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Review

Aging, Angiotensin System and Dopaminergic Degeneration in the Substantia Nigra

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ABSTRACT: For years, the renin-angiotensin system (RAS) was described as a circulating humoral system that regulates blood pressure and water homeostasis. Angiotensin II (AII) is the most important effector peptide. However, in addition to the "classical" humoral RAS there exist local RAS in many tissues and locally formed AII 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. Accordingly, disruption of AII receptors promotes longevity in mice. The brain has an independent local RAS, which was also initially associated with the central control of blood pressure. However, more recent studies have involved brain RAS in brain disorders, including neurodegenerative diseases. The interaction between AII and dopamine is particularly interesting. Recent evidence suggests that dopamine and AII systems directly counterregulate each other in renal cells as well as in the striatum and substantia nigra. Dopamine depletion may induce RAS upregulation as a potential compensatory mechanism. However, RAS hyperactivation also exacerbates NADPH-oxidase activity, oxidative stress and the microglial inflammatory response and contribute to progression of dopaminergic neuron loss, as observed in recent studies with animal models of Parkinson's disease (PD). Aging is the most prominent risk factor for PD and other neurodegenerative diseases. Interestingly, we observed increased activation of the NADPH oxidase complex and increased levels of the pro-inflammatory cytokines in the nigra of aged male rats, which was associated with increased RAS activity and was reduced by treatment with AII antagonists. We also observed that the lack of oestrogen may act as an additional factor for increasing RAS activity in the nigra in aged females, which was significantly reduced by treatment with AII antagonists. Manipulation of the brain RAS may constitute an effective neuroprotective strategy against the aging-related risk of dopaminergic degeneration.

Key words: Dopamine; Menopause; Neurodegeneration; Neuroinflammation; Oxidative stress; Parkinson; Renin-angiotensin-system

Dopaminergic degeneration in the substantia nigra and Parkinson's disease

Alterations in dopaminergic innervation are known to be involved in a number of diseases including depression, attention deficit disorders, schizophrenia, epilepsy, pituitary tumours, Huntington's disease and, particularly, Parkinson's disease (PD).

The neurotransmitter dopamine (DA) is synthesized by mesencephalic neurons in the substantia nigra compacta (SNc) and ventral tegmental area (VTA), and by some other groups of neurons such as hypothalamic neurons in the arcuate and periventricular nuclei [1]. SNc neurons innervate the striatum through the nigrostriatal pathway. DA acts as a neuromodulator that controls important

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physiological functions such as voluntary movements, motivated behaviour, learning and hormone production.

PD is the second most common neurodegenerative disease after Alzheimer's disease. The incidence of PD is estimated to be 8-18 per 100000 person-years and affects more than 1% of those older than 60 years and up to 4% of those older than 80 years [2]. PD is a neurodegenerative disease characterized by progressive degeneration of DA-containing neurons in the SNc and by the presence of intraneuronal proteinaceous cytoplasmic inclusions known as Lewy bodies. This leads to a marked deficiency in striatal DA, which causes the major clinical symptoms of PD: akinesia, muscular rigidity and resting tremor [3, 4]. In PD, clinical signs are usually detected when approximately 50% of nigral neurons and 80% of striatal DA are lost. It is known, however, that other areas of the brain may be affected in PD, and that lesions also occur outside the central nervous system (CNS) such as in the enteric nervous system [5].

Although the clinical phenotype of PD is relatively homogeneous the pathogenic mechanism appears to be multifactorial. It has been shown that several genes are mutated or deleted in familial PD. However, the aetiology of sporadic, idiopathic PD, which accounts for most cases of PD cases, is still unclear. A number of mechanisms have been involved in DA neuron degeneration in PD, including mitochondrial dysfunction, oxidative stress, inflammation, and impairment of the ubiquitin-proteosome system [6]. 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 DA neuron degeneration. Several studies have provided evidence that oxidative stress (OS) plays a major role in all forms of PD [7, 8], and there has been some discussion as to whether OS is a primary event or a consequence of other pathogenic factors. However, DA degeneration is unquestionably mediated by overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS are generated as a result of normal metabolism. However, OS occurs when ROS or RNS are produced in excess or are insufficiently degraded and overwhelm the protective defence mechanisms of a cell, leading to functional impairment and finally cell death [8]. The brain is particularly susceptible to OS, since it consumes about 20% of the total body O_2, and DA nigrostriatal neurons appear particularly vulnerable to OS-derived cell death [9, 10]. A number of factors are thought to be involved, including increased iron content, reduced antioxidant capacity or factors associated with the DA synthesized, released and metabolized in these neurons. The protective defence mechanisms for DA neurons may be overwhelmed by additional deleterious factors in neurons already subjected to DA-derived toxicity thus leading to DA neuron death (i.e. a "synergistic effect hypothesis").

