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Review Article

Brain angiotensin and dopaminergic degeneration: relevance to Parkinson's disease

Jose L Labandeira-Garcia, Jannette Rodriguez-Pallares, Ana I Rodríguez-Perez, Pablo Garrido-Gil, Begoña Villar-Cheda, Rita Valenzuela, Maria J Guerra

Laboratory of Neuroanatomy and Experimental Neurology, Department of Morphological Sciences, Faculty of Medicine, University of Santiago de Compostela, Santiago de Compostela; Spain. Networking Research Center on Neurodegenerative Diseases (CIBERNED), Spain

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Abstract: The pathogenic mechanism of Parkinson's disease (PD) appears to be multifactorial. However, oxidative stress and neuroinflammation, including activation of NADPH-dependent oxidases, play a major role in the progression of dopaminergic cell death. The renin-angiotensin system (RAS) was described as a circulating humoral system that regulates blood pressure and water homeostasis. However, there exist local RAS in many tissues, and locally formed angiotensin activates NADPH-dependent oxidases, which are a major source of superoxide and are upregulated in major aging-related diseases such as hypertension, diabetes and atherosclerosis. Furthermore, an intracellular or intracrine RAS, with still unknown functions, has been identified in several cell types. The brain has an independent local RAS, which has been involved in several brain disorders, including neurodegenerative diseases. It is particularly interesting for PD the important interaction observed between angiotensin and dopamine, which counterregulate each other in renal cells and also in the striatum and substantia nigra. In recent studies, we have observed both a local and an intracellular RAS in the rodent, monkey and human substantia nigra, and that dopamine depletion induced RAS upregulation possibly as a compensatory mechanism. However, RAS hyperactivation also exacerbated oxidative stress and neuroinflammation, which contributed to progression of dopaminergic degeneration. In addition, we observed increased RAS activity in the nigra of animals with higher vulnerability of dopaminergic neurons to degeneration, such as aged males, menopausal females and rats subjected to chronic brain hypoperfusion. RAS activity and dopaminergic vulnerability were significantly reduced by treatment with angiotensin type I receptor antagonists. Manipulation of the brain RAS may constitute an effective neuroprotective strategy against dopaminergic degeneration in PD.

Keywords: Aging, angiotensin, degenerative disease, dopamine, menopause, neurodegeneration, neuroinflammation, oxidative stress, Parkinson, renin-angiotensin system

The renin-angiotensin system (RAS)

The renin-angiotensin system (RAS) was initially considered as a circulating humoral system, which function is the regulation of blood pressure and sodium and water homeostasis. This circulating RAS induces vasoconstriction by enhancing norepinephrine release from sympathetic terminals, and also activates the release of aldosterone from the adrenal cortex and antidiuretic hormone from the neurohypophysis. 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 receptors: AII type 1 and 2 (AT1 and AT2) receptors [1, 2]. The AT1 receptor belongs to the superfamily of seven transmembrane domain, and the human AT1 gene is located in chromosome 3q and codes for a protein of 40-42KDa (359 amino acids). AT1 receptors mediate most of the classical peripheral actions of AII, including vasoconstriction, renal water and salt retention and facilitation of sympathetic transmission. The AT2 receptor consists of a protein made up of 363 aminoacids with seven hydrophobic transmembrane domains [3, 4] and the human AT2 gene is located on the X chromosome [5]. However, the function

of AT2 receptors remains more elusive and controversial. It is known that AT2 is ubiquitously expressed in developing fetal tissues, including brain, and decreases after birth to remain at lower levels in adult tissues. AT2 has been associated with modulation of cell proliferation, cell differentiation, apoptosis and regenerative processes [6-8]. Several recent studies have observed that AT2 receptors are expressed at a low density in many healthy adult tissues, but are upregulated in pathological circumstances. It is generally considered that AII, via AT2 receptor, exerts actions directly opposed to those mediated by AT1 receptors thus antagonizing many of the effects of the latter [9, 10]. However, the relationships between AT1 and AT2 are probably more complex and remain to be totally clarified. The classical circulating RAS has been considered phylogenetically one of the oldest hormone systems, which played a major role in the survival of mammals and in human evolution [11, 12], and renin was one of the first substances shown to exert physiological effects [13-15].

Over the last 2 decades, it has been shown that in addition to the "classical" humoral RAS there exists a second RAS or local or tissular RAS in many tissues, including brain tissue [16, 17]. This local system contains the different components previously described for the circulating RAS. The locally formed AII plays an important functional role in these tissues, and is particularly involved in local pathological changes (see below), as the local AII regulates many substances such as growth factors and cytokines, which are involved in processes such as cell growth/apoptosis and inflammation [18, 19]. Furthermore, it has been shown that reactive oxygen species (ROS) play a crucial role in the signaling of AII, via AT1 receptors, in several cell types [20, 21]. Local AII, via AT_1 receptors, is known to contribute to oxidative stress (OS) damage as a major activator of the NADPH-oxidase complex in several types of cells and tissues [20, 22]. The NADPH oxidase complex is the most important intracellular source of ROS other than mitochondria [23, 24]. Furthermore, ROS originated by NADPH oxidases favour their own production via mitochondria, intracellular iron uptake and other intracellular sources [25]. In addition, a number of studies have shown a ROS-mediated relationship (i.e. cross-talk signalling) between the NADPH oxidase complex and the mitochondria [26-28]. These feed-forward mechanisms form a vicious circle and may amplify and sustain ROS thus contributing to cell death. NADPH-dependent oxidases are upregulated in major aging-related diseases such as hypertension, diabetes and atherosclerosis [29, 30]. It is usually considered that AT2 receptor activation inhibits NADPH-oxidase activation and counteracts the deleterious effects of AT1 activation.

