Angiotensin type-1-receptor antagonists reduce 6-hydroxydopamine toxicity for dopaminergic neurons

P. Rey,[a] A. Lopez-Real,[a] S. Sanchez-Iglesias,[b] A. Muñoz[a], R. Soto-Otero,[b] and J. L. Labandeira-Garcia[a]

Laboratory of Neuroanatomy and Experimental Neurology, Dept. of Morphological Sciences (a) and Laboratory of Neurochemistry, Dept. of Biochemistry and Molecular Biology (b), Faculty of Medicine, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain.

Correspondence:

Jose L. Labandeira-Garcia M.D., Ph. D.

Dept. of Morphological Sciences, Faculty of Medicine, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain.

Tel. +34-981563100; Fax: +34-981547078
E. mail: cmlaband@usc.es

Abstract

Angiotensin II activates (via type 1 receptors) NAD(P)H-dependent oxidases, which are a major source of superoxide, and is relevant in the pathogenesis of several cardiovascular diseases and certain degenerative changes associated with ageing. Given that there is a brain renin-angiotensin system and that oxidative stress is a key contributor to Parkinson's disease, we investigated the effects of angiotensin II and angiotensin type 1 (AT_1) receptor antagonists in the 6-hydroxydopamine model of Parkinson's disease. Rats subjected to intraventricular injection of 6-hydroxydopamine showed bilateral reduction in the number of dopaminergic neurons and terminals. Injection of angiotensin alone did not induce any significant effect. However, angiotensin increased the toxic effect of 6-hydroxydopamine. Rats treated with the AT_1 receptor antagonist ZD 7155 and then 6-hydroxydopamine (with or without exogenous administration of angiotensin) showed a significant reduction in 6-hydroxydopamine-induced oxidative stress (lipid peroxidation and protein oxidation) and dopaminergic degeneration. Dopaminergic degeneration was also reduced by the NAD(P)H inhibitor Apocynin. Angiotensin may play a pivotal role, via AT_1 receptors, in increasing the oxidative damage of dopaminergic cells, and treatment with AT_1 antagonists may reduce the progression of Parkinson's disease.

Keywords: angiotensin; basal ganglia; dopamine; neuroprotection; NAD(P)H-oxidase; Parkinson's disease; oxidative stress, 6-hydroxydopamine

1. Introduction

The renin-angiotensin system (RAS) was originally described as a circulating humoral system that regulates blood pressure, aldosterone release and sodium reabsorption. Angiotensin II (AII) is the most important effector peptide, and is formed by the sequential action of two enzymes, renin and angiotensin converting enzyme (ACE), on the precursor glycoprotein angiotensinogen. The actions of AII are mediated by two main cell surface receptors: AII type 1 and 2 (AT_1 and AT_2) receptors [2,63]. However, numerous clinical and laboratory data provide evidence that AII is relevant in the pathogenesis of several diseases, particularly of the cardiovascular system, and certain degenerative changes associated with ageing [3,11,56]. It has been also reported that AT_1 antagonists reduce age-related mitochondrial dysfunction [10]. It is now generally accepted that in addition to the "classical" humoral RAS there exist local RAS in many tissues, including brain tissue [2, 46], and that locally formed AII regulates many substances such as growth factors and cytokines that are involved in processes such as cell growth/apoptosis or inflammation [56]. Interestingly, it has recently been shown that in several cell types reactive oxygen species (ROS) play a crucial role in the signaling of AII via AT_1 receptors, and that AII activates NAD(P)H-dependent oxidases, which are a major source of superoxide (O_2^-) and are upregulated in major ageing-related diseases such as hypertension, diabetes and atherosclerosis [23,48]. Accordingly, AT_1 receptor antagonists induce inhibition of oxidase, reduction of oxidative stress and improvement in endothelial dysfunction [55,67].

It is now established that the brain possesses a local angiotensin system, and that the local AII exerts multiple actions [2,46]. All components of the RAS have been observed in the striatum [1,2,8,52], and AII modulates dopamine (DA) release from the striatal DA terminals via their AT_1 receptors [7,47]. Furthermore, it has recently been

shown that NAD(P)H-oxidase has a wide distribution throughout the brain, including striatum and substantia nigra [30,57], and that NAD(P)H-oxidase-derived ROS play a major role in AII signaling in neurons [49,65]. There is growing evidence indicating that the oxidative stress is a key contributor to the pathogenesis and progression of Parkinson's disease (PD) and ageing-related loss of DA neurons [29,51]. In addition, increased ACE activity in the cerebrospinal fluid [32], association between genetic polymorphism of the ACE gene and the disease [39], and increased expression of NAD(P)H:quinone oxidoreductase in neurons [64] have been observed in PD patients.

Altogether this suggests that, as previously observed in cardiovascular diseases, the brain RAS may play a major role in the pathogenesis and progression of PD and ageing-related loss of DA neurons, and that manipulation of RAS components may be useful for neuroprotection in PD patients. In the present study, we treated rats with intraventricular injections of the DA neurotoxin 6-hydroxydopamine (6-OHDA), AII, the AT_1 receptor antagonist ZD 7155 or the NAD(P)H inhibitor Apocynin, to study the effects of the RAS on the 6-OHDA-induced degeneration of DA neurons and the level of 6-OHDA-induced oxidative stress in the ventral midbrain and striatum.

2. Materials and methods

Experimental design

Male adult Sprague-Dawley rats (weighing about 200 g) were used in the present experiments. All experiments were carried out in accordance with the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) and approved by the corresponding committee at the University of Santiago de Compostela. The rats were divided into 10 groups (A-J; see Table 1). Rats in group A were used as normal (i.e. non-lesioned) controls, and received the corresponding intraventricular injections of

vehicle (saline containing 0.2% ascorbic acid, 3 μl; see below). Rats in group B were injected in the third ventricle with Angiotensin II (AII; 5 μg; Sigma) in 3 μl of sterile ascorbate saline 24 h before, 25 min before and 24 h after ascorbate saline injection. Rats in group C were injected in the third ventricle with 3 μl of sterile ascorbate saline 24 h before, 25 min before and 24 h after 6-OHDA injection (200 μg of 6-OHDA in 3 μl of sterile ascorbate saline). Rats in group D were injected intraventricularly with AII, as in group-B rats, and 3 μl ascorbate saline containing 6-OHDA. Rats in groups E and H were injected as those in group D but were pretreated with the AT_1 receptor antagonist ZD 7155 hydrochloride (Tocris; 50μg/3 μl, intraventricularly; group E) or Apocynin (Fluka; 750μg /5 μl, intraventricularly; group H) 20 min before each AII injection. Rats in group F and I were injected intraventricularly as those in group D but were pretreated with ZD 7155 (group F) or Apocynin (group I) rather than AII (i.e. without exogenous administration of AII). Finally, rats in groups G and J were injected with ZD 7155 (group G) or Apocynin (group J) alone (i.e. without administration of 6-OHDA or AII). The injections were performed using a single cannula placed in the third ventricle during the whole injection period (stereotaxic coordinates: 0.8 mm posterior to bregma, midline, 6.5 mm ventral to the dura, and tooth bar at 0). The solution was injected with a 10 μl Hamilton syringe coupled to a motorized injector (Stoelting), at 0.5 μl/min. All surgery was performed under ketamine/xylazine anesthesia, and thirty minutes prior to injection of 6-OHDA, rats received desipramine (Sigma, 25 mg/kg i.p.) to prevent uptake of 6-OHDA by noradrenergic terminals. The accuracy of the injections and cannula placement were confirmed by post-mortem analysis.