In the present review article we suggest that the brain renin angiotensin system (RAS) plays a major role in this process, since several major factors involved in DA degeneration (i.e. main sources of ROS such as NADPH-oxidase complex and inflammation) have been shown to be enhanced by RAS activation in various peripheral tissues, and more recently in the SNc in several studies in our laboratory.

NADPH complex activation, neuroinflammation and dopaminergic degeneration

The NADPH oxidase complex is the most important intracellular source of ROS other than mitochondria [11, 12]. Furthermore, ROS originated by NADPH oxidases favour their own production via mitochondria, intracellular iron uptake and other intracellular sources [13]. 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 [14-16]. These feed-forward mechanisms form a vicious circle and may amplify and sustain ROS thus contributing to cell death. NADPH oxidases are upregulated in several age-related diseases such as hypertension, diabetes and atherosclerosis [17-20], and RAS is a major activator of the NADPH-oxidase complex (see below). Recent studies have shown the presence of NADPH oxidase in neurons and glial cells [21-24] and also that microglial activation and NADPH-derived free radicals play major roles in cell death induced by DA neurotoxins [25-28] and possibly in the toxicity of environmental pesticides and other factors which can induce PD.

NADPH oxidase is a multi-component enzyme composed of 3 cytosolic proteins or subunits (p40, p47, and p67) and at least 2 membrane proteins (gp91 and p22). These components are spatially separated and the complex is inactive. The NADPH complex becomes assembled and activated following stimulation. The best known NADPH oxidase is that of phagocytes-neutrophiles and monocytes. In these cells and microglial cells in the nervous system, the enzyme produces large quantities of extracellular superoxide and other reactive oxidants that are used for the purpose of killing invading microorganisms or cells [11, 12]. Superoxide may exert its toxicity by converting to highly reactive tissue damaging species such as hydrogen peroxide, and peroxynitrite after reacting with NO.

NADPH-derived ROS may also act indirectly by inducing the release of proteases [29]. In addition, NADPH produces intracellular ROS that act on intracellular signalling pathways to induce microglial activation and amplify the production of proinflammatory molecules [30]. Recent studies have shown that a number of cell types (including neurons and glial cells) contain NADPH oxidases. However, in contrast to the high production of oxidants by phagocytes, other cell types produce ROS at low rate and have a signalling function. It has been suggested that NADPH-induced ROS may initially have developed as an intracellular signalling mechanism common to all cell types, and later as a specialized defence system in macrophage cells [11, 12].

Neuroinflammation plays a major role in the progression of DA cell death. A marked microglial reaction has been observed in the nigra and striatum of brains from both PD patients [31] and PD animal models [28, 32, 33]. It has been suggested that this may be a response to DA cell death in order to eliminate dead neurons and other debris, and that the inflammatory response around the dead neurons and terminals may induce non-specific damage to other neurons and contribute to the long term progression of DA cell death, as observed in some autoimmune diseases [34]. However, several experimental studies have indicated that microglial activation and NADPH-derived ROS constitute an early component of DA cell death and that both factors act synergistically with other factors to induce DA cell death at early stages of the lesion process [25-28].

Dopaminergic degeneration and aging

Aging is the most prominent risk factor for PD and other neurodegenerative diseases [35-37]. Furthermore, the progressive motor impairment that occurs during normal aging has been associated with nigrostriatal dysfunction, and several studies have shown that the dopaminergic (DA) system is altered during normal aging [38]. Some early studies have suggested that there is a progressive loss of neurons in the SNc of human brains with advancing age, and losses of more than one-third may occur between 20 and 90 years of age, at a rate of 5-10% loss per decade [39, 40]. Thus, PD was once considered to be a form of accelerated aging, so that exogenous insults that damage DA neurons may exacerbate the progressive age-related loss of neurons. Recent studies suggest, however, that aging does not induce a significant loss of DA neurons but rather induces changes in soma size, tyrosine hydroxylase (TH) expression and DA activity, which may increase the vulnerability of DA neurons to toxic damage and increase the risk of developing PD [35, 41]. There is no consensus about how advancing age may affect PD. Several recent studies suggest that aging-induced changes may increase the vulnerability of DA neurons to toxic damage and increase the risk of developing PD [35, 41]. Several factors such as neurotoxicity derived from DA metabolism (i.e. the "dopamine oxidative stress hypothesis") or an aging-related decrease in neurotrophic factors may be involved. We have recently observed age-related decreases in microvascularization and angiogenic factors such as VEGF (vascular endothelial growth factor) in the nigra of aged rats, which interestingly may be reduced by physical exercise [42]. In summary, several recent studies suggest that in the nigra, as in other tissues, normal aging is associated with a proinflammatory, pro-oxidant state that may favour an exaggerated response to injury and degenerative diseases [43-45], and act synergistically with other factors to induce DA cell death. Here, we suggest that aging-enhanced activity of nigral RAS plays a major role in this process.