A better knowledge of the local RAS has led to identification of a number of new components of the RAS and new mechanisms involved in the RAS function. In addition to ACE, some homologue components such as ACE2 and Chymase have been described in several cell types [31-33]. In addition to AII, several angiotensin peptides such as angiotensin (1-7), angiotensin III and angiotensin IV have been involved in the functional effects of RAS [10]. Angiotensin IV has been suggested to exert functional effects via specific AT4 receptors [34], and angiotensin (1-7) appears to act via a new G-protein coupled receptor, Mas [35], which may counteract or downregulate the effects of stimulation of AT1 via AII, at least in some types of cells [36, 37]. The recent identification of a specific receptor for renin and its precursor prorenin (PRR) is particularly interesting [38, 39]. The receptor is expressed at relatively high levels in heart, brain, placenta and adipocytes, and at lower levels in other tissues [40, 41]. The presence of PRRs may explain that inhibition of AII was not sufficient to block the RAS activity entirely in several experimental situations [42, 43]. This receptor exerts dual molecular functions [38, 44]: (i) AII-dependent actions: binding of renin to its receptor increases the catalytic activity of renin by about 4-5 times, and binding of the precursor prorenin induces catalytic activity similar to that of renin to hydrolyse angiotensinogen into angiotensin, and (ii) AII-independent actions by triggering its own intracellular signaling cascade to induce effects similar to those demonstrated for AT1 receptors [45, 46]. A peptide called "handle region peptide" (HRP), which mimics part of the prosegment of prorenin is a potential inhibitor of PRRs [47, 48].

In addition to the "classical" humoral RAS and the local or tissue RAS, a number of recent studies support the existence of third level of RAS in several types of cells [49]: the intracellular or intracrine RAS. Several transmembrane receptors are known to accumulate in nuclei, particu-

larly in nuclear membranes, and in the cytoplasm. Cells such as cardiomyocytes possess AII receptors that couple to nuclear signaling pathways and regulate transcription. The observed intracellular location supports the possibility of an intracellular function for AII, in addition to the effects induced by activation of cell surface AT1 and AT2 receptors. Extracellular AII may act intracellularly by binding to AT1 receptors, which are subsequently internalized, or AII may be synthesized within the cell. AT1R-dependent internalization of AII has been described in a number of different cell types [50-52]. However, a number of recent observations in several types of cells suggest some AII may be formed and act intracellularly [49]. Furthermore, AII has been suggested to induce transcription of angiotensinogen and renin in response to binding to nuclear AT1 receptors in some cell types [51]. The existence of functional intracellular RAS opens up new perspectives for understanding the effects of the RAS and for the management of RAS-related diseases [53, 54].

The brain renin-angiotensin system

The effects of the circulating RAS on the brain were initially associated with areas involved in the central control of blood pressure and sodium and water homeostasis [55-58]. As active components of RAS, particularly AII, do not cross the barrier [59], AII receptors identified in circumventricular organs, which lack the blood-brain barrier, and in cerebrovascular endothelial cells, were considered responsible for a number of central responses induced by peripheral or circulating AII. However, AII receptors were also located in neurons and glial cells inside the blood-brain barrier, which suggested that brain has an independent local or tissue RAS. Over the last two decades, all components of the classical RAS have been identified in the brain [55-58]. It has been suggested that brain levels of AII are higher than circulating levels [60], and RAS components such as ACE, AT1, AT2, and AT4 receptors have been observed in different brain areas (see for review: 55-58). It is known that the precursor protein angiotensinogen is mainly produced by astrocytes [61, 62], although it is also produced at low levels in neurons [63, 64]. The existence of brain renin has been, a controversial matter since it was initially reported by Ganten in 1971 [65]. The controversial results were probably due to the low expression levels of renin, which were below the detection threshold of some immunohistochemical studies and other standard assays. However, immunoreactive renin has been observed in neurons and glial cells in numerous areas of mouse and rat brain [66, 67], and in all areas examined in the human brain, including basal ganglia [68]. Expression of renin mRNA by in situ hybridization was also observed in the brain [69, 70]. More recently the expression of renin in neurons and glial cells was clearly confirmed by the use of transgenic models [71-74]. However, it has been suggested that brain levels of AII may be too high in comparison with the levels of renin. This may now be explained by the recent location of prorenin/renin receptor (PRR) in the brain. High levels of PRR mRNA expression were initially observed in brain homogenates [38], and we have recently shown by in situ hybridization and immunofluorescent labeling abundant PRR in dopaminergic and non dopaminergic neurons and glial cells in the monkey and rat brain [75]. Binding of prorenin (i.e. a previously considered inactive precursor of renin) activates its catalytic activity, and prorenin to renin ratios are 5-10 times higher, and even up to 20-200 times higher in pathological conditions [76]. Finally, other components involved in the effects of AII observed in several peripheral tissues such as NADPH-oxidase have been shown to be widely distributed throughout the brain, and it was also observed that NADPH-oxidase-derived ROS also play a major role in AII signaling in neurons [77, 78] and glial cells [79, 80].