Five rats of each group were used to study the level of 6-OHDA-induced oxidative stress, which was estimated by determination of lipid peroxidation (TBARS formation) and protein oxidation (carbonyl content) in the ventral midbrain (i.e. from

bregma -4.5 to bregma -6.5, and ventral +6.5 to ventral +9.5; see ref. 50) and the striatum (from bregma +2.5 to bregma -1). For biochemical studies on oxidative stress rats were killed 48 h after lesion, because we observed the highest levels of 6-OHDA-induced oxidative stress indicators at this time in preliminary experiments (unpublished data). Six rats of each group were used for immunohistochemical studies. Given that previous studies on the time course of the DA degeneration in intraventricular 6-OHDA lesion model have shown that the DA lesion is complete or practically complete 1 week after 6-OHDA injection [53], the rats processed for immunohistochemistry (see below) were killed 1 week postlesion. Furthermore, in preliminary experiments the doses of 6-OHDA were reduced to the minimal levels that induced a significant loss of nigral DA neurons and high survival-rate.

Immunohistochemistry and Cresyl violet staining

One week post-lesion, the animals were killed by chloral hydrate overdose and perfused first with 0.9% saline and then with cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed and subsequently washed and cryoprotected in the same buffer containing 20% sucrose, and finally cut on a freezing microtome. Sections were processed for tyrosine hydroxylase (TH) immunohistochemistry (as follows). After incubation for 1 hour in 10% normal swine serum with 0.25% Triton-X-100 in 0.02 M potassium phosphate-buffered saline containing 1% bovine serum albumin (KPBS-BSA), sections were incubated overnight at room temperature with rabbit polyclonal antiserum to TH (Peel-Freez; 1:500 in KPBS-BSA containing 2% normal swine serum and 0.25% Triton-X-100). The sections were subsequently incubated first for 90 min with the corresponding biotinylated secondary antibody (Vector, USA; diluted 1:200) and then for 90 min with an avidin-

biotin-peroxidase complex (ABC, Vector, USA). Finally, the labeling was visualized with 0.04% hydrogen peroxide and 0.05% 3-3' diaminobenzidine (DAB, Sigma).

Estimation of the total TH-immunoreactive (TH-ir) neurons was made in the substantia nigra compacta (SNc; see ref. 50). We used an unbiased stereology method (i.e. the optical fractionator). Uniform randomly chosen 40-μm sections through the entire substantia nigra (i.e. every fourth section from the rostral tip to the caudal end) were analyzed for the total number of TH-ir cells by means of a stereological grid (fractionator). Sampling was carried out using the CAST-Grid system (Computer Assisted Stereological Toolbox; Olympus, Denmark), which comprised an Olympus IX51 microscope, a ProScan II X-Y motorized stage (Prior Scientific, UK) run by a PC computer, a microcator (MT1201, Heidenhain, Germany) connected to the stage and that feeds the computer with the distance information in the Z axis, and a JVC color video camera (Japan). The CAST-Grid software (version 2.1.5.9) was used to delineate the SNc as observed with a 4X objective, and to generate counting areas. A counting frame (1800 μm^2) was placed at random on the first counting area and systematically moved through all counting areas until the entire delineated area was sampled. The sampling frequency was chosen so that a minimum of 150 TH-positive neurons were counted in each nigra. Counting was done using a 100X oil objective (NA 1.4). Guard volumes (5 μm from the top and the bottom of the section) were excluded from both surfaces to avoid the problem of lost caps, and only the profiles that came into focus within the counting volume (with a depth of 12 μm) were counted. The total number of neurons was calculated according to the optical fractionator formula [24, 66]. The coefficient of error associated with the estimation was calculated according to and was less than 0.10. The nigral volume was estimated according to Cavalieri's method [24].

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) on a personal computer coupled to a videocamera (CCD-72, MTI) and a constant illumination light table (Northern Light, St. Catharines, Canada). At least four sections through the central striatum (i.e. from bregma +1 to bregma +0; see ref. 50) of each rat were measured (both the right and left striatum), and for each section optical densities were corrected by subtraction of background, as observed in the corpus callosum.

In order to confirm that 6-OHDA induces cell death and not just phenotypic down-regulation in TH activity, series of sections through the entire substantia nigra of control rats and rats treated with 6-OHDA (groups A and C) were counterstained with Cresyl violet, and the total number of neurons in the SNc was estimated using the unbiased stereology method described above for TH-ir cells. Neurons were distinguished from glial cells on morphological grounds, and neurons with visible nuclei were counted as above

Determination of TBARS

The TBARS determination was performed spectrophotometrically. The striata or ventral midbrain tissue were homogenized with three volumes (w/v) of a Na_2PO_4/KH_2PO_4 buffer (pH 7.4) isotonized with KCl and containing butylated hydroxytoluene (200 μM) and desferrioxamine (200 μM). An aliquot of the resulting sample (200 μl) was treated with SDS (8%, w/v) followed by acetic acid (20%) and the mixture vortexed for 1 min. Thiobarbituric acid (0.8%) was then added and the resulting mixture incubated at 95 ºC for 60 min. After cooling to room temperature, 3 ml of *n*-butanol were added and the mixture shaken vigorously. After centrifugation at 4000 r.p.m. for 5 min, the absorbance of the supernatant (organic layer) was measured at 532 nm using an Ultrospec III

spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). For calibration, a standard curve (5-150 nM) was generated using the malonodialdehyde (MDA) derived by acid hydrolysis (SO_4H_2; 1.5%, v/v) of 1,1,3,3-tetraethoxypropane and the TBARS results expressed as nmol MDA/mg protein. The protein concentration of the sample obtained was determined according to a previously published method [45], using BSA as the standard.