The renin angiotensin system (RAS)

The RAS is phylogenetically one of the oldest hormone systems, and renin was one of the first substances shown to exert physiological effects. In 1898, Tigersted and Bergman [46] observed that injection of renal extracts exerted profound effects on blood pressure. The octapeptide angiotensin II (AII), a product of the catalytic action of renin was isolated in 1940 by Baun-Menendez et al. [47] and Page and Helmer [48]. For years, the renin-angiotensin system (RAS) was described as a circulating humoral system that regulates blood pressure and sodium and water homeostasis. The 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. The RAS, therefore, appears to have played a major role in the survival of mammals and in human evolution [49, 50]. 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 (AT_1 and AT_2) receptors [51, 52]. The AT_1 receptor belongs to the superfamily of seven transmembrane domain, and the human AT_1 gene is located in chromosome 3q and codes for a protein of 40-42KDa (359 amino acids). Most species express a single autosomal AT_1 gene, but two related AT_1a and

AT_1b receptor genes are expressed in rodents (95% identical in their aminoacid sequences). Like other G-protein coupled receptors, AT_1 receptors are desensitized and internalized after agonist stimulation [53, 54]. AT_1 receptors mediate most of the classical peripheral actions of AII, including vasoconstriction, renal water and salt retention and facilitation of sympathetic transmission.

The AT_2 receptor consists of a protein made up of 363 aminoacids with seven hydrophobic transmembrane domains [55, 56] and the human AT_2 gene is located on the X chromosome [57]. However, the function of AT_2 receptors remains more elusive and controversial. It is known that AT_2 is ubiquitously expressed in developing fetal tissues, including brain, and decreases after birth to remain at lower levels in adult tissues. AT_2 has therefore been associated with modulation of cell proliferation, cell differentiation, apoptosis and regenerative processes [58-60]. However, several recent studies have observed that AT_2 receptors are expressed at a low density in many healthy adult tissues, but are upregulated in pathological circumstances. In adult tissues, AT_2 receptors exert actions directly opposed to those mediated by AT_1 receptors thus antagonizing many of the effects of the latter [61, 62].

The local or tissue renin angiotensin system

It is now generally accepted that in addition to the "classical" humoral RAS there exist local RAS in many tissues, including brain tissue [63, 64], and that locally formed AII regulates many substances such as growth factors and cytokines, which are involved in processes such as cell growth/apoptosis and inflammation [65, 66]. Interestingly, it has been shown that reactive oxygen species (ROS) play a crucial role in the signalling of AII, via AT_1 receptors, in several cell types [21, 67]. Local AII, via AT_1 receptors, activates NADPH-dependent oxidases (see above) [21, 22], which are a major source of superoxide (O_2^-) and are upregulated in major aging-related diseases such as hypertension, diabetes and atherosclerosis [17, 19]. However, AT_2 receptor activation inhibits NADPH-oxidase activation. Local or tissue effects of RAS (i.e. tissue RAS) were initially reported in the arterial wall and the link between local RAS and the pathophysiology of the vascular disease is well established. It appears that AII acts on at least two levels in this process. Firstly, AII acts on the resident vascular cells (i.e. endothelial cells, smooth muscle cells); on these cells, AII via AT_1 receptors stimulates production of ROS by activation of NADPH, and produces chemokines, cytokines, and adhesion molecules which contribute to the migration of inflammatory cells into the injured tissue. Secondly, AII acts on inflammatory cells; it has been suggested that during the activation of inflammatory cells there is an activation of their RAS [68, 69], and that cells such as neutrophils, monocytes and lymphocytes have AT_1 receptors and are activated by AII to induce inflammatory responses and to release high levels of ROS mainly by activation of their NADPH complex [21, 30].

