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Short communication

Age-related changes of the nitric oxide system in the rat brain

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Abstract

This work examines the age-related changes of the NO pathway in the central nervous system (CNS), analyzing nitric oxide synthase (NOS) isoform expression, the level of nitrotyrosine-modified proteins, and the NOS activity in the cerebral cortex, decorticated brain (basal ganglia, thalamus, hypothalamus, tegtum and tegmentum) and cerebellum of young, adult and aged rats. Our data demonstrate that the different NOS isoforms are not uniformly expressed across the CNS. In this sense, the nNOS and eNOS isoenzymes are expressed mainly in the cerebellum and decorticated brain, respectively, while the iNOS isoenzyme shows the highest level in cerebellum. Concerning age, in the cerebral cortex nNOS significantly increased its expression only in adult animals; meanwhile, in the cerebellum the eNOS expression decreased whereas iNOS increased in adult and aged rats. No age-related changes in any isoform were found in decorticated brain. NOS activity, determined by nitrate plus nitrite quantification, registered the highest levels in the cerebellum, where the significant increase detected with aging was probably related to iNOS activity. The number of nitrotyrosine-modified immunoreactive bands differed among regions; thus, the highest number was detected in the decorticated brain while the cerebellum showed the least number of bands. Finally, bulk protein nitration increased in cerebral cortex only in adult animal. No changes were found in the decorticated brain, and the decrease detected in the cerebellum of aged animals was not significant. According to these results, the NO pathway is differently modified with age in the three CNS regions analyzed.

Theme: Development and regeneration

Topic: Aging process

Keywords: Aging; nNOS; iNOS; eNOS; Nitrotyrosine-modified protein; Nitrate/nitrite quantification

Aging is a complex process that finally results in morphological and biochemical neurodegenerative changes [31]. The cumulative damage caused by free radicals to many cellular components has been proposed as the main mechanism underlying the aging process [20]. In fact, all oxidative damage impairs the signal-transduction pathway, as proposed for the aged brain [10].

Nitric oxide (NO) is a free radical, which acts as a neuromodulator in the CNS. This diffusible gas is involved in different mechanisms of synaptic plasticity, including those playing a key role in learning and memory both in the brain and cerebellum [7,48]. In addition, NO also maintains the basal vascular tone and regulates cerebral microcirculation [12,19,23].

NO is formed from L-arginine by NO synthase (NOS), which oxidizes the terminal guanidino nitrogen of L-arginine, releasing NO and citrulline [28]. At least three distinct isoforms of NOS have been cloned and located [25]. Endothelial NOS (eNOS) and neuronal NOS (nNOS) isoenzymes have been described as constitutive proteins whose activities are regulated by calcium increases. Inducible NOS (iNOS) has been detected at low levels under basal conditions but is induced by endotoxins and cytokines producing toxic levels of NO; such activity does not depend on calcium increases [28].

Current studies point to different and sometimes contro-

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versial hypotheses concerning the role played by NO during cerebral aging. On the one hand, some authors conjecture that NO may decline with age; in this sense, Noda et al. [29], Yamada et al. [46], and Yamada and Nabeshima [45] suggest that the lower production of NO may affect learning and memory. As NO maintains basal vascular tone, and regulates blood flow, a reduction in eNOS may alter cerebral circulation [27]. It has also been proposed that the use of the NOS inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME), affects the motor activity in the CNS and peripheral nervous system [2]. In addition, the lower level of L-arginine and L-citrulline in the brain of old Wistar rats could also be related to the depressed NOS activity in the aging process [39].

On the other hand, other authors have related aging with a boost in NO production, arguing that this molecule may act as a free radical contributing to the senescence process [10,14,26]. Greater NO production may also be related to hearing impairment with advancing age [34], to neuronal loss in cognitive related brain areas, and to a rise in neuronal apoptosis [44]. This hypothesis is supported by the significant decline detected with aging in the concentration of an endogenous NOS inhibitor asymmetrical dimethyl arginine (ADMA) described by Abe [1]. Moreover, it is known that the free radical superoxide (O_2^-) reacts with NO, producing the powerful oxidant peroxynitrite anion ($ONOO^-$) [5]. Peroxynitrite is a nitrating agent capable of attacking and modifying proteins, lipids and DNA as well as depleting antioxidant defenses [42]. Thus, apart from the functional implications for enzymatic activity and the assembly of cytoskeletal proteins, nitrated proteins are good markers of the cumulative exposure to NO [18,43].