In the basal ganglia, the presence of RAS components has been reported in several studies over the last decades. Autoradiographic studies reported AT_1 receptors in dopaminergic neurons, both in cell bodies in the substantia nigra compacta (SNc) and their terminal fields in the striatum of different mammals, including humans [1, 81-83]. It was suggested that the density of AT_1 receptors is very high in human striatum and substantia nigra, in comparison with those in rats and other mammals [1, 81]. In a series of recent studies [75, 84, 85], we demonstrated, by immunofluorescence and laser confocal microscopy, the presence of AT_1 and AT_2 receptors in nigral dopaminergic neurons and glial cells (i.e. astrocytes and microglia) in rodents and primates, including human [86], as well as in primary mesencephalic cell cultures [7, 84, 85]. The presence of AT_1 and AT_2 mRNA was also confirmed by in situ hybridization and

real time quantitative PCR [84, 85]. High concentrations of ACE have been observed in the striatum and substantia nigra of mammals including rats and humans and angiotensinogen was observed in astrocytes [81, 87-89]. Furthermore, we demonstrated, by immunofluorescence and biochemical methods, the presence of different cytoplasmatic and membrane subunits of the NADPH complex in mesencephalic dopaminergic neurons, astrocytes and microglia, as well as NADPH-complex activity in the nigra and striatum [84, 85, 90]. Recently, we have described for the first time prorenin receptors (PRRs) in nigral dopaminergic neurons and microglial cells in monkeys and rats by use of immunofluorescence and in situ hybridization [75]. Interestingly, the labelling for PRR, AT_1 and AT_2 receptors was located not only at the cell surface but also intracellularly in dopaminergic neurons and glial cells in the substantia nigra of mammals, including monkeys and humans [86]. Therefore, our observations support the existence of an intracellular/intracrine RAS in the brain, and in the SNc in particular, as previously been suggested for other cell types [73, 91].

The brain renin angiotensin system in aging and disease

Recent studies in different tissues have shown that normal aging is associated with a proinflammatory and pro-oxidant state that may favour an exaggerated response to injury and degenerative diseases [92-94], and that local RAS, via AT_1 receptors, is involved in age related degenerative changes [95-98]. Under normal physiological conditions, the capacity of AII to promote ROS appears to be tightly regulated [22, 99, 100]. However aging has been shown to be associated with overactivation of RAS in a number of tissues [101-103]. In accordance with this, recent studies with AT_1 receptor deficient mice indicate that disruption of AT_1 promotes longevity through attenuation of OS and additional mechanisms such as upregulation of the prosurvival gene sirtuin 3 and mitochondrial protection [100, 104, 105]. Similarly, the absence of AT_1 receptors has been shown to protect against the aging-related progression of atherosclerosis [106]. NADPH oxidases are upregulated in several age-related diseases such as hypertension, diabetes, atherosclerosis, cardiac fibrosis, and renal disease [29, 30, 77, 107], and RAS is a major activator of the NADPH-oxidase complex (see above). In addition, AII, via AT_1 receptors, mediates several key events in inflammatory processes that play a major role in most of these diseases [20, 94, 108-110].

Similarly, numerous recent studies have involved brain RAS in disorders such as anxiety and stress [111], depressive illness [112], cognitive dysfunctions, and alcohol intake [113]. Inhibition of AT_1 receptors has been reported to improve learning, spatial working memory and motor performance in aged rats [114, 115]. In addition, the presence of NADPH oxidase has been shown in neurons and glial cells [20, 22, 116, 117]. Several studies have shown that, as observed in peripheral organs [18, 19], AT_1 receptor blockers and ACE inhibitors also decreased the inflammatory response in the central nervous system (CNS) [118, 119]. In accordance with their inhibitory effect on brain inflammation, beneficial effects AT1 inhibition have been observed in a number of processes mediated by microglial activation and neuroinflammation, including animal models of Alzheimer's disease [120, 121], brain ischemia [122, 123] and multiple sclerosis [118, 119]. In addition, we have obtained a considerable amount of experimental data that suggest a major role for the brain RAS in Parkinson's disease (PD), as detailed below.