Estimation of protein carbonyl content

The protein carbonyl content was assessed spectrophotometrically. An aliquot of the sample obtained after the homogenization of brain tissue for TBARS (200 μl) was submitted to precipitation of nucleic acids with 1% streptomycin sulphate (1:9, v/v) followed by centrifugation at 13000 r.p.m. The pellet was then discarded and the supernatant treated with trichloroacetic acid (1 M) followed consecutively by sonication (Branson Sonic Corp., Danbury, USA) and centrifugation in a microcentrifuge (model E, Beckman Instruments) at 13000 r.p.m. for 5 min. The resulting pellet was reconstituted in NaOH (0.5 M) with vigorous vortexing for 3 min. Ten mM 2,4-dinitrophenylhydrazine in 2 M chloric acid was then added and the mixture incubated at room temperature for 1 hr, in darkness, and with continuous agitation. After the addition of trichloroacetic acid (1 M), the resulting mixture was centrifuged at 13000 r.p.m. for 5 min. The resulting pellet was washed twice with ethyl acetate:ethanol (1:1, v/v), and the washed pellet was then reconstituted with 6 M guanidine in a 20 mM KH_2PO_4 buffer (pH 2.3) and the absorbance of the resulting solution measured at 370 nm. The carbonyl content was calculated from the absorbance data using $\varepsilon = 22000\ M^{-1} \cdot cm^{-1}$ as the value of the absorption coefficient for dinitrophenylhydrazone and expressing this parameter as nmol carbonyls/mg protein. Because of the numerous washing steps, the protein

content in the final pellet was estimated on an HCl blank pellet processed simultaneously, using a BSA standard curve in 6 M guanidine, and reading the absorbance at 280 nm.

Chemicals used in experiments on oxidative stress processes

Thiobarbituric acid, butylated hydroxytoluene crystalline, 2,4-dinitrophenylhydrazine hydrochloride, desferrioxamine, 1,1,3,3-tetraethoxypropane, sodium dodecylsulfate (SDS), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, USA). Guanidine hydrochloride was from Aldrich Chemical Co. (Milwaukee, USA). The water used for the preparations of solutions was of Milli-RiOs/Q-A10 grade (Millipore Corp., Bedford, USA). All remaining chemicals used were of analytical grade and were purchased from Fluka Chemie AG (Buchs, Switzerland).

Statistical analysis

Statistical differences were tested using one-way ANOVA followed by a post-hoc Student-Newman-Keuls test ($P<0.05$). Normality of populations and homogeneity of variances were verified before each ANOVA. All statistical analyses were performed using Sigmastat 3.0 from Jandel Scientific (San Diego CA, USA).

3. Results

3.1. Effects of Angiotensin II and AT_1 receptor antagonists on the degeneration of DA neurons in the substantia nigra and DA terminals in the striatum

In control rats (i.e. injected with vehicle only; group-A rats), the dopaminergic neurons in the substantia nigra compacta (SNc, see ref. 50; a total of 16973 ± 910 neurons in both SNc) were intensely immunoreactive to TH (Figs. 1A, 2A). Furthermore, additional groups of TH-ir neurons were observed in their usual locations throughout the brain. As expected, the number of neurons counted using Cresyl-violet stained sections (18659 ± 335) was slightly higher than that of TH-immunoreactive (TH-ir) neurons since some non-dopaminergic neurons located in the SNc are also included. Control rats showed dense and evenly distributed TH immunoreactivity through the striatum, which indicated the presence of a dense network of nigrostriatal dopaminergic terminals (Fig. 3A,F). In rats subjected to intraventricular injection of AII alone (group-B rats), the apparent reduction observed in the number of DA neurons in the SNc and in the density of DA striatal terminals with respect to the control rats was not statistically significant (Fig. 4). Rats subjected to intraventricular injection of 6-OHDA (group-C rats), showed a bilateral reduction in the number of TH-ir neurons in the SNc and a marked reduction in the TH-ir in both striata (Figs. 1B, 2B, 3B,G and 4). Other groups of DA neurons were also affected by the neurotoxin (see ref. 53 for details). Sections from group-C rats showed a significant reduction in the number of Cresyl-violet stained neurons in the SNc (13191 ± 1234). Rats subjected to intraventricular injection of 6-OHDA and AII (group-D rats) showed a bilateral reduction in the number of TH-ir neurons in the SNc and a bilateral reduction in the density of striatal DA terminals, and this reduction was significantly higher than that observed in group-C rats treated with 6-OHDA alone (Figs. 1C, 2C, 3C,H and 4). However, the neurotoxic effect of treatment with 6-OHDA + AII was significantly reduced by pretreatment with the AT_1 receptor antagonist ZD 7155 (group-E rats; Figs. 3E,J and 4). Finally, it is particularly interesting to remark that rats treated with the AT_1

receptor antagonist ZD 7155 and then 6-OHDA (i.e. without administration of exogenous AII; group-F rats) showed a significant reduction in the 6-OHDA induced degeneration of DA neurons and striatal DA terminals (Figs. 1D, 2D, 3D,I and 4). No significant changes in the number of TH-ir neurons or terminals were observed in rats treated with ZD 7155 alone (group-G rats).

3.2. Effects of the NAD(P)H inhibitor Apocynin on the DA degeneration induced by 6-OHDA and angiotensin.

As observed after treatment with the AT_1 receptor antagonist ZD 7155, pretreatment with the NAD(P)H inhibitor Apocynin significantly decreased the neurotoxic effect of 6-OHDA and AII on the DA terminals in the striatum and DA neurons in the SNc (Fig. 4). With the doses of 6-OHDA used in the present experiments, to obtain a decrease of approximately 30% in the number of TH-ir neurons, pretreatment with Apocynin reduced the lesion induced by 6-OHDA + AII (about 50% decrease in TH-ir neurons) to levels that were not significantly different from control levels (group-H rats). This indicates that Apocynin not only reduces the effect of exogenous AII but also the effect of 6-OHDA administration. In agreement, rats treated with Apocynin and then 6-OHDA (i.e. without administration of exogenous AII; group-I rats) showed a significant reduction in the 6-OHDA-induced degeneration of DA neurons and striatal DA terminals. No significant changes in the number of TH-ir neurons or terminals were observed in rats treated with Apocynin alone (group-J rats)

3.3. Effects of Angiotensin II and AT_1 receptor antagonists on the indices of lipid peroxidation and protein oxidation

The concentration of TBARS was used as an index of lipid peroxidation in the striatum and ventral midbrain (Fig. 5A,B). Intraventricular administration of 6-OHDA (200 μg) induced a significant increase in TBARS concentration in both striatum and ventral midbrain. No significant increase with respect to control animals was observed after administration of AII alone. Forty-eight hours after 6-OHDA injection, analysis of levels of lipid peroxidation in the striatum and the ventral midbrain did not reveal any significant difference between the effects of administration of 6-OHDA + AII and administration of 6-OHDA alone. However, pre-treatment with the AT_1 receptor antagonist ZD 7155 induced a marked decrease in the levels of lipid peroxidation induced by 6-OHDA when compared with the levels obtained after administration of 6-OHDA alone. After treatment with ZD 7155, the 6-OHDA-induced increase in TBARS concentration showed a reduction of about 75% in the striatum and 60% in the ventral midbrain (Fig. 5A). In agreement, administration of ZD 7155 alone (group-G rats) induced a significant decrease in TBARS concentration with respect to the control rats.