These conflicting reports may reflect the partial focus of the studies, which fail to include all NOS isoforms, evaluate only NOS-expression or only NOS-activity, analyze only restricted brain zones, or even use different analytical methods. These partial studies make the definitive conclusions about NO implications during aging incomplete and sometimes inconsistent Moreover, these studies do not cover the different key stages from youth to advanced age that enable the tracking of changes in the NO-system over a lifespan. Consequently, in the present study, in order to elucidate the role of the NO pathway in cerebral aging, we propose to analyze the expression of the different NOS isoforms and nitrated proteins, as well as the NO synthase activity in cerebral cortex, cerebellum and decorticated brain in 2-, 4- and 30-month old rats.

The study was performed on young (2-month-old), adult (4-month-old) and aged (30-month-old) male albino Wistar rats, kept under standard conditions of light and temperature and allowed ad libitum access to commercial rat chow. All the experiments were carried out according to E.U. guidelines on the use of animals for biochemical research (86/609/EU).

Anti-neuronal NOS, a rabbit polyclonal antiserum raised against the full-length recombinant rat brain nNOS [35] (a gift from V. Riveros-Moreno of Welcome Research Laboratories, Beckenham, UK) was further characterized by Western blot of rat liver and brain homogenates [36,43]. Anti-nitrotyrosine is a polyclonal antiserum produced by our group at the Instituto Cajal (Madrid, Spain), and further characterized by Western blot of rat liver and brain homogenates [43]. Anti-endothelial and anti-inducible NOS were mouse monoclonal antisera purchased from Transduction Laboratories (Lexington, KY-Madrid, Spain) and anti-α-tubulin was a monoclonal antiserum obtained from Sigma (St Louis, MO-Madrid, Spain).

For Western blot analysis, a total of 15 rats, 5 of each age were killed by cervical dislocation and the brains were immediately removed. The cerebral cortex, decorticated brain (this piece includes: basal ganglia, thalamus, hypothalamus, tegtum and tegmentum) and cerebellum [40] were dissected, rinsed in saline solution and stored at −80 °C until used. Brain tissues were homogenized in 1/3 (w/v) of 30 mM Tris–HCl, pH 7.4 containing 0.5 mM DTT, 1 mM EDTA, 1% SDS and protease inhibitors. The resulting homogenates were centrifuged for 1 h at 100,000×*g*. All the operations were performed at 0–4 °C. Protein concentrations in the supernatants were determined by the Bradford method [8], with bovine serum albumin as a standard.

Equal amounts of the denatured proteins per lane (iNOS, eNOS, nitrotyrosine: 60 μg; nNOS: 40 μg), regardless of CNS region or age, were loaded and separated on a 7.5%/10% (NOS/nitrotirosyne) SDS–polyacrylamide gel (Mini Protean II, BioRad), as described by Laemmli [22]. Afterwards, proteins were transferred to a PVDF membrane (Immobilon P, Millipore). The membranes were blocked with 5% powdered non-fat milk in 25 mM Tris–HCl, pH 7.6; 137 mM NaCl, 2.6 mM KCl, 0.1% Tween-20, and incubated overnight at 4 °C with diluted monoclonal anti-iNOS and anti-eNOS (1/1000), rabbit polyclonal anti-nNOS (1:3000) and anti-nitrotyrosine (1:1000) in blocking solution. Bound antibody was revealed by means of an enhanced chemiluminescence kit (Amersham) according to the manufacturer's instructions. After immunodetection, membranes were probed with anti α-tubulin (Sigma) as a loading control. The relative amount of the proteins in each sample was quantified by densitometric scanning.

For comparative analyses of the expression of NOS isoforms in the different CNS regions, equal amounts of protein from the 5 animals of the same age group and region were mixed and loaded in the same gel. The following steps of the Western blot were carried out as described above.

