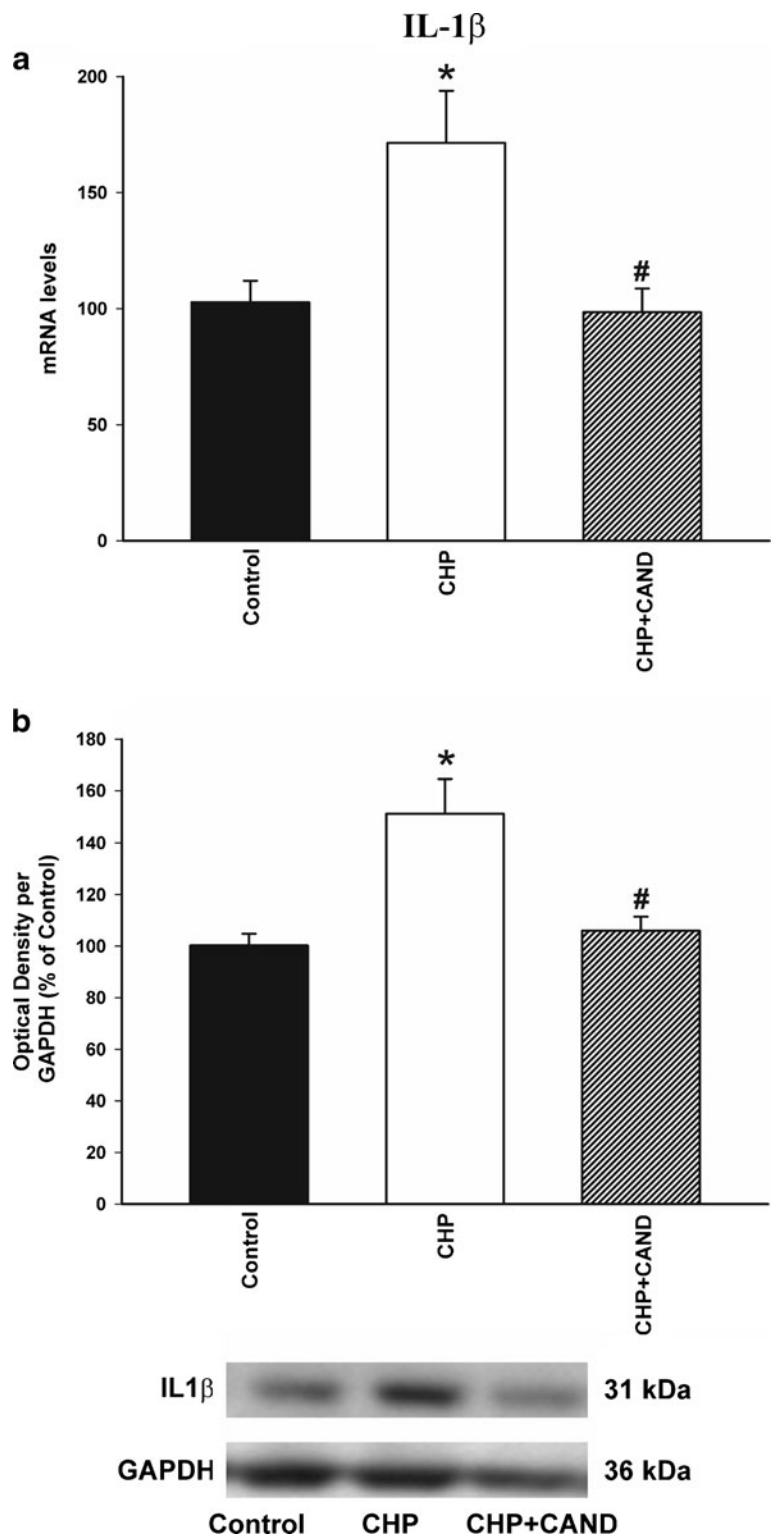


Fig. 7 Real-time quantitative RT-PCR (**a**) and Western blot (WB; **b**) analysis of interleukin-1 β (IL-1 β) expression in control rats as compared with rats subjected to CHP or chronic cerebral hypoperfusion and treatment with the AT1 blocker candesartan (CHP+cand). Protein expression was obtained relative to the GAPDH band value and the expression of each gene was obtained relative to the housekeeping transcripts (β -actin). The results were then normalized to values for control group (100 %). Data are mean values \pm SEM. * p < 0.05 relative to the control group, # p < 0.05 relative to the group subjected to chronic hypoperfusion (one-way ANOVA and Bonferroni post hoc test)