Interaction between dopamine and angiotensin for regulation of peripheral tissue and brain functions

It is well known that the neurotransmitter dopamine is synthesized by mesencephalic neurons in the SNc and ventral tegmental area, and by some other groups of neurons such as hypothalamic neurons in the arcuate and periventricular nuclei [124]. SNc neurons innervate the striatum through the nigrostriatal pathway. Dopamine acts as a neuromodulator that controls important physiological functions such as voluntary movements, motivated behavior, learning and hormone production. Alterations in dopaminergic innervation are known to be involved in a number of diseases including depression, attention deficit disorders, schizophrenia, epilepsy, pituitary tumors, Huntington's disease and, particularly, PD. However, is usually not taken into account by neuroscientists that dopamine and dopamine receptors are located in a large number of peripheral tissues where they also play important functions. The interaction

between the RAS and the dopaminergic system is particularly interesting with regard to the regulation of renal sodium excretion and several cardiovascular functions [125-127]. Recent evidence suggests that dopamine and angiotensin systems directly counterregulate each other in renal cells [126] and that abnormal counterregulatory interactions between dopamine and AII play a major role in renal degenerative changes and hypertension [128]. In renal proximal tubule cells, important interactions between several types of dopamine receptors and AT1 receptors, as well as dimerization of AT1 receptors and dopamine receptors such as D1, D3 or D5 have been observed [125-127].

In the brain, an interaction between AII and dopamine was initially suggested by the results of early microdialysis studies, which showed that acute AII perfusion induces dopamine release, which was blocked by AT_1 antagonists [129, 130]. The mechanism responsible for the AII-induced dopamine release has not been clarified, although the possible involvement of D_2 autoreceptors has been suggested [129]. This suggestion is supported by a number of recent studies in peripheral tissues in which direct counter-regulatory interaction between AT_1 receptors and D_2 dopamine receptors has been observed [131, 132]. Interestingly, chronic inhibition of RAS by the use of ACE inhibitors or AT1 blockers resulted in increased dopamine levels [133-135], possibly as a consequence of compensatory changes in dopamine or AII receptors that remain to be clarified [133, 134]. In a recent study [136], we have shown similar functional interactions and counterregulatory mechanisms in the striatum and substantia nigra of rodents. We studied the effect of transitory reserpine-induced dopamine depletion and chronic 6-hydroxydopamine (6-OHDA)-induced dopaminergic degeneration on the expression of AII receptors and NADPH complex activation in the nigra and striatum. Depletion of dopamine with reserpine induced a significant increase in the expression of AT_1, AT_2 receptors and the NADPH-oxidase complex activity, which decreased as the dopamine function was restored. Similarly, 6-OHDA-induced chronic dopaminergic denervation led to significant increase in expression of AT_1, AT_2, receptors and NADPH-oxidase complex activity, which decreased with administration of L-dopa. Our data [136] suggest that the AT_1 receptor expression is closely linked to dopamine levels. In accordance with previous studies [9, 137, 138], oxidative stress induced via AT_1 receptors was apparently counteracted by protective counterregulatory AT_2 upregulation. Therefore, an upregulation of AT_1 receptors in the substantia nigra and striatum after decrease in dopamine levels (i.e. initial stages of PD) may be related to counterregulatory mechanisms to increase dopamine levels. However, the resulting RAS hyperactivation may also exacerbate the oxidative stress and microglial inflammatory response and contribute to further progression of dopaminergic neuron loss (see below).

Brain RAS and dopaminergic degeneration

In addition to the above mentioned interaction between RAS and dopamine in the basal ganglia, a number of data suggest that alteration in interactions between both systems may play a major role in PD. A number of recent studies suggest that neuroinflammation and oxidative stress play a pivotal role at least in the progression of PD, and RAS plays a key role in the initiation and perpetuation of inflammation and oxidative damage in several tissues (see above) [29, 18, 19, 21]. The pathogenic mechanism of PD appears to be multifactorial. It has been shown that several genes are mutated or deleted in familial PD. However, the etiology of sporadic, idiopathic PD, which accounts for most cases of PD cases, is still unclear. A number of mechanisms have been involved in dopaminergic neuron degeneration in PD, including mitochondrial dysfunction, oxidative stress, inflammation, and impairment of the ubiquitin-proteosome system [139]. These pathogenic factors are not mutually exclusive, and one of the key aims of current PD research is to discover the mechanisms involved in possible interactions between these pathways, which result in dopaminergic neuron degeneration. Several studies have provided evidence that OS plays a major role in all forms of PD [140-143]. There has been some discussion as to whether OS is a primary event or a consequence of other pathogenic factors. However, dopaminergic degeneration is unquestionably mediated by overproduction of ROS and reactive nitrogen species (RNS). A number of factors are thought to be involved in the higher vulnerability of dopaminergic neurons to OS, including increased iron content, reduced antioxidant capacity or factors associated with the dopamine synthesized, released and metabolized in these neurons. The protective defense mechanisms for dopaminergic neurons may be overwhelmed by

additional deleterious factors in neurons already particularly vulnerable (i.e. a "synergistic effect hypothesis"). Furthermore, neuroinflammation plays a major role in the progression of dopaminergic cell death, since a marked microglial reaction has been observed in the nigra and striatum of brains from both PD patients [144] and PD animal models [90, 145, 146]. It has been suggested that this may be a response to dopaminergic cell death in order to eliminate dead neurons and other debris, as observed in several autoimmune diseases [147]. However, several experimental studies have shown that microglial activation and microglial NADPH-derived ROS constitute an early component of dopaminergic cell death and that both factors act synergistically with other factors to induce dopaminergic cell death at early stages of the lesion process [79, 80, 90, 148]. We suggest that the brain RAS plays a major role in this process, since several major factors involved in dopaminergic degeneration (i.e. main sources of ROS such as NADPH-oxidase complex and inflammation) have been shown to be enhanced by RAS activation in several peripheral tissues, and more recently in the SNc, as detailed below.