NO production in the different CNS regions was determined by tissular accumulation of nitrite and nitrate. Briefly, a portion of tissue specimens from the 5 animals of each aged group and region were homogenized in 3 volumes (w/v) of PBS (pH 7.6) at 4 °C. Homogenates

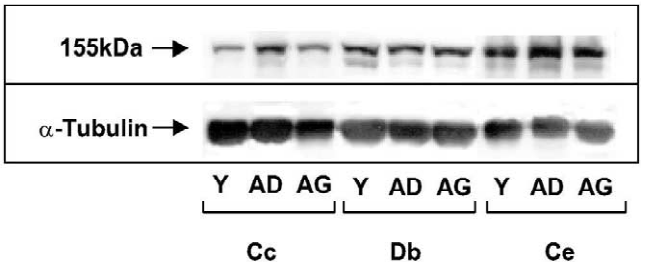

Fig. 1. Age-related comparative analysis of nNOS expression in the different CNS regions studied. Upper panel: Autoradiographies of the 155 kDa band (nNOS) on a typical Western blot. Equal amounts of protein from the 5 animals of the same age group (Y: young, AD: adult, and AG: aged) and region (Cc: cerebral cortex, Db: decorticated brain, Ce: cerebellum) were mixed and loaded in the same gel. Bottom panel: α-Tubulin immunodetection was also included as a protein-loading control.

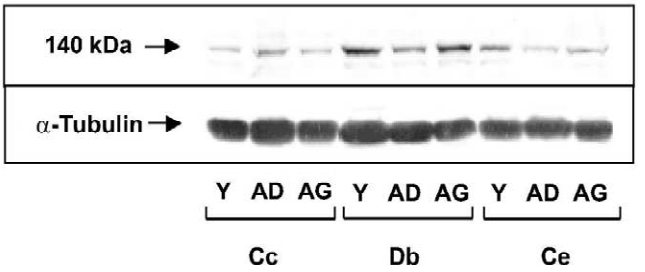

Fig. 3. Age-related comparative analysis of eNOS expression in the different CNS regions studied. Upper panel: autoradiographies of the 140-kDa band (eNOS) on a typical Western blot. Equal amounts of protein from the 5 animals of the same age group (Y: young, AD: adult, and AG: aged) and region (Cc: cerebral cortex, Db: decorticated brain, Ce: cerebellum) were mixed and loaded in the same gel. Bottom panel: α-Tubulin immunodetection was also included as a protein-loading control.

were then sonicated and centrifuged at 100,000×*g* for 60 min at 4 °C. The nitrate plus nitrite determination (NO_x) was carried out in the supernatants using a colorimetric kit according to the manufacture's instructions (Nitrate/Nitrite colorimetric Assay Kit, Cayman Chemical).

Western blot analysis of denatured homogenates from cerebral cortex; decorticated brain and cerebellum revealed that all regions contained a 155-kDa protein, detected by the nNOS antibody. Comparing the nNOS expression among the three regions, the protein level was significantly higher in the cerebellum, particularly in adult and aged animals (Fig. 1).

The relative densities of the nNOS bands in each region are shown in Fig. 2. A nNOS overexpression was found in the cerebral cortex of adult rats compared with the young ones ($P < 0.05$). This expression diminished in the cerebral cortex of aged rats although the change was not statistically significant. In the decorticated brain and cerebellum, no age-related changes were observed.

The eNOS immunoreactivity in cerebral cortex, decorticated brain and cerebellum was detected as a 140-kDa protein band. In addition, eNOS protein, regardless of the age of the animal, was clearly higher in decorticated brain than in the cerebral cortex or cerebellum (Fig. 3).

The relative densities of the eNOS bands in each region are shown in Fig. 4. No age differences in the expression level were found in either the cerebral cortex or decorticated brain. However, in the cerebellum, eNOS expression was significantly higher in young animals than in adult ($P < 0.05$) and aged ($P < 0.05$) ones. Nevertheless, this trend did not continue at later stages of age, as the eNOS level was similar in adult and aged rats.

The iNOS expression by means of Western blot analysis showed a slight 130 kDa immunoreactive band in the three CNS regions which proved stronger in the cerebellum of adult and aged animals (Fig. 5).

The relative densities of the iNOS bands in each region are shown in Fig. 6. No significant differences in the level of iNOS protein were detected in cerebral cortex or decorticated brain at any age of the animal. However, in cerebellum, there was an increase from young to adult and aged animals.