Protein oxidation was assessed by the determination of protein carbonyl content in the samples of striatum and ventral midbrain (Fig. 5C,D). Intraventricular injection of 6-OHDA induced a significant increase in the protein oxidation detected in both striatum and ventral midbrain. No significant increase with respect to control animals was observed after administration of AII alone. Forty-eight hours after 6-OHDA injection, analysis of levels of protein carbonyl content in the striatum or the ventral midbrain did not reveal any significant difference between administration of 6-OHDA + AII and administration of 6-OHDA alone. After pre-treatment with the AT_1 antagonist ZD 7155, however, the increase in protein carbonyl content induced by 6-OHDA showed a 50% reduction in the striatum and 60% reduction in the ventral midbrain (Fig.

5C,D). Interestingly, administration of ZD 7155 alone (group-J rats) induced a significant decrease in protein oxidation with respect to the control rats.

4. Discussion

It is well established that the brain possesses a local RAS, and the majority of the known actions of AII in the adult brain appear to be mediated by AT_1 receptors [2,21,46]. During development, AT_2 receptors are much more abundant than AT_1 receptors, and they decrease sharply in abundance after birth, suggesting that AT_2 receptors may play a major role in brain development and differentiation [21, 54]. AII is formed from the precursor glycoprotein angiotensinogen by the sequential action of the enzyme renin, which generates AI, and ACE, which generates AII from AI. All of these components have been observed in the striatum. Angiotensinogen protein and mRNA have been found in the striatum, and several studies have shown that astrocytes are the major source of this glycoprotein [26,44,61]. ACE occurs widely in the brain and is associated with the endothelium of cerebral blood vessels, epithelial cells of choroids plexus and the plasma membranes of astrocytes [12]. Furthermore, ACE occurs in neurons, and high concentrations of ACE have been observed in the striatum and substantia nigra of mammals including rats [14] and humans [13]. Immunohistochemical location of AII, as well as of AII-ir terminals, has been reported in the human and rat striatum [8, 52]. AT_1 receptors occur in DA neurons, both in cell bodies in the substantia nigra and their terminal fields in the striatum of different mammals, including rats and humans, as revealed by autoradiography [1,2,15,58] and subsequently confirmed by immunohistochemistry [4,54] and in situ hybridization

histochemistry [38]. Interestingly, the density of AT_1 receptors is very high in human striatum and substantia nigra in comparison with rats and other mammals [2,38].

Some findings suggest that, as previously observed in cardiovascular diseases, the RAS may play a major role in the pathogenesis and progression of PD, and that manipulation of the RAS components may be useful for neuroprotection of PD patients. In the striatum of PD patients, a marked reduction in AT_1 receptors attributed to the loss of DA terminals [20], as well as increased ACE activity in the cerebrospinal fluid [32] and an association between genetic polymorphism of the ACE gene and PD [39] have been observed. Furthermore, in previous studies, we have observed that 6-OHDA-induced degeneration of DA neurons was decreased by ACE inhibitors [41,42]. However, independently of their effect on the RAS, ACE inhibitors themselves may have antioxidant properties [5], and may also hydrolyze several other neuropeptides such as bradykinin [40], which may be behind the neuroprotective effects. Therefore, AII and AII receptors may not be involved in the neuroprotective effects observed after administration of ACE inhibitors. The results of the present study suggest, however, the involvement of AII and AII receptors in the neuroprotective effects.

In the present experiments, administration of angiotensin alone induced an apparent reduction in the number of DA neurons and DA striatal terminals, although the reduction was not statistically significant. The dose of AII used in the present study (5 μg, 4.8 nM) has previously been observed to affect striatal function. Intrastriatal injection of AII at doses of 0.3 to 10 nM induced striatal DA release in rats [6,28,36], and functional effects have been detected after intraventricular injections of doses as low as 10 ng [16,35]. The bioactivity of the dose of AII used in the present study was also revealed by the increasing effect induced on the 6-OHDA-induced loss of DA cells in the SNc and DA terminals in the striatum. Furthermore, the effects of the

administration of too high concentrations of AII may be counteracted by downregulation of AT_1 receptors [25]. The results suggest, therefore, that administration of exogenous AII is not sufficient by itself to induce a significant loss of DA neurons, at least after a 7 day survival period, but it is sufficient to induce a detectable amplifying effect on the 6-OHDA toxicity. More interestingly, the inhibition of the effect of the endogenous and /or exogenous AII on the AT_1 receptors by ZD 7155 decreased the 6-OHDA-induced loss of DA neurons. The neuroprotective effect induced by administration of the AT_1 antagonist appears, however, more marked than the neurotoxic effect induced by administration of exogenous AII (see below).

6-OHDA is a selective catecholaminergic neurotoxin widely used to investigate the pathogenesis and progression of PD. In the present study, administration of 6-OHDA induced a loss of TH-ir nigral neurons and TH-ir striatal terminals. Changes in TH expression do not necessarily imply neuronal death. However, the animal model used in the present study (i.e. injection of 6-OHDA in the third ventricle) has recently been described in detail [53], and it has been shown that the lesion is complete or practically complete 1 week after injection, and that the loss of TH immunoreactivity in neurons corresponds with neuronal death, which was also confirmed in the present experiments by using Cresyl-violet stained sections. The specificity of 6-OHDA neurotoxicity has been associated with its uptake and accumulation by a transport mechanism specific for catecholaminergic neurons [27]. It has been observed that 6-OHDA is readily oxidized, thereby producing a variety of cytotoxic radical products [33,60], and its neurotoxicity has been shown to be related to the production of reactive oxygen species. However, it has been suggested that 6-OHDA also acts directly by inhibiting the mitochondrial respiratory chain at the level of complex I of the electron transport chain [22]. Therefore oxidative stress is a major molecular mechanism

underlying both the DA degeneration induced by 6-OHDA and that observed in PD patients [51,60].