Emerging aspects of the renin angiotensin system

In addition to the above mentioned major components of the RAS, several other components have been found to be involved in secondary mechanisms of RAS function. ACE_2 is a more recently discovered homologue of ACE, with 56% homology with the terminal domain of ACE [70]. In contrast to the wide distribution of ACE, ACE_2 expression was initially though to be restricted to a few cell types. However, a more widespread distribution is emerging [71]. AII levels may also be regulated by chymase as an alternative pathway to ACE in some cell types, particularly in disease conditions [72]. In addition to AII, several angiotensin peptides such as angiotensin (1-7), angiotensin III and angiotensin IV have been recognised as important [62]. AT_4 receptors are known to be identical to insulin-regulated amino peptidase and angiotensin IV and AT_4 ligands activate several intracellular second messenger systems [73]. Angiotensin (1-7) appears to act via a new G-protein coupled receptor, Mas [74], and it has been suggested that this system may counteract or downregulate the effects of stimulation of AT_1 via AII, at least in some types of cells [75, 76].

The identification of a specific receptor for renin and its precursor prorenin (PRR) is particularly interesting [77, 78]. The receptor is expressed at relatively high levels in heart, brain, placenta and adipocytes, and at lower levels in other tissues [79, 80]. Blockage of AII generation and signalling has been widely used to prevent progression of organ damage in cardiovascular and renal diseases. However, it has been reported that progression of the disease is delayed, but not totally abolished, and that inhibition of AII is not sufficient to block the RAS activity entirely [81, 82]. This and other unclear aspects of the function of tissue RAS may be explained by the discovery of the prorenin receptor (PRR). This receptor exerts dual molecular functions [77, 83]: (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 pro-renin induces catalytic activity similar to that of renin to hydrolyse angiotensinogen into angiotensin, and (ii) AII-independent actions by triggering its own intracellular

signalling cascade to induce effects similar to those demonstrated for AT_1 receptors [84, 85]. A peptide called "handle region peptide" (HRP), which mimics part of the prosegment of prorenin is a potential inhibitor of PRRs [86, 87].

Finally, a number of recent studies support the existence of an intracellular/intracrine RAS in several types of cells [88, 89]. Therefore, in addition to the "classical" humoral RAS and the local or tissue RAS, 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 [90, 91].

The renin angiotensin system in disease and aging

As commented above, AII, via AT_1 receptors, is known to contribute to oxidative stress damage as a major activator of the NADPH-oxidase complex in several types of cells and tissues [21, 22]. Upregulation of NADPH-oxidases plays a main role in the pathogenesis of major aging-related diseases such as hypertension, diabetes, arteriosclerosis, cardiac fibrosis, renal disease, neurodegenerative diseases (see below) and others [17, 19, 20]. In addition, AII, via AT_1 receptors, mediates several key events in inflammatory processes that play a major role in most of these diseases [21, 30, 45, 92, 93].

Numerous recent studies in different tissues have shown that normal aging is associated with a proinflammatory, pro-oxidant state that may favour an exaggerated response to injury and degenerative diseases [43-45], and that local RAS, via AT_1 receptors, is involved in age related degenerative changes [94-97]. Under normal physiological conditions, the capacity of AII to promote ROS appears to be tightly regulated [22, 98, 99]. However, aging has been shown to be associated with overactivation of RAS in a number of tissues [100-102]. 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 [99, 103, 104]. Similarly, the absence of AT_1 (AT_{1A}) receptors has been shown to protect against the age-related progression of atherosclerosis [105].

The brain renin angiotensin system

The AII receptors in circumventricular organs, which lack the blood-brain barrier, and in cerebrovascular endothelial cells respond to the peripheral or circulating AII. However, the brain AII receptors located in neurons and glial cells inside the blood-brain barrier respond to the existence of an endogenous RAS system, since active components of RAS, such as AII, do not cross the barrier [106]. In accordance with this, beyond the actions of the peripheral RAS components, a number of studies have shown that the 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 [107-110], although a considerable number of questions remain to be clarified. It is well known that the astroglia is the main site of angiotensinogen synthesis in the brain [111, 112], although it is probably produced at low levels in neurons [113, 114]. It has been suggested that brain levels of AII are higher than circulating levels [115], and classical RAS components such as ACE, AT_1, AT_2, and AT_4 receptors have been observed in different brain areas (see for review: 107-110). ACE occurs widely in the brain and is associated with the endothelium of cerebral blood vessels, epithelial cells of the choroids plexus and the plasma membranes of astrocytes and neurons. Furthermore, it has recently been shown that NADPH-oxidase is widely distributed throughout the brain, and that NADPH-oxidase-derived ROS play a major role in AII signalling in neurons [18, 116] and in oxidative damage induced by microglial cells [26, 27].