The levels of NO_x in the cerebral cortex, decorticated brain and cerebellum of young, adult and aged rats are presented in Table 1. The NO_x levels did not differ with age, either in cerebral cortex or decorticated brain. In addition, the NO_x quantitative measurements were comparable between the two regions, indicating a similar activity of NOS isoforms in these brain zones. In the cerebellum, an age-dependent rise in the NO_x level was found,

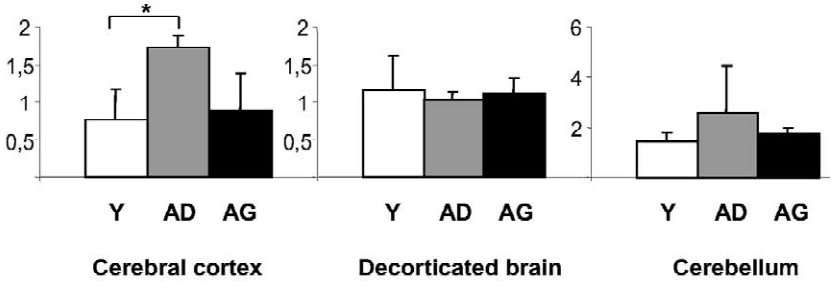

Fig. 2. Densitometric quantifications of nNOS expression in the CNS of young (Y), adult (A) and aged (AG) rats. Three independent Western blots for each CNS region (cerebral cortex, decorticated brain and cerebellum) were carried out to evaluate nNOS immunoreactivity. Results are average values of three experimental animals. (*$P < 0.05$).

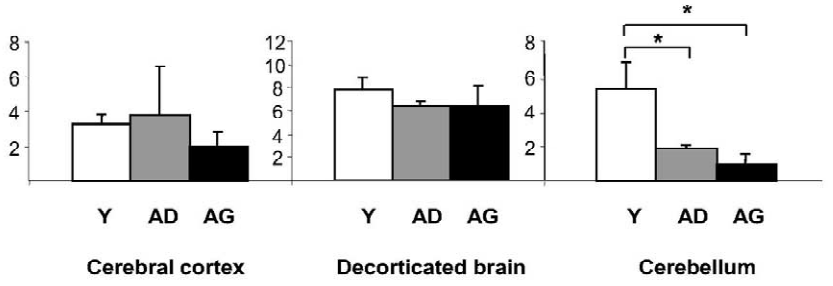

Fig. 4. Densitometric quantifications of eNOS expression in the CNS of young (Y), adult (A) and aged (AG) rats. Three independent Western-blots for each CNS region (cerebral cortex, decorticated brain and cerebellum) were carried out to evaluate eNOS immunoreactivity. Results are average values of three experimental animals. (*$P < 0.05$).

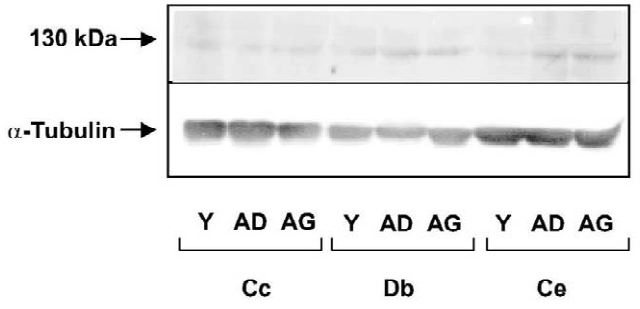

Fig. 5. Age-related comparative analysis of iNOS expression in the different CNS regions studied. Upper panel: autoradiographies of the 130 kDa band (iNOS) on a typical Western blot. Equal amounts of protein from the 5 animals of the same age group (Y: young, AD: adult, and AG: aged) and region (Cc: cerebral cortex, Db: decorticated brain, Ce: cerebellum) were mixed and loaded in the same gel. Bottom panel: α-Tubulin immunodetection was also included as a protein-loading control.

Table 1
Nitrate/nitrite levels in the cerebral cortex, decorticate brain and cerebellum of young, adult and aged rats

Experimental group	μmoles/mg		
	Cerebral cortex	Decorticated brain	Cerebellum
Young	1.31±0.32	1.36±0.41	1.33±0.29
Adult	1.27±0.11	1.07±0.09	2.11±0.09*
Aged	1.33±0.21	1.53±0.69	2.84±0.54*

Data are the means (±S.D.) of five determinations.
*NO_x levels significantly greater than in young experimental group ($P < 0.02$)

although with statistical significance only from young to adult ($P < 0.02$) and aged ($P < 0.02$) groups. The non-significant variations detected between adult and aged rats were probably due to the high interindividual variability of the oldest animals. Moreover, comparisons of the NO_x levels in the cerebellum and in the other two brain regions revealed that, although in young animals these levels were similar in the three regions analyzed, in the cerebellum values rose in adults and nearly doubled in aged animals.