In a series of studies in animal models of PD and cultures of dopaminergic neurons or glial cells we have shown that AII, via AT_1 receptors, exacerbates dopaminergic cell death and may play a synergistic role in the pathogenesis and progression of PD. Firstly, we treated animal models of PD (rats lesioned with the dopaminergic neurotoxin 6-OHDA and mice lesioned with the dopaminergic neurotoxin MPTP) with ACE inhibitors (ACEi) [149, 150]. The animals treated with ACEi showed a significant decrease in the loss of dopaminergic neurons in the nigra and dopaminergic terminals in the striatum, as well as a significant decrease in the levels of oxidative stress indicators (lipid peroxidation and protein oxidation) induced by the dopaminergic neurotoxins in the ventral mesencephalon and striatum. Secondly, rats lesioned with 6-OHDA and mice lesioned with MPTP were treated with angiotensin and AT_1 or AT_2 receptor antagonists [84, 85, 151]. We observed that AII increased the neurotoxic effect induced by dopaminergic neurotoxins, and that blockage of AT_1 receptors led to significant reduction in the loss of dopaminergic neurons and levels of protein oxidation and lipid peroxidation induced by the neurotoxins. Interestingly, the neuronal loss was also reduced by apocynin, an inhibitor of the NADPH-oxidase activation, which suggested that NADPH activation and NADPH-derived ROS were involved in the dopaminergic neuron death. This was confirmed in subsequent experiments focused on the mechanisms involved in the observed effects of AII and detailed below.

In contrast with the considerable amount of recent experimental data from our laboratory and others [152, 153] supporting the involvement of brain RAS in dopaminergic degeneration, data from clinical studies are still scarce. Early neuropathological studies reported a marked reduction in AT_1 receptors in the striatum of PD patients, which was attributed to the loss of dopaminergic terminals [1, 81], although our data in animal models treated with L-dopa [136] suggest that it was possibly more closely related to the L-dopa treatment received by those patients. More interestingly, increased ACE activity in the cerebrospinal fluid of patients with PD has been reported [154], as well as an association between genetic polymorphism of the ACE gene and PD [155]. The use of several types of antihypertensive drugs and risk of PD was evaluated in a case-control analysis [156]. It was concluded that the risk was not materially altered for users of ACE inhibitors, and that the exposure to AT1 antagonists only was too low for a meaningful analysis. However, the methodology of this study has been questioned as the authors focused their main analyses on "current use" of antihypertensives (at least one prescription during the 90 days preceding the date of the first recording of a diagnosis of PD), and not during a relevant period of exposure [157]. Other studies in Parkinson's disease patients treated with the ACE inhibitor Perindopril revealed positive effects and improved motor responses to L-dopa [135], and positive or negative effects of ACE inhibitors or AT1 antagonists have been observed in single case reports [158]. Additional clinical studies with a more robust design are necessary.

Enhanced RAS activity and dopaminergic vulnerability. Aging, menopause and brain hypoperfusion

Brain RAS, aging and dopaminergic vulnerability

In additional series of experiments, we studied if enhanced RAS activity in the nigra may be involved in the increased vulnerability of dopa-

minergic neurons to degeneration observed in aging, post-menopause or chronic cerebral hypoperfusion. Aging is the most prominent risk factor for PD and other neurodegenerative diseases [159-161]. Furthermore, the progressive motor impairment that occurs during normal aging has been associated with nigrostriatal dysfunction, and several studies have shown that the dopaminergic system is altered during normal aging [159, 162]. There is no consensus about how advancing age may affect PD. Several factors such as neurotoxicity derived from dopamine metabolism (i.e. the "dopamine oxidative stress hypothesis") or an aging-related decrease in neurotrophic factors may be involved. In summary, several recent studies suggest that in the nigra, as in other tissues (see above), normal aging is associated with a proinflammatory and pro-oxidant state that may favour an exaggerated response to injury and degenerative diseases [92-94], and act synergistically with other factors to induce dopaminergic cell death. Aging has been shown to be associated with overactivation of RAS in a number of tissues [101-103], and AII, via AT_1 receptors, contributes to OS damage and inflammatory responses in several types of cells and tissues [20, 22, 95-98]. Here, we suggest that aging-enhanced activity of nigral RAS plays a major role in this process, which was confirmed in animal models of PD.