The NAD(P)H oxidase complex is the most important intracellular source of ROS other than mitochondria. Furthermore, earlier studies demonstrated that extramitochondrial ROS can induce mitochondrial DNA damage, thereby destroying respiratory enzymes to produce more reactive oxygen species [37]. Thus, ROS originated by NAD(P)H oxidases favour their own production via mitochondria, intracellular iron uptake and other intracellular sources [9]. These feed-forward mechanisms form a vicious circle and may amplify and sustain ROS production induced by low doses of 6-OHDA or other neurotoxins, contributing to DA cell death. Furthermore, it has been recently reported that AII also stimulates mitochondrial ROS production through the opening of mitochondrial ATP-sensitive potassium channels [31] Recent studies have shown the presence of NAD(P)H oxidase in neurons and glial cells [30,57], and that in these cells, NAD(P)H oxidase and ROS are involved in signaling by AII via AT_1 receptors [49,65]. In addition, it has been observed that in experimental stroke ischemic injury is modulated by AII via neuronal AT_1 receptors, and that AT_1 antagonists reduced the expression of NAD(P)H and lipid peroxidation, lessened cerebral infarction and improved the neurological outcome [34,43]. In agreement, in a series of interesting studies using mesencephalic cell cultures, Gao et al. [17-19] have shown that several well-known dopaminergic toxins (i.e. MPTP, rotenone and inflammogen lipopolysaccharide) induce NAD(P)H oxidase-mediated generation of ROS in microglial cells, and suggested that the release of superoxide from activated microglia greatly enhanced the neurotoxin-induced DA degeneration. Interestingly, it was observed that low and apparently non-toxic doses of two neurotoxins can act synergistically to induce DA degeneration, and that NAD(P)H oxidase-mediated

generation of ROS appeared to be a key contributor to synergistic DA neurotoxicity [17,19]. The present results suggest that endogenous AII increases the neurotoxic effect of 6-OHDA on DA neurons, as previously observed after simultaneous administration of low doses of two DA neurotoxins to DA cell cultures [17,19]. This effect was blocked by administration of the AT_1 antagonist ZD 7155, suggesting that this increasing effect of AII on 6-OHDA neurotoxicity is exerted via AT_1 receptors. Furthermore, pretreatment with the NAD(P)H inhibitor Apocynin inhibited the neurotoxic effect of 6-OHDA + AII, suggesting that NAD(P)H oxidase-mediated superoxide production plays a major role in the synergistic neurotoxicity of 6-OHDA and AII. Moreover, treatment with ZD 7155 or Apocynin not only reduced the increase in DA cell loss induced by administration of AII, but also counteracted the neurotoxic effect of the 6-OHDA in rats treated with 6-OHDA + AII, which was confirmed in rats treated with 6-OHDA and apocynin or ZD 7155 alone (i.e. without administration of exogenous AII).

The present studies on the levels of major oxidative stress indicators (i.e. lipid peroxidation and protein oxidation) induced by 6-OHDA in the main areas of DA system degeneration (i.e. the striatum and the ventral midbrain) showed that inhibition of AT_1 receptor activity lead to a marked reduction in 6-OHDA-induced increase in lipid peroxidation (75 and 60% reduction, respectively) and protein oxidation (50 and 60% reduction, respectively). In agreement, administration of ZD 7155 alone (i.e. without 6-OHDA) induced a significant reduction in the levels of lipid peroxidation and protein oxidation in comparison with controls. This is consistent with the neuroprotective effect of the AT_1 antagonist ZD 7155 observed in the immunohistochemical studies. Forty-eight hours after treatment with 6-OHDA and AII no significant increase in lipid peroxidation or protein oxidation was found in

comparison with rats treated with 6-OHDA alone, despite the immunohistochemical data showing that administration of exogenous AII increased 6-OHDA-induced lesion 1 week after treatment. For biochemical studies, rats were killed 48 h after lesion because we observed the highest levels of 6-OHDA-induced oxidative stress indicators at this time point in preliminary experiments. However, 48 h after the 6-OHDA injection may be an inadequate time point for detecting the increasing effect of the AII injection on the 6-OHDA-induced ROS (or the increase in ROS induced by AII alone, group-B rats). Firstly, 48 h after the 6-OHDA injection is probably not the time point corresponding to the maximal effect of the injected AII on the ROS levels. Secondly, an increase in ROS induced by the injection of AII may be non-significant at the time when maximal levels of ROS induced by 6-OHDA occur (i.e. 48 h). In any case, the neuroprotective effect induced by administration of the AT_1 antagonist appears more marked than the neurotoxic effect induced by administration of exogenous AII. In different cell types, it has been reported that AII-mediated NAD(P)H oxidase activation occurs via AT1 receptors, whereas the AT_2 receptor appears to inhibit oxidase activation and have protective effects [11,59], and it has been suggested that AT1 receptor antagonists may enable endogenous angiotensin II to stimulate cell survival via activation of AT_2 receptors. However, administration of exogenous AII would act on both AT_1 and AT_2 receptors. We have observed both AT_1 and AT_2 receptors in mesencephalic DA neurons in vitro, and that AII increased differentiation of DA neurons via AT_2 receptors [54]. However, it is not clear if there are AT_2 receptors in DA neurons and terminals in the adult brain, since conflicting results have been reported [15, 20]. In addition, the effect of the administration of exogenous AII may be partially counteracted by downregulation of AT_1 receptors [25]. Finally, possible indirect effects of AII via glial cell receptors may also be investigated in futures studies [17-19].

The most interesting point is, therefore, that pre-treatment with ZD 7155 induced a significant decrease in the DA neurotoxicity and oxidative stress induced by 6-OHDA alone, and that treatment with ZD 7155 alone induced a significant reduction of oxidative stress indicators in comparison with untreated controls. As indicated above, ROS originated by NAD(P)H oxidases may amplify and sustain the effect of low doses of 6-OHDA or other neurotoxins in animal models of PD or PD patients. Since AII is a major activator of NAD(P)H oxidase complex via AT_1 receptors, treatment with AT_1 antagonists may reduce the amplifying effect of NAD(P)H oxidase-derived ROS on DA neurotoxins. In preliminary experiments (A. Muñoz and J.L. Labandeira-Garcia, unpublished observations), we used radioimmunoassay (RIA; Bühlmann Laboratories AG, Switzerland) to estimate AII levels in striatal homogenates from control rats and 6-OHDA lesioned rats (i.e. rats treated as those in groups A and C in the present study). Rats subjected to partial DA denervation using intraventricular 6-OHDA showed a slight increase (around 20% increase) in striatal AII levels with respect to controls. It is known that AII induces DA release from striatal DA terminals via AT_1 receptors [7,15,47], and increased AII levels after partial DA denervation may constitute a compensatory response induced by a decrease in striatal DA levels. However, an increase in activation of AT_1 receptors may lead to a vicious circle and amplify ROS production and DA cell degeneration. It is not known if there is an increase in striatal AII levels in PD. However, increased ACE activity in the parkinsonian cerebrospinal fluid [32] and an association between genetic polymorphisms of the ACE gene [39] and PD have been observed. Additional experiments are necessary to clarify the effects of the DA denervation on the levels of AII and other components of the striatal RAS.