However, the existence of brain renin has been a controversial matter since it was initially reported by Ganten in 1971 [117]. 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 [118, 119] and in all areas examined in the human brain, including basal ganglia [120]. Expression of renin mRNA by hybridization histochemistry was also observed in the brain [121, 122]. More recently the expression of renin in neurons and glial cells was clearly confirmed by the use of transgenic models [123-126]. However, it has been suggested that brain levels of AII may be too high in comparison with the levels of renin. This may be explained by the recent location of prorenin/renin receptor (PRR) in the brain. High levels of PRR mRNA expression were initially reported [77], and we have recently observed by in situ hybridization and immunofluorescent labelling abundant PRR in dopaminergic and non dopaminergic neurons and glial cells in the monkey and rat brain [127]. 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 [128].

The brain renin angiotensin system and disease

The brain RAS was initially associated with areas involved in the central control of blood pressure and sodium and water homeostasis [107-110]. However, more recent studies have involved brain RAS in additional brain functions and disorders such as anxiety and stress [129], depressive illness [130], cognitive functions, and alcohol intake [131]. Inhibition of AT_1 receptors has been reported to improve learning, spatial working memory and motor performance in aged rats [132, 133]. In addition, several studies have shown that AT_1 receptor blockers and ACE inhibitors prevent the onset of stroke [134], as well as the subsequent brain damage by reducing the inflammatory response [135, 136]. Interestingly, the results of a number of molecular, genetic and clinical studies support a relation between RAS and Alzheimer's disease (for review see: 137-139). In recent years, we have obtained a considerable amount of experimental data that suggest a major role for the brain RAS in Parkinson's disease, as detailed below.

The brain renin angiotensin system and the dopaminergic system

Over the last decades, several studies have reported the presence of RAS components in the basal ganglia and high concentrations of ACE have been observed in the striatum and substantia nigra of mammals including rats and humans [140-143]. Autoradiographic studies reported AT_1 receptors in DA neurons, both in cell bodies in the substantia nigra and their terminal fields in the striatum of different mammals, including humans [51, 143-145]. Furthermore, some of these studies have 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 [51, 143]. In a series of recent studies [127, 146, 147], 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 (unpublished), as well as in primary mesencephalic cell cultures [59, 146, 147]. The presence of AT_1 and AT_2 mRNA and protein was also confirmed by in situ hybridization and real time quantitative PCR [146, 147]. Angiotensinogen was observed in astrocytes. 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.

More 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 [127]. Interestingly, the labelling for PRR was located not only at the cell surface but also intracellularly. PRR labelling was mainly observed at the nuclear level, which was confirmed by use of Hoechst stain as a nuclear marker, although extranuclear labelling was also observed. Several studies have reported that PRRs are located at the cell surface [77]. However, a detailed study of the subcellular location of PRR, by use of fractionated protein isolation followed by Western blotting of HeLa-S3 cells, revealed a preferential intracellular presence of PRRs, particularly at the level of the nuclear envelope and the endoplasmic reticulum, in addition to the cell surface location [84]. The discrepancy may be explained by cell-type differences. PRRs may internalize renin and prorenin, or a nonsecreted (i.e. intracellular) renin may interact directly with the intracellular PRRs. Several transmembrane receptors are known to accumulate in nuclei and, particularly, nuclear membranes. Cells such as cardiomyocytes possess AII receptors that couple to nuclear signaling pathways and regulate transcription. This supports the possibility of an intracellular function for AII, in addition to that induced by activation of cell surface AT_1 and AT_2 receptors. Extracellular AII may act intracellularly by binding to the AT_1 receptors, which are subsequently internalized, or AII may be synthesized within the cell. AT_1-dependent internalization of AII has been described in a number of different cell types, including neurons [148-150]. However, the demonstration of intracellular location of PRRs in DA neurons suggests that some AII may be formed intraneuronally, as previously suggested for heart cells [88]. Intracellular AII has been suggested to induce transcription of angiotensinogen and renin in response to binding to nuclear AT_1 receptors in some cell types [148]. In accordance, intraneuronal angiotensinogen and intraneuronal forms of renin have been observed [114, 125]. Therefore, our observation of intracellular PRRs supports the existence in the brain, and in DA neurons in particular, of an intracellular/intracrine RAS, which has previously been suggested for several cell types [89, 125].