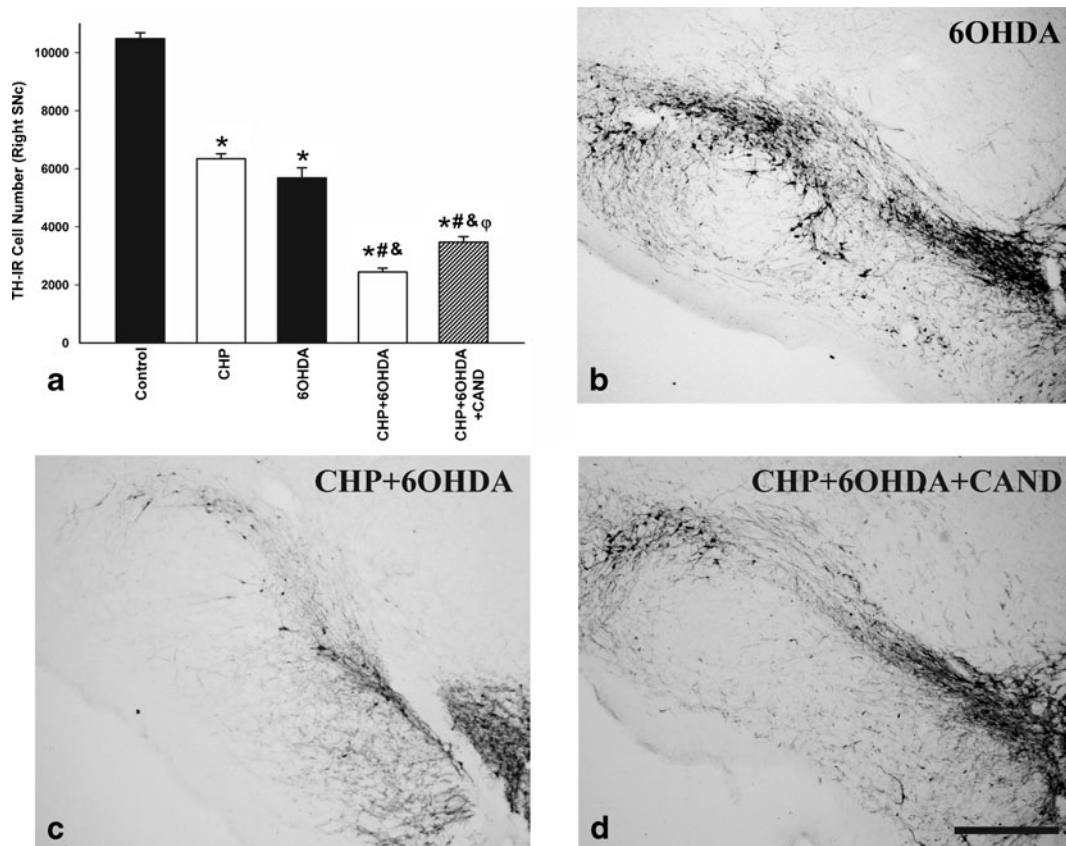


Fig. 8 Dopaminergic (TH-ir) neurons in the SNc of control (sham-operated) rats and rats subjected to CHP alone, intra-atrial injection of 6-OHDA alone, chronic cerebral hypoperfusion and intra-atrial injection of 6-OHDA (CHP+6-OHDA), or chronic cerebral hypoperfusion, intra-atrial injection of 6-OHDA and treatment with candesartan (CHP+6-OHDA+cand). Representative photomicrographs of the SNc of different groups of rats are shown in **b–d**. The SNc of control rats and rats

subjected to CHP alone is shown in Fig. 2**b, c**. The dopaminergic neurons were quantified as the total number of TH-ir neurons in the ipsilateral SNc (**a**). Data are means \pm SEM. * p <0.05 relative to the control group, # p <0.05 relative to the CHP group, & p <0.05 relative to the group treated with 6-OHDA alone, ϕ p <0.05 relative to the group subjected to CHP and 6-OHDA (one-way ANOVA and Bonferroni post hoc test). Scale bar 500 μ m