There is growing evidence indicating that the ageing-related loss of DA neurons and development of PD may be the result of interaction of multiple factors that act on

the DA neurons, which are particularly vulnerable to oxidative stress because of a number of intrinsic characteristics, including a reduced antioxidant capacity [29]. This combines to initiate the slow acting yet long term neurodegenerative process of PD. Genetic predisposition, environmental toxins, neuroinflammation and DA toxicity may be potential factors. In addition, the results of the present experiments suggest that striatal AII, via AT_1 receptors, may play a pivotal role in increasing the oxidative damage to DA cells, and that manipulation of the RAS components, and particularly treatment with AT_1 antagonists, widely used in cardiovascular therapy, may reduce the progression of PD.

Acknowledgements

This work was financially supported by grants from the Spanish Ministry of Education (MEC) and Galician Government (XUGA). The authors thank Pilar Aldrey for her excellent technical assistance.

Disclosure Statement

We have no actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted that could inappropriately influence (bias) our work.

All experiments were carried out in accordance with the “Principles of laboratory animal care” (NIH publication No. 86-23, revised 1985) and approved by the corresponding committee at the University of Santiago de Compostela.

References

[1] Allen AM, MacGregor DP, Chai SY, Donnan GA, Kaczymarzyk S, Richardson K, Kalnins R, Ireton J, Mendelsohn FAO. Angiotensin II receptor binding associated with nigrostriatal dopaminergic neurons in human basal ganglia. Ann Neurol 1992; 32: 339-4.

[2] Allen AM, Moeller I, Jenkins A, Zhuo J, Aldred GP, Chai SY, Mendelsohn FAO. Angiotensin receptors in the nervous system. Brain Res Bull 1998; 47: 17-8.

[3] Basso N, Paglia N, Stella I, Cavanagh EMV, Ferder L, Lores-Arnaiz MR, Inserra F. Protective effect of the inhibition of the renin-angiotensin system on ageing. Regul Pept 2005; 128: 247-52.

[4] Benarroch E, Schmeichel AM. Immunohistochemical localization of angiotensin type 1 receptor in human hypothalamus and brainstem. Brain Res 1998; 812: 292-96.

[5] Benzie IFF, Tomlinson B. Antioxidant power of angiotensin-converting enzyme inhibitors in vitro. Br J Clin Pharmacol 1998; 45: 168-9.

[6] Borawska MH, Kucharewicz G, Wisniewski K. The role of dopamine in the facilitatory effect of angiotensin II on impaired learning in rats chronically treated with ethanol. Psychopharmacology 1988; 96:500-04

[7] Brown D C, Steward LJ, Ge J, Barnes NM. Ability of angiotensin II to modulate striatal dopamine release via AT_1 receptor in vitro and in vivo. British J Pharmacol 1996; 118: 414-20

[8] Brownfield MS, Reid IA, Ganten D, Ganong WF. Differential distribution of immunoreactive angiotensin and angiotensin-converting enzyme in rat brain. Neuroscience 1982; 7: 1759-69.

[9] Cai H. NAD(P)H oxidase-dependent self-propagation of hydrogen peroxide and vascular disease. Circ Res 2005; 96: 818-22.

[10] Cavanagh E, Piotrkowski B, Basso N, Stella I, Inserra F, Ferder L, Fraga CG. Enalapril and losartan attenuate mitochondrial dysfunction in aged rats. FASEB J 2003; 1096-98.

[11] Chabrashvili T, Kitiyakara C, Blau J, Karber A, Islam S, Welch WJ, Wilcox CF. Effect of Ang II type 1 and 2 receptors on oxidative stress, renal NAD(P)H oxidase, and SOD expresión. Am J Physisiol Regu Integr Comp Physiol 2003; 285: R117-24.

[12] Chai SY, Johnston CL. Tissue distribution of angiotensin converting enzyme. In: LaragJH, Brenner BM (Ed). Hypertension: Pathophysiology, Diagnosis and Management. New York: Raven Press 1995; pp 1683-93.

[13] Chai SY, McKenzie JS, McKinley MJ, Mendelsonhn FAO. Angiotensin converting enzyme in human basal forebrain and midbrain visualized by in vitro autoradiography. J Comp Neurol 1990; 291: 179-94

[14] Chai SY, Mendelsohn FAO, Paxinos G. Angiotensin converting enzyme in rat brain visualized by quantitative in vitro autoradiography. Neuroscience 1987; 20: 615-27.

[15] Daubert DL, Meadows GG, Wang JH, Sanchez PJ, Speth RC. Changes in angiotensin II receptors in dopamine-rich regions of the mouse brain with age and ethanol consumption. Brain Res 1999; 816: 8-16.

[16] Dogan DM, Sumners C, Broxson CS, Clark N, Tümer N. Central angiotensin II increases biosynthesis of tyrosine hydroxylase in the rat adrenal medulla. Biochem Biophys Res Com 2004; 313: 623-26.

[17] Gao HM, Liu B, Hong JS. Critical role for microglial NADPH oxidase in rotenone-induced degeneration of dopaminergic neurons. J Neurosci 2003; 23: 6181-87.

[18] Gao HM, Liu B, Zhang W, Hong JS. Synergistic dopaminergic neurotoxicity of MPTP and inflammogen lipopolysaccharide: relevance to the etiology of Parkinson´s disease. FASEB J 2003; 17: 1957-59.

[19] Gao HM, Liu B, Zhang W, Hong JS. Synergistic dopaminergic neurotoxicity of MPTP and inflamogen lipopolysaccharide: relevance to the etiology of Parkinson's disease. FASEB J 2003; 17:1957-59.

[20] Ge J, Barnes NM. Alterations in angiotensin AT_1 and AT_2 receptor subtype levels in brain regions from patients with neurodegenetative disorders. Eur J Pharmacol 1996; 297: 299-306.

[21] Gendron L, Payet MD, Gallo-Payet N. The angiotensin type 2 receptor of angiotensin II and neuronal differentiation: from observations to mechanisms. J. Mol. Endocrinol 2003; 31: 359-72.

[22] Glinka Y, Tipton KF, Youdim MBH. Mechanism of mitochondrial respiratory complex I by 6-hydroxydopamine and its prevention by desferrioxamine. Eur J Pharmacol 1998; 351: 121-29.

[23] Griendling KK, Sorescu D, Ushio-Fukai M. NADPH oxidase. Role in cardiovascular biology and disease. Circulation Res 2000; 86: 494-501.

[24] Gundersen HJG, Bendsen TF, Korbo L, Marcussen N, Moller A, Nielsen K. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. APIMS 1988; 96: 379-94.

[25] Ichiki T, Takeda K, Tokunou T, Funakoshi Y, Ito K, Iino N, Takeshita A. Reactive species-mediated homologous downregulation of angiotensin II type 1 receptor mRNA by angiotensin II. Hypertension 2000; 37: 535-40.