In a recent study with aged male rats [163], we have confirmed that aging enhances the dopaminergic cell death induced by dopaminergic neurotoxins [94, 159, 161, 164], and that nigral RAS is involved. We observed increased activation of the NADPH oxidase complex and increased levels of the pro-inflammatory cytokines IL-1β and TNF-α in aged rat, which indicated a pro-oxidative and pro-inflammatory state in the nigra. This was associated with increased expression of AT_1 receptors and decreased expression of AT_2 receptors, and was reduced by treatment with the AT_1 antagonist candesartan. The observed upregulation of AT_1 receptors in aged rats may contribute to increased dopaminergic cell vulnerability to degeneration. This is supported by experiments with PD animal models [84, 85, 151], in which we have observed that AII enhanced neuroinflammation, NADPH-derived OS and dopaminergic cell death via AT_1 receptors. However, it is also interesting that we observed decreased expression of AT_2 receptors in aged rats. It is known that AT_2 receptors counterbalance the deleterious effect of AT_1 receptor stimulation, and functional interactions between the two receptor subtypes may determine the AII-induced effects [165]. In aged rats, there was an apparent absence of a counterregulatory increase in AT_2 expression (i.e. the expression of AT_2 mRNA and protein was decreased) despite increased expression of AT_1 receptors and increased NADPH activation [136, 163]. Interestingly AT2 expression was increased by treatment with candesartan. A decreased expression of AT2 receptors in aged animals may contribute to further enhancement of a pro-oxidative, pro-inflammatory state and dopaminergic cell vulnerability in aged animals. However, changes in AT2 receptor expression may be involved in unknown mechanisms that remain to be clarified. The mechanism responsible for the increased RAS activity in the nigra of aged animals has not been clarified. Interestingly, several studies have shown that there is an aging-related decrease in dopamine release, which cannot be totally counteracted by functional compensatory changes and results in a progressive decrease in motor activity [160, 166]. Furthermore, dopamine and AII systems directly counterregulate each other and there is a negative reciprocity between dopamine and AT_1 receptors [136]. Therefore, the upregulation of AT_1 receptors that we observed in aged rats [163] may be part of the compensatory changes to increase dopamine levels. However, increased RAS activity via AT_1 receptors may also induce the above mentioned pro-inflammatory, pro-oxidative state, which may be further enhanced by a lack of compensatory upregulation of AT_2 receptors in aged rats. Other mechanisms may also be involved in aging-related enhanced RAS activity, since increased RAS activity has been observed in other aged tissues (i.e. apparently non dopamine-related tissues) [95-97].

Brain RAS, menopause and dopaminergic vulnerability

In addition to aging, menopause has also been identified as a prominent risk factor for PD. Numerous experimental studies have shown that oestrogen exerts protective effects against dopaminergic cell degeneration [167, 168], and a number of epidemiological studies have reported that the incidence and prevalence of PD is higher in postmenopausal than in premeno-

pausal women of similar age [169-171]. However, controversial effects of estrogen replacement therapy have been also reported [172, 173], and the age of the women receiving the treatment appears to be a major factor in the discrepancies. The mechanism by which estrogen protect dopaminergic neurons has not been clarified, although recent studies have suggested that modulation of the glial neuroinflammatory response by estrogen is involved [174, 175]. Interestingly, estrogen-induced regulation of the RAS mediates beneficial effects of oestrogen in several tissues [176-178], and interactions between oestrogen and AII receptors have also been observed [179-182]. Therefore, the lack of oestrogen may act as an additional factor for increasing RAS activity in the nigra in aged females. In a recent study [183], we used young ovariectomized rats to investigate this question (i.e. in the absence of other potential aging-related factors). We studied the effect of ovariectomy and estrogen replacement on the nigral RAS and on dopaminergic degeneration induced by intrastriatal injection of 6-OHDA, and observed a marked loss of dopaminergic neurons in ovariectomized rats lesioned with 6-OHDA, which was significantly reduced by oestrogen replacement or treatment with the AT_1 receptor antagonist candesartan. We also observed that estrogen replacement induced significant downregulation of the ACE activity as well as downregulation of AT_1 receptors, upregulation of AT_2 receptors and downregulation of the NADPH complex activity in the substantia nigra in comparison with untreated young ovariectomized rats. Together the results confirm that the lack of oestrogen may act as an additional factor for increasing RAS activity in the nigra in females. In aged females, however, additional factors may come into play. In recent experiments [184], we compared the above mentioned results in young ovariectomized rats (i.e. early surgical menopause) with those obtained in aged rats (i.e. natural menopause). Interestingly, both groups of menopausal rats showed increased RAS activity. However, oestrogen therapy significantly reduced 6-OHDA-induced dopaminergic cell loss in young rats but not in aged rats, and the changes in RAS activity were not restored in aged rats by oestrogen to levels observed in young menopausal rats treated with oestrogen. Treatment with the AT_1 antagonist candesartan significantly reduced RAS activity and dopaminergic neuron loss in both groups of menopausal rats. These results may explain the reason for the discrepancies between some experimental studies undertaken in young ovariectomized animals and epidemiological studies in aged menopausal women. It may also explain the discrepancies between observational studies that have supported the concept that oestrogen therapy in postmenopausal women protects against aging-related diseases, including PD, and several randomized controlled trials that reported no or even detrimental effects [185-187]. The vast majority of women who engaged in these trials were on average 65 years or older, and 12 years postmenopause before oestrogen therapy [188, 189]; on the contrary, most women initiated replacement therapy in their perimenopausal period in observational studies that reported beneficial effects [190-192].