Angiotensin and dopamine interaction

RAS appears to be involved in several brain functions. However, the interaction between AII and DA is particularly interesting. An important interaction between DA and AII receptors in peripheral tissues has been demonstrated in several recent studies, particularly with regard to the regulation of renal sodium excretion and cardiovascular function [151-153]. Recent evidence

suggests that DA and AII systems directly counterregulate each other in renal cells [152] and that abnormal counterregulatory interactions between DA and AII play a major role in degenerative changes and hypertension [154]. In a recent study [155], 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 DA depletion and chronic 6-hydroxydopamine (6-OHDA)-induced DA 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 and AT_2, receptors and NADPH-oxidase complex activity, which decreased with administration of L-dopa.

A significant reduction in expression of AT_1 mRNA was also observed after administration of dopamine to cultures of microglial cells [155]. The observed changes in AT_1 mRNA expression in microglia cultures with respect to controls lacking DA are consistent with the above mentioned results of in vivo experiments: AT_1 expression (increased by DA depletion) decreased with L-DOPA administration to levels of controls containing DA, and also decreased as the dopamine function was restored in rats treated with reserpine. However, the response of microglial cells is particularly interesting since we have shown that AT_1 activation induces microglial activation, and microglial activation contributes to progression of DA degeneration (see below). Furthermore, the effects of microglial DA receptor stimulation on microglial AT_1 receptor expression in pure microglial cultures reveals direct crosstalk/interaction between dopamine and angiotensin receptors. It has been shown that the precursor of AII angiotensinogen is produced by astrocytes, and no significant changes in levels of extracellular AII can be expected in the absence of astrocytes. This is in accordance with several recent studies on non neural cells, particularly renal proximal tubule cells, which have shown important and direct effects/interactions between several dopamine receptors and AT_1 receptors, including possible dimerization of AT_1 receptors and several dopamine receptor subtypes such as D_1-D_3-D_5 [151-153]. However, we have also observed that changes in AII levels may affect AII receptor expression (i.e. an indirect mechanism). Transgenic rats with very low levels of brain AII showed increased AT_1 expression, and administration of AII (100nM) to primary mesencephalic cultures decreased the expression of AT_1 [155]. In summary, our data suggest that the AT_1 receptor expression is closely linked to DA levels and that both direct (interaction between DA and AT_1 receptors) and indirect (i.e. due to changes in AII levels) mechanisms may be involved.

In accordance with previous studies [61, 156, 157], oxidative stress induced via AT_1 receptors was counteracted by protective counterregulatory AT_2 upregulation, which induces decreased NADPH complex activity. The upregulation of AT_1 receptors in DA neurons and terminals after DA depletion may be related to counter-regulatory mechanisms to increase DA levels. AII-induced DA release is supported by the results of earlier microdialysis studies showing that acute AII perfusion induces DA release, which is blocked by AT_1 antagonists [158, 159]. The mechanism responsible for the DA release has not been clarified, although the possible involvement of D_2 autoreceptors has been suggested [158]. This suggestion is supported by a number of recent studies in which direct counter-regulatory interaction between AT_1 receptors and D_2 dopamine receptors has been observed [160, 161]. Interestingly, chronic inhibition of RAS by use of ACE inhibitors resulted in increased DA levels [162-164], possibly as a consequence of compensatory changes such as those mentioned above, although compensatory changes in striatal enkephalin or tachyquinin levels have also been suggested [162, 163]. In summary, DA depletion may induce RAS upregulation as a potential compensatory mechanism. However, RAS hyperactivation may also exacerbate the OS and microglial inflammatory response and contribute to further progression of DA neuron loss as described below.

RAS hyperactivity in the basal ganglia and dopaminergic degeneration

As with early cardiovascular studies, which only focused on the functional control of blood pressure by RAS (in part by vasoconstriction induced by the release of catecholamines by neuronal terminals), a possible effect of AII on death/survival of DA neurons was not considered in early studies on CNS and basal ganglia RAS. However, a number of findings suggest that, as previously observed in cardiovascular diseases, RAS dysfunction 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 DA neurons. A number of recent studies suggest that neuroinflammation and oxidative stress play a pivotal role at least in the progression of PD, and that RAS plays

a key role in the initiation and perpetuation of inflammation and oxidative damage in several tissues (see above) [17, 65-67]. In addition, some data suggested the potential involvement of RAS in PD. A marked reduction in AT_1 receptors has been observed in the striatum of PD patients, and attributed to the loss of DA terminals [51, 143], although we believe that it was possibly more closely related to the L-dopa treatment [155]. More interestingly, increased ACE activity in the cerebrospinal fluid of patients with PD has been reported [165], as well as an association between genetic polymorphism of the ACE gene and PD [166]. On this basis, in a series of recent studies in animal models of PD and cultures of DA neurons or glial cells we have shown that AII, via AT_1 receptors, exhacerbates DA cell death and may play a synergistic role in the pathogenesis and progression of PD.