[26] Intebi AD, Flaxman MS, Ganong WF, Deschepper CF. Angiotensinogen production by rat astroglial cells in vitro and in vivo. Neuroscience 1990; 34: 545-54.

[27] Jellinger K, Linert L, Kienzl E, Herlinger E, Youdim MBH. Chemical evidence for 6-hydroxydopamine to be an endogenous toxic factor in the pathogenesis of Parkinson's disease. J Neural Transm 1995; 46: 297-314.

[28] Jenkins TA, Chai SY, Howells DW, Mendelsohn FAO. Intrastriatal angiotensin II induces turning behaviour in 6-hydroxydopamine lesioned rats. Brain Res 1995; 691: 213-16.

[29] Jenner P. Oxidative stress in Parkinson´s disease. Ann Neurol 2003; 53: S26-38.

[30] Kim AM, Shin K-S, Chung Y-B, Jung KW, Cha CI, Shin DH. Immunohistochemical study of $p47^{Phox}$ and $gp91^{Phox}$ distributions in rat brain. Brain Res 2005; 1040: 178-86.

[31] Kimura S, Zhang G-X, Nishiyama A, Shokoji T, Yao L, Fan Y-Y, Rahman M, Abe Y. Mitochondria-derived reactive oxygen species and vascular MAP kinases. Comparison of Angiotensin II and Diazoxide. Hypertension. 2005; 45: 438-44.

[32] Konings CH, Kuiper MA, Bergmans PL, Grijpma AM, van Kamp GJ, Wolters EC. Increased angiotensin-converting enzyme activity in cerebrospinal fluid of treated patients with Parkinson´s disease. Clin Chim Acta 1994; 23: 101-06.

[33] Kumar R, Agarwal AK, Seth PK. Free radical-generated neurotoxicity of 6-hydroxydopamine. J Neurochem 1995; 64: 1703-07.

[34] Kusaka I, Kusaka G, Zhou C, Ishikawa M, Nanda A, Granger DN, Zhang JH, Tang J. Role of AT_1 receptors and NAD(P)H oxidase in diabetes-aggravated ischemic brain injury. Am J Physiol Heart Circ Physiol 2004; 286: H2442-51.

[35] Lebrun CJ, Mollenhoff E, Bao G, Culman J, Unger T. Angiotensin II induces the expression of c-fos mRNA in the central nervous system of the rat. Clin Exp Hypertens 1995; 17: 877-93.

[36] Lee J, Adachi K, Gionhaku N, Fujita S, Uchida T, Gerstner GE, Koshikawa N. Evidence that angiotensin II enhances apomorphine-induced jaw movements via AT1 receptors in the ventrolateral striatum: studies by magnet-sensing system in freely moving rats. Methods Find Exp Clin Pharmacol 2004; 26:195-99

[37] Lenaz G. Role of mitochondria in oxidative stress and ageing. Biochim Biophys Acta 1998; 1366: 53-67.

[38] Lenkei Z, Palkovits M, Corvol P, Llorens-Cortes C. Expression of angiotensin type-1 (AT_1) and type 2 (AT_2) receptor mRNAs in the adult rat brain: A functional neuroanatomical review. Front Neuroendocrinol 18, 383-39.

[39] Lin J-J, Yueh K-C, Chang D-C, Lin S-Z. Association between genetic polymorphism of angiotensin-converting enzyme gene and Parkinson disease. J Neurol Sci 2002; 199: 25-9.

[40] Liu YH, Yang XP, Mehta D, Bulagannawar M, Scicli GM, Carretero OA. Role of kinins in chronic heart failure and in the therapeutic effect of ACE inhibitors in kinogen-deficient rats. Am J Physiol Heart Circ Physiol 2000; 278: H507-14.

[41] Lopez-Real A, Rey P, Soto-Otero R, Mendez-Alvarez E, Labandeira-Garcia JL. Angiotensin-converting enzyme inhibitors reduce oxidative stress and protect dopaminergic neurons in a 6-hydroxydopamine rat model of parkinsonism. J Neurosci Res 2005; 81: 865-73.

[42] Lopez-Real A. Influencia del sistema renina-angiotensina (RAS) sobre la degeneracion del sistema nigroestriatal dopaminergico inducida por 6-OHDA. In: Analisis experimental de nuevas estrategias terapeuticas para la enfermedad de

Parkinson. Ph. D. Thesis, University of Santiago de Compostela, Spain; 2003, p. 173-87.

[43] Lou M, Blume A, Zhao Y, Gohlke P, Deuschl G, Herdegen T, Culman J. Sustained blockade of brain AT_1 receptors before and after focal cerebral ischemia alleviates neurologic deficits and reduces neuronal injury, apoptosis, and inflammatory responses in the rat. J Cereb Blood Flow Metab 2004; 24: 536-47.

[44] Lynch KR, Hawelu-Johnson CL, Guyenet PG. Localization of brain angiotensinogen mRNA by hybridization histochemistry. Mol Brain Res 1987; 2: 149-58.

[45] Markwell MAK, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 1978; 87: 206-210.

[46] McKinley MJ, Albiston AL, Allen AM, Mathai ML, May CN, McAllen RM, Oldfield BJ, Mendelsohn FAO, Chai SY. The brain reenin-angiotensin system: location and physiological roles. Int J Biochem Cell Biol 2003; 35: 901-18.

[47] Mendelsohn FAO, Jenkins TA, Berkovic SF. Effects of angiotensin II on dopamine and serotonin turnover in the striatum of conscious rat. Brain Res 1993; 613: 221-9.

[48] Münzel T, Keany JF. Are ACE inhibitors a “magic bullet” against oxidative stress? Circulation 2001; 104: 1571-77.

[49] Noh KM, Koh JY. Induction and activation by Zinc of NADH oxidase in cultured cortical neurons and astrocytes. J Neurosci 2000; 20: RC111 (1-5).

[50] Paxinos G, Watson C. The rat brain in stereotaxic coordinates. Academic Press, New York; 1986.

[51] Przedborski S, Jackson-Lewis V. Experimental developments in movement disorders: uptake on proposed free radical mechanisms. Curr Opin Neurol 1998; 11: 335-39.

[52] Quinlan JT, Phillips MI. Immunoreactivity for angiotensin II-like peptide in the human brain. Brain Res 1981; 205: 212-18.

[53] Rodriguez M, Barroso-Chinea P, Abdala P, Obeso J, Gonzalez-Hernandez T. Dopamine cell degeneration induced by intraventricular administration of 6-hydroxydopamine in the rat: similarities with cell loss in Parkinson's disease. Exp Neurol 2001; 169: 163-81.