Discussion

Chronic cerebral hypoperfusion is directly responsible for dopaminergic degeneration. Chronic cerebral hypoperfusion can act synergistically with other factors to induce progression of dopaminergic neuron loss

Data from several clinical studies suggest an interaction between aging-related cerebrovascular disease/brain hypoperfusion and dopaminergic degeneration. Dopaminergic cell loss and parkinsonian signs have been observed in elders without PD (Buchman et al. 2012), presynaptic dopaminergic function is reduced in the majority of patients with VP (Zijlmans et al.

2007), and a subset of patients with clinically suspected VP were found to have a good therapeutic response to L-dopa (Thanvi et al. 2005; Zijlmans et al. 2004a, b). The present model of chronic brain hypoperfusion shows that chronic hypoperfusion induces a significant loss of dopaminergic neurons and a significant decrease in striatal dopaminergic terminals and striatal dopamine levels. Furthermore, the loss of dopaminergic neurons and striatal TH and dopamine levels were significantly reduced by treatment with candesartan, which was initiated 20 days after the bilateral occlusion of the carotid arteries, thus excluding the possibility that loss of dopaminergic neurons

was produced in the hypoxic–ischemic acute phase and confirming the long-term loss of dopaminergic neurons in the chronic phase.

A second important question addressed in the present study is whether the hypoperfusion derived from aging and/or vascular disease, acting synergistically with factors that induce PD, may increase the risk of development of PD (i.e., accelerate the onset of a latent PD) or exacerbate the progression and severity of already established PD. It is usually recognized that the presence of age-associated white matter lesions or a subclinical vascular impairment may contribute to dementia and possibly other symptoms such as postural stability and gait dysfunctions in PD as a consequence of additive or overlapping mechanisms (Bohnen and Albin 2011; Rektor et al. 2006, 2009). However, the present study shows that hypoperfusion may also lead to increased dopaminergic cell death by enhancing the deleterious effects of other factors (such as the low doses of the dopaminergic neurotoxin 6-OHDA in the present experiments).

Aging, cerebral hypoperfusion, and Parkinson's disease

It is well known that advancing age itself is one of the most significant risk factors for the development of neurodegenerative diseases such as PD and that prevalence of both PD and cerebrovascular disease increases with age. A recent study revealed that nigral pathology is common (almost 40 %) in elders without PD (Buchman et al. 2012), and we have recently observed an age-dependent decrease in nigral vascularization and nigral vascular endothelial growth factor levels (Villar-Cheda et al. 2009). The present results suggest that aging-related cerebrovascular disease and hypoperfusion contribute to the degenerative changes in dopaminergic neurons, and the subsequent neuron death. Interestingly, rats subjected to hypoperfusion showed a significant decrease in striatal levels of dopamine and DOPAC with no significant change in dopamine turnover (DOPAC/dopamine ratio). It has been observed that the compensatory increase in dopamine turnover observed in young animals after acute dopaminergic lesions is completely absent in aged animals (Collier et al. 2007), and the present results suggest that the aging-related chronic hypoperfusion may also be involved in this effect. Hypoperfusion may affect the compensatory response in the remaining DA neurons; however, other

mechanisms may be involved in the lack of compensatory DA turnover observed after chronic hypoperfusion (Ben et al. 1999; Nandhagopal et al. 2011).