In a first set of experiments, we lesioned rats with the DA neurotoxin 6-OHDA and mice with the DA neurotoxin MPTP, and treated the animals with ACE inhibitors (ACEi) [167, 168]. The animals treated with ACEi showed a significant decrease in the loss of DA neurons in the nigra and DA 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 DA neurotoxins in the ventral mesencephalon and striatum. However, independently of their effect on the RAS, ACE inhibitors themselves may have antioxidant properties, and ACE may also be involved in hydrolyzation of several other neuropeptides such as bradykinin, which may be involved in the neuroprotective effects. Therefore, in additional studies we treated rats with low doses of 6-OHDA together with angiotensin (through an intraventricular cannula, because 6-OHDA and AII do not cross the blood-brain barrier) and AT_1 or AT_2 receptor antagonists [169]. We observed that AII increased the neurotoxic effect induced by 6-OHDA. Furthermore, blockage of AT_1 receptors led to significant reduction in the loss of DA neurons and levels of protein oxidation and lipid peroxidation induced by the DA neurotoxin 6-OHDA and AII. Interestingly, the neuronal loss induced by 6-OHDA and AII 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 lesion. This was confirmed in more recent experiments detailed below.

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 [146, 147]. We first treated the cultures with low doses of 6-OHDA or MPP^+, which did not induce a significant loss of DA 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 DA 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 [147]. The effects of AII and AII receptor antagonists on NADPH-oxidase activation in DA neurons and glial cells were studied by detection of intracellular superoxide anion with dihydroethidium, after treatment of primary mesencephalic cultures with DA neurotoxins (i.e. 6-OHDA or MPP^+). Levels of intracellular superoxide increased in DA neurons and microglial cells after treatment with AII and decreased after treatment with AT_1 antagonists or the NADPH-oxidase inhibitor apocynin [146, 147].

As AII receptors and NADPH subunits were observed in both DA neurons and glial cells, AII may induce DA degeneration through several mechanisms, as previously observed in the vessel wall (i.e. acting on the resident cells and/or the inflammatory cells). Firstly, AII may increase levels of oxidative stress in DA neurons by acting via neuronal AT_1 receptors and the neuronal NADPH complex [116, 170]. ROS derived from neuronal NADPH may act synergistically with ROS derived from intraneuronal 6-OHDA or MPTP, or other factors in PD, to increase OS in DA neurons. However, in cultures in which glial cells were eliminated (i.e. neuron enriched cultures), AII did not significantly increase the effect of low doses of neurotoxins, suggesting that glial cells play a major role in the increasing effect of AII on dopaminergic cell loss and that ROS derived from activation of intraneuronal NADPH are not sufficient, acting synergistically with the neurotoxins, to induce significant DA cell loss. Extracellular ROS derived from glial cells therefore play a major role in this effect. Both AII receptors and NADPH subunits were observed in astrocytes and microglial cells. However, several previous studies have shown that microglia, but not astroglia, significantly enhance the progression of DA degeneration [25, 26]. It is known that microglial NADPH-derived superoxide plays a critical role in DA neuron degeneration induced by DA neurotoxins such as rotenone and MPTP [25-27], and that microglial NADPH oxidase activation and superoxide production enhance not only extracellular levels of ROS but also the release of pro-inflammatory factors from microglia [30]. Data from in vivo experiments with animal models of PD also suggested a

major role for AII-enhanced microglial activation in AII-enhanced DA cell death [146, 147]. Treatment with different AT_1 receptor antagonists decreased neurotoxin-induced DA cell death, as well as the nigral and striatal microglial activation induced by the neurotoxins (i.e. 6-OHDA or MPTP), which confirmed in animal models the involvement of the microglial cells observed in the in vitro experiments.

In summary, our data suggest that RAS hyperactivity is involved in degeneration of DA neurons and the progression of PD, and that microglial derived oxidative stress and neuroinflammation play a major role in this effect. This does not exclude the possibility that AII may act on DA neurons by additional mechanisms similar to those recently observed in other tissues, and that are currently under investigation in our laboratory. In fact, we have recently shown the presence of prorenin/renin receptors (PRRs) in neurons and microglial cells of the SN in primates and rats [127] (see above). In primary rat mesencephalic cultures, we observed that PRRs contribute to the previously reported effects of RAS on DA neuron degeneration and potentially to progression of PD. Administration of the PRR blocker HRP (handle region peptide) has been found to lead to a significant decrease in 6-OHDA-induced dopaminergic cell death in cultures, which may be due to decreased generation of AII. 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 signalling 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.