[54] Rodriguez-Pallares J, Quiroz CR, Parga JA, Guerra MJ, Labandeira-Garcia JL. Angiotensin II Increases Differentiation of Dopaminergic Neurons from Mesencephalic Precursors via Angiotensin Type 2 Receptors. Eur J Neurosci 2004; 20: 1489-98.

[55] Rueckschloss U, Quinn MT, Holtz J, Morawietz H. Dose–dependent regulation of NAD(P)H oxidase expression by angiotensin II in human endothelial cells. Protective effect of angiotensin II type 1 receptor blockade in patients with coronary artery disease. Artrioscler Thromb Vasc Biol 2002; 22: 1845-51.

[56] Ruiz-Ortega M, Lorenzo O, Ruperez M, Esteban V, Suzuki Y, Mezzano S, Plaza JJ, Egido J. Role of the renin-angiotensin system in vascular diseases. Expanding the field. Hypertension 2001; 38: 1382-87.

[57] Serrano F, Kolluri NS, Wientjes FB, Card JP, Klann E. NADPH oxidase immunoreactivity in the mouse brain. Brain Res 2003; 998: 193-98.

[58] Simonnet G, Giorguieff-Chesselet MF, Carayon A, Bioulac B, Cesselin F, Glowinski J, Vincent JD. Angiotensin II and the nigrostriatal system. J Physiol 1981; 77: 71-79.

[59] Sohn HY, Raff U, Hoffmann A, Gloe T, Heermeier K, Galle J, Pohl U. Differential role of angiotensin II receptor subtypes on endothelial superoxide formation. Br J Pharmacol. 2000;131:667-72

[60] Soto-Otero R, Méndez-Álvarez E, Hermida-Ameijeiras A, Muñoz-Patiño A, Labandeira-García JL. Autoxidation and neurotoxicity of 6-hydroxydopamine in the presence of some antioxidants: potential implication in relation to the pathogenesis of Parkinson´s disease. J Neurochem 2000; 74: 1605-12.

[61] Stornetta RL, Hawelu-Johnson CL, Guyenet PG, Lynch KR. Astrocytes synthesize angiotensinogen in brain. Science 1988; 242: 1444-46.

[62] Touyz RM. Reactive oxygen species and angiotensin II signaling in vascular cells - implications in cardiovascular disease. Braz J Med Biol Res 2004; 37: 1263-73.

[63] Unger T, Chung O, Csikos T, Culman J, Gallinat S, Gohlke P, Höhle S, Meffert S, Stoll M, Stroth U, Zhu Y-Z. Angiotensin receptors. J Hypertens 1996; 14 (suppl. 5): S95-103.

[64] Van Muiswinkel FL, de Vos RAI, Bol JGJM, Andringa G, Jansen Steur ENH, Ross D, Siegel D, Drukarch B. Expression of NAD(P)H:quinine oxidoreductase in the normal and Parkinsonian substantia nigra. Neurobiol Aging 2004; 25: 1253-62.

[65] Wang G, Anrather J, Huang J, Speth RC, Pickel V, Iadecola C. NADPH oxidase contributes to angiotensin II signalling in the nucleus tractus solitarius. J Neurosci 2004; 24: 5516-24.

[66] West MJ, Slomianka L, Gundersen HJG. Unbiased stereological estimation of the total number of neurons of the rat hippocampus using the optical fractionator. Anat Rec 1991; 231: 482-97.

[67] Zalba G, San Jose G, Moreno MU, Fortuño MA, Fortuño A, Beaumont FJ, Diez J. Oxidative stress in arterial hypertension. Role of NADPH oxidase. Hypertension 2001; 38: 1395-99.

FIGURE LEGENDS

Fig. 1. Microphotographs showing changes in tyrosine hydroxylase-immunoreactivity (TH-ir) 1 week post-lesion at rostral, central and caudal levels of the substantia nigra in group-A rats injected intraventricularly with vehicle (i.e. controls; A), with 6-OHDA + vehicle (B), with 6-OHDA + Angiotensin II (AII; C) or with 6-OHDA + ZD 7155 (D). The number of TH-ir cells (i.e. spared dopaminergic neurons) was higher in the treated group (D) than in the rats that did not receive ZD 7155 (B, C). Scale bar = 600 μm

Fig. 2. Microphotographs showing changes in the number of tyrosine hydroxylase-immunoreactive (TH-ir) cells 1 week post-lesion at rostral levels of the substantia nigra in group-A rats injected intraventricularly with vehicle (i.e. controls; A), with 6-OHDA + vehicle (B), with 6-OHDA + Angiotensin II (AII; C) or with 6-OHDA + ZD 7155 (D). The number and immunoreactivity of TH-ir cells (i.e. spared dopaminergic neurons) was higher in the treated group (D) than in the rats that did not receive ZD 7155 (B, C). Scale bar = 75 μm

Fig. 3. Microphotographs showing changes in striatal tyrosine hydroxylase-immunoreactive (TH-ir) terminal density 1 week post-lesion in group-A rats injected intraventricularly with vehicle (i.e. controls; A, magnified in F), or with 6-OHDA + vehicle (B, G), or with 6-OHDA + Angiotensin II (AII; C, H), or with 6-OHDA + ZD 7155 (D, I), or with 6-OHDA + AII + ZD 7155 (E, J) . TH-ir terminal density (i.e.

spared dopaminergic terminals) was higher in the treated groups (D, E) than in the rats that did not receive ZD 7155 (B,C). Scale bar = 1 mm (A-E) and 30 μm (F-J).

Fig. 4. Dopaminergic neurons in the substantia nigra compacta (A) and density of striatal dopaminergic terminals estimated as optical density (B), 1 week post-surgery in the different experimental groups. Optical densities (B) are expressed as percentages of the values obtained in the control groups. Data are means ± S.E.M. obtained from six animals (n = 6). Means that differ significantly are indicated by a different letter. P< 0.05, one-way ANOVA and post-hoc Student-Newman-Keuls test. 6-OHDA, 6-hydroxydopamine; AII, angiotensin II; apo, Apocynin; DA, dopamine; TH, tyrosine hydroxylase; ZD, ZD 7155.

Fig. 5. Levels of lipid peroxidation (assessed by the formation of TBARS; A, B) and protein carbonyl content (C, D) induced in the striatum (A, C) and ventral midbrain (B, D) in control rats and rats treated with intraventricular administration of angiotensin II, or ZD 7155, or 6-OHDA + vehicle, or 6-OHDA + angiotensin II, or 6-OHDA + ZD 7155, or 6-OHDA + AII + ZD. Data are means ± S.E.M. from five rats (*n*= 5). Statistical significance at *P*<0.05, one-way ANOVA and post-hoc Student-Newman-Keuls test. 6-OHDA, 6-hydroxydopamine; AII, angiotensin II; ZD, ZD 7155.