Role of the brain renin–angiotensin system as a potential link between cerebrovascular disease and Parkinson's disease

The mechanistic links between hypoperfusion/vascular disease and neurodegeneration are unknown. In the present study chronic hypoperfusion led to dopaminergic cell death as well as increased expression of inflammatory markers such as IL-1 β and increased levels of oxidative stress markers such as NADPH activity, which have been shown to be involved in progression of dopaminergic cell death in animal models of PD and PD patients (Koprach et al. 2008; Wu et al. 2003). NADPH oxidases are a major source of superoxide (O₂⁻) and are upregulated in major aging-related diseases such as hypertension, diabetes and atherosclerosis (Griendling et al. 2000; Münzel and Keany 2001). Hypoperfusion-induced dopaminergic cell death, IL-1 β expression, and NADPH activity were reduced by treatment with the AT₁ antagonist candesartan, which shows that the nigral RAS is involved in this process. In animals subjected to chronic hypoperfusion, the increased RAS activity in the substantia nigra was confirmed by observation of increased expression of AT₁ receptors and decreased expression of AT₂ receptors. AT₁ and AT₂ receptors have opposing effects and AT₂ receptors counterbalance the deleterious effect of AT₁ receptor stimulation, so that functional interactions between the two receptor subtypes and their specific distribution determine the AII-induced effects (Sohn et al. 2000). AT₁ receptor activation upregulates NADPH oxidase activity, which is inhibited by AT₂ receptor activation. In addition to these major components of the RAS, several emerging components of the RAS such as angiotensin (1–7) (Clark et al. 2001) or prorenin receptors (Valenzuela et al. 2010) may also modulate brain RAS activity (Labandeira-Garcia et al. 2011). In the case of rats with chronic hypoperfusion, changes in local RAS activity creates a pro-oxidative and pro-inflammatory state, as suggested by the increased NADPH activity and IL-1 β expression relative to control rats.

Recent studies have shown that the local brain RAS is involved in several brain functions and disorders

(Labandeira-Garcia et al. 2011; Phillips and de Oliveira 2008; Wright and Harding 2011). In several animal models of PD, we have previously shown that there is a local RAS in the substantia nigra and that dopaminergic cell loss is enhanced by AII via AT1 receptors, activation of the microglial NADPH oxidase complex (Joglar et al. 2009; Rey et al. 2007; Rodriguez-Pallares et al. 2008) and subsequent mitochondrial-derived oxidative stress (Rodriguez-Pallares et al. 2012). Interestingly, we have also observed increased RAS activity (including increased AT1 expression, decreased AT2 expression, increased NADPH activity and increased IL-1 β levels) in the nigra of aged rats together with increased vulnerability of dopaminergic neurons to neurotoxins, which were inhibited by treatment with the AT1 antagonist candesartan (Rodriguez-Perez et al. 2012; Villar-Cheda et al. 2012). The mechanism responsible for the increased RAS activity in the nigra of aged animals has not been clarified. However, the present results suggest that the increase may be at least partially due to the chronic hypoperfusion observed in the substantia nigra of aged animals (Villar-Cheda et al. 2012).

In conclusion, this study details the effects of brain hypoperfusion on dopaminergic neurons in the rat. Brain hypoperfusion induces dopaminergic neuron loss and enhances the effect of the dopaminergic neurotoxin 6-hydroxydopamine. The AT1 receptor antagonist candesartan has a neuroprotective effect, which suggests that increased angiotensin II activity participates in dopaminergic degeneration during brain hypoperfusion. The present results suggest that the presence of vascular disease or aging-derived hypoperfusion may not only increase the severity of PD by aggravating some symptoms (i.e., an additive or overlapping effect of PD and VP), but may lead to significant loss of dopaminergic neurons and terminals and, particularly, act synergistically with other factors to accelerate the onset of PD or exacerbate the progression and severity of the already established PD. Therefore, early treatment of vascular disease should be considered for the treatment of aged PD patients and PD patients with cerebrovascular risk factors. The results also suggest that inhibition of RAS activity with low doses of AT1 antagonists such as candesartan may constitute a useful neuroprotective strategy as may inhibit nigral inflammation and oxidative stress and also improve hypoperfusion by acting on cerebral microvessels.

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