Aging-related RAS hyperactivity and dopaminergic degeneration

An age-related proinflammatory, pro-oxidant state in the nigra may increase the vulnerability of dopaminergic neurons to additional damage, which may explain why aging is the most prominent risk factor for PD. Local RAS via AT_1 receptors is involved in age-related degenerative changes in several tissues [94-97]. We therefore wondered if enhanced RAS activity in the nigra may be involved in the increased vulnerability of DA neurons in relation to aging. In a recent study with aged male rats [171], we have confirmed that aging enhances the DA cell death induced by DA neurotoxins [35, 37, 45, 172], 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 rats, which indicate pro-oxidative, 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 DA cell vulnerability. This is supported by the above described experiments with PD animal models [146, 147, 169], in which we have observed that AII enhanced neuroinflammation, NADPH-derived OS and DA cell death via AT_1 receptors. This was also confirmed in aged rats because AT_1 receptor inhibition with candesartan decreased neuroinflammatory and OS markers, and DA cell death. 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 [173]. In aged rats, we observed the absence of a counter-regulatory increase in AT_2 expression, and expression of AT_2 mRNA and protein decreased, despite increased expression of AT_1 receptors and increased NADPH activation [155, 171]. This may contribute to further enhancement of a pro-oxidative, pro-inflammatory state and DA cell vulnerability in aged animals.

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 DA release, which cannot be totally counteracted by functional compensatory changes and results in a progressive decrease in motor activity [35, 174]. Furthermore, DA and AII systems directly counterregulate each other and there is a negative reciprocity between DA and AT_1 receptors [155] (see above). Therefore, the upregulation of AT_1 receptors that we observed in aged rats [171] may be part of the compensatory changes to increase DA 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 it has been observed in other tissues (i.e. apparently non DA-related tissues) so that RAS appears to play a major role in longevity (see above).

Menopause, RAS hyperactivity and dopaminergic degeneration

The above described results were obtained in aged male rats. In aged menopausal females, the hormonal changes, and particularly the lack of oestrogen must be taken into account as an additional factor involved in the increased DA cell vulnerability and potential changes in RAS activity. Numerous experimental studies have shown that oestrogen exerts protective effects against dopaminergic cell degeneration [175, 176], and a number of epidemiological studies have reported that the incidence and prevalence of PD is higher in postmenopausal than in premenopausal women of similar age [177-179]. However, controversial effects of oestrogen replacement therapy (ORT) have been also reported [180-181], and the age of the women receiving the treatment appears to be a major factor in the discrepancies. The mechanism by which oestrogen protects DA neurons has not been clarified, although recent studies have suggested that modulation of the glial neuroinflammatory response by oestrogen is involved [182, 183]. Interestingly, oestrogen-induced regulation of the RAS mediates beneficial effects of oestrogen in several tissues [184-186], and interactions between oestrogen and AII receptors have also been observed [187-190]. 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 [191], we used young ovariectomized rats to investigate this question (i.e. in the absence of other potential aging-related factors above reported for aged males). We studied the effect of ovariectomy and oestrogen replacement on the nigral RAS and on dopaminergic degeneration induced by intrastriatal injection of the dopaminergic neurotoxin 6-OHDA. The neurotoxin induced a marked loss of dopaminergic neurons in ovariectomized rats, which was significantly reduced by oestrogen replacement or treatment with the AT_1 receptor antagonist candesartan. We also observed that oestrogen 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 (Rodriguez-Perez and Labandeira-Garcia, unpublished), 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 DA 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 [192-194]. The vast majority of women who engaged in these trials were on average 65 years or older, and 12 years postmenopause before oestrogen therapy [195-196]; on the contrary, most women initiated ORT in their perimenopausal period in observational studies that reported beneficial effects [197-199].

Conclusions

We suggest that brain RAS is involved in degeneration of DA neurons and the progression of PD, and that RAS-induced activation of microglia and NADPH-oxidase complex plays a major role in this effect. Aging-related increase in RAS activity is involved in pro-inflammatory and pro-oxidative changes in the nigra, and in the increased susceptibility of DA neurons to degeneration. Increased RAS activity may constitute a major factor in the increased risk of PD with aging, and the lack of oestrogen may act as an additional factor for increasing RAS activity in the nigra in menopausal women. Manipulation of the brain RAS may constitute an effective neuroprotective strategy against the aging-related risk of dopaminergic degeneration, and for the prevention or coadjutant treatment of PD in oestrogen-deficient women, together with or instead of oestrogen replacement therapy.

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