Brain RAS, brain hypoperfusion and dopaminergic vulnerability

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 (almost 40%) [193], presynaptic dopaminergic function is reduced in the majority of patients with vascular parkinsonism [194], and a subset of patients with clinically suspected vascular parkinsonism were found to have a good therapeutic response to L-dopa [195, 196]. These clinical observations have experimentally been confirmed in a recent study with animal models of chronic brain hypoperfusion [197], in which we have shown that chronic hypoperfusion induces a significant loss of dopaminergic neurons and a significant decrease in striatal dopaminergic terminals and striatal dopamine levels. Furthermore, we observed that hypoperfusion led to increased dopaminergic cell death by enhancing the deleterious effects of other factors (such as the low doses of the dopaminergic neurotoxins), which suggests that 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.

The mechanistic links between hypoperfusion/vascular disease and neurodegeneration are unknown. However, we observed an age-

dependent decrease in nigral vascularisation and nigral vascular endothelial growth factor (VEGF) levels [198], and that that chronic hypoperfusion led to increased expression of inflammatory markers such as IL-1β and increased levels of oxidative stress markers such as NADPH activity [197], which have been shown to be involved in progression of dopaminergic cell death in animal models of PD and PD patients [80, 199]. Interestingly, these changes were accompanied by increased RAS activity in the substantia nigra, and chronic treatment with the AT1 receptor antagonist candesartan significantly reduced OS and inflammatory markers as well as the loss of dopaminergic neurons, striatal dopaminergic terminals and striatal dopamine levels [197].

Mechanisms involved in the effects of brain RAS on dopaminergic degeneration

We used 6-OHDA or MPTP models of parkinsonism and primary cultures of dopaminergic neurons to study the possible mechanisms involved in the above mentioned effects [84, 85, 200, 201]. We first treated the cultures with low doses of 6-OHDA or MPP^+, which did not induce a significant loss of dopaminergic neurons, and observed that the loss of neurons increased significantly when the cultures were simultaneously treated with AII. This effect was blocked by treatment with AT_1 antagonists but not with AT_2 antagonists. Interestingly the enhancing effect AII on dopaminergic cell death in cultures was also reversed by apocynin, indicating that NADPH activation and NADPH-derived superoxide anion and ROS are involved. This was also confirmed by real time quantitative PCR, which revealed that treatment with AII induced an increased expression of NADPH subunits via protein kinase C [85]. The effects of AII and AII receptor antagonists on NADPH-oxidase activation in dopaminergic neurons and glial cells were studied by detection of intracellular superoxide anion with dihydroethidium, after treatment of primary mesencephalic cultures with dopaminergic neurotoxins (i.e. 6-OHDA or MPP^+). Levels of intracellular superoxide increased in dopaminergic neurons and microglial cells after treatment with AII and decreased after treatment with AT_1 antagonists or the NADPH-oxidase inhibitor apocynin [84, 85].

As AII receptors and NADPH subunits were observed in both dopaminergic neurons and glial cells, AII may induce dopaminergic degeneration through several mechanisms, as previously observed in the vessel wall, where this question has been extensively studied as chronic inflammation is the hallmark of atherosclerosis. AII acts in this process on at least two levels [18, 19]. Firstly, AII acts on the resident vascular cells (i.e. endothelial cells, smooth muscle cells, or neurons in the brain), in which via AT1 receptors stimulates production of low levels of intracellular ROS by activation of NADPH oxidase. ROS act as second messengers on several signalling pathways, including those involved in triggering the inflammatory response and the migration of inflammatory cells into the lesioned area. Secondly, AII acts on inflammatory cells (such as microglial cells in the brain), in which NADPH oxidase produces ROS with dual functions: i) high concentrations of ROS are released extracellularly for killing invading microorganisms or cells; ii) low levels of intracellular ROS act as a second messenger in several signalling pathways involved in the inflammatory response [24, 108]. As observed for vascular tissues, the presence of NADPH oxidase and AT1 and AT2 receptors was observed in nigral microglia and dopaminergic neurons [75, 84, 85]. It was also shown that AII via AT1 receptors, activates the microglial NADPH-complex and exacerbates the glial inflammatory response [84, 85, 151]. In neurons and other non-inflammatory cells, activation of the NADPH oxidase complex produces low levels of ROS for signalling function [24]; these ROS also modulate neuronal levels of ROS by interaction with mitochondria-derived ROS, and with ROS from other sources such as neurotoxins or activated microglia. Cross-talk signaling between NADPH oxidase and mitochondria has been observed in several types of cells. This not only includes an upstream role for NADPH oxidase in the modulation of mitochondrial superoxide [202, 203] but also that mitochondrial superoxide stimulates extramitochondrial NADPH oxidase activity in a feed-forward fashion [204, 205]. This interaction has recently been confirmed in a dopaminergic cell line treated with MPP^+ and angiotensin [153]. Treatment with MPP^+ induced mitochondrial release of ROS, which induced a second wave of NADPH oxidase-derived ROS; the latter was reduced by treatment with the AT1 antagonist candesartan [153]. Using primary cultures of mesencephalic cells, we have recently shown that mitochondrial ATP-sensitive potassium channels play a major role in the

interaction between NADPH-derived ROS and mitochondria after treatment with AII and/or dopaminergic neurotoxins such as MPP^+ and 6-OHDA [206, 207].

AT1, AT2 receptors and NADPH oxidase are present in dopaminergic neurons as well as in microglia, and inhibition of neuronal AT1 receptors may reduce ROS derived from neuronal NADPH, as indicated above. This may lead to direct inhibition of dopaminergic neuron death, followed by a subsequent reduction in microglial activation. However, this possibility is not supported by our studies. Using neuron-enriched primary mesencephalic cultures we have observed that only high doses of neurotoxins can induce dopaminergic neuron death in the absence of glia [84, 90, 206, 207]. This has been confirmed in a recent study with a dopaminergic cell line (i.e. in the absence of glia [153], as significant cell death was only observed after treatment with very high doses of MPP^+ (300μM). However, in our studies, we investigated the effects of very low or sublethal doses of neurotoxins, because the effects of these low doses may be more similar to the effects caused by environmental neurotoxins or by other deleterious factors involved in PD. Low or sublethal doses of neurotoxins do not induce significant neuron death in pure neuronal cultures. However, sublethal insults can induce neuron derived proinflammatory signals which, in the presence of glia, trigger microglial activation and the subsequent increase in microglia-derived ROS and cytokines, which induce the progression of neuronal death [108, 208]. Furthermore, other studies have shown that microglial activation and free radicals derived from microglial NADPH play a major role in the toxicity of MPTP and possibly in PD, and that lesioned dopaminergic neurons are particularly vulnerable to microglial NADPH-derived ROS [79, 80, 148].

A number of recent studies have revealed additional details on cellular mechanisms that mediate or are involved the AII-induced effects described above. Firstly, we have recently shown the presence of prorenin/renin receptors in neurons and microglial cells of the SNc in primates and rats [75], and in primary rat mesencephalic cultures, we observed that PRRs contribute to dopaminergic neuron degeneration and potentially to progression of PD. This may be due to the above mentioned role of PPRs in generation of AII by binding renin and prorenin. However, administration of renin with simultaneous blockage of AT_1 and AT_2 receptors has also been found to lead to an increase in cell death induced by low doses of 6-OHDA [127]. This suggests that AII-independent PRR intracellular signaling also contributes to exacerbation of dopaminergic cell death, and that potential neuroprotective strategies to decrease RAS activity should address AII generation and/or signalling and PRR signalling. Recent studies with AT1 antagonist telmisartan and AT1 –deficient mice have shown that activation of peroxisome proliferator-activated receptor gamma (PPAR-γ) mediates the neuroprotective and anti-inflammatory effects of AT1 receptor inhibition in animal models of PD [200]. It has also been shown that activation of the RhoA/ROCK pathway is involved in the MPTP-induced dopaminergic degeneration, and in the enhancing effect of AII/AT1 activation on the microglial response and dopaminergic degeneration [201]. It is known that RhoA/ROCK is an important regulator of the actin cytoskeleton, which is particularly important for migration of inflammatory cells into inflamed areas [209, 210], including microglia [211]. It has been shown that during activation of inflammatory cells Rho/ROCK induces changes in the actin cytoskeleton that results in process retraction, cell spreading and changes in cell motility characteristics of activation of inflammatory cells such as microglia [212]. Finally, we have recently shown that, in addition to the presence of a local or tissular RAS in the substantia nigra, there is an intracellular or intracrine RAS in dopaminergic neurons and glial cells of mammals, including monkeys and humans [86]. The functional role of the intracellular RAS and the functional interactions between both systems remain to be clarified.

Conclusions

Local brain RAS activation is involved in exacerbation of oxidative stress and neuroinflammation, which leads to progression of dopaminergic degeneration and Parkinson's disease. Increased RAS activity was observed in the substantia nigra of animals with high vulnerability of dopaminergic neurons to degeneration, such as aged males, menopausal females and rats subjected to chronic brain hypoperfusion. Increased RAS activity may constitute a major factor in the increased risk of developing PD in these population groups. Manipulation of the brain RAS may constitute an effective neuropro-

tective strategy in population groups at high risk of developing PD, or for coadjutant treatment to reduce the progression of PD.

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Address correspondence to: Dr. Jose L Labandeira-Garcia, Department of Morphological Sciences, Faculty of Medicine, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain. Tel: +34 -881812223; Fax: +34-881812338; E-mail: joseluis.labandeira@usc.es

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