We detected five nitrotyrosine immunoreactive bands corresponding to proteins of 88, 73, 50.4, 42 and 34.7 kDa; this latter band was the predominant regardless of the region studied (Fig. 7). With the exception of the decorticated brain, not all these proteins were present in the other two brain regions analyzed. Thus, irrespective of the age of the animal, the 73 kDa band was absent in the cerebral cortex and cerebellum; in addition, neither the 88 nor the 42 kDa bands were detected in the cerebellum. Finally, in the cerebral cortex of young animals, the 73 and 42 kDa bands were absent and the 34.7 and 88 kDa bands were less immunoreactive than those of adult and aged animals (Fig. 7).

The quantitative evaluation of bulk-nitrated proteins (Fig. 8) demonstrated the degree of nitrotyrosine alteration as function of the region analyzed and the age. In the cerebral cortex, the immunoreactivity was consistently lower in young animals; however, no differences in the nitration were found when adult and aged rats were compared. In the decorticated brain, although it was the

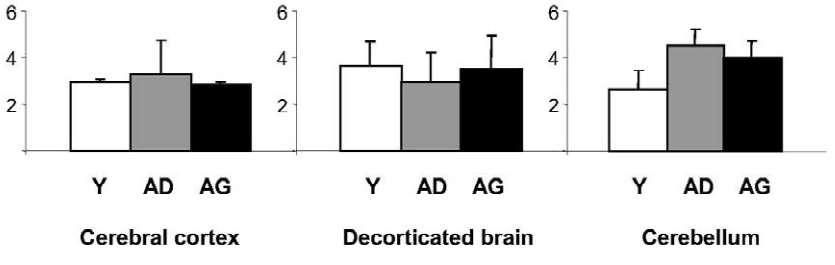

Fig. 6. Densitometric quantifications of iNOS expression in the CNS of young (Y), adult (A) and aged (AG) rats. Three independent Western blots for each CNS region (cerebral cortex, decorticated brain and cerebellum) were carried out to evaluate iNOS immunoreactivity. Results are average values of three experimental animals.

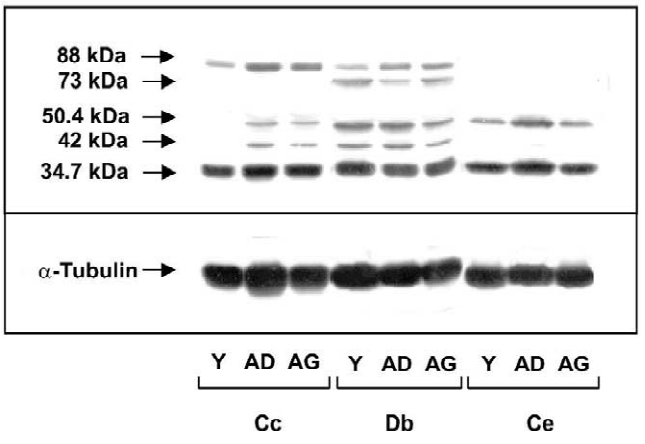

Fig. 7. Age-related comparative analysis of the level of nitrotyrosine modified proteins in the three CNS regions studied. Upper panel: autoradiography of the different bands (88, 73, 50, 42, 34.7 kDa) obtained. Equal amounts of protein from 5 animals of the same age group (Y: young, AD: adult, and AG: aged) and regions (Cc: cerebral cortex, Db: decorticated brain, Ce: cerebellum) were mixed and loaded in the same gel. Bottom panel: α-Tubulin immunodetection was also included as a protein-loading control.

region where a higher number of reactive bands were detected, neither changed with age. In the cerebellum, where the least number of nitrotyrosine-modified proteins were found, a non-significant decline with aging was found.

Current literature contains conflicting data concerning the dual role (neuroprotector/neurotoxic) that the NO may play in the aging CNS [24,32]. In the present study, we have investigated the NO pathway in an integrated way, exploring possible modifications of the NO in aging. Thus, we have studied the expression of all NOS isoforms, NO production and the rate of nitrated proteins in the cerebrum and cerebellum of young, adult and aged rats. Our data provide evidence of the heterogeneous distribution of NOS isoenzymes and NO production across the three studied regions of the CNS and how these changes occur differently in each region, depending on the age of the animals.

In terms of methodology, nNOS and iNOS were reported first as soluble enzymes, and only eNOS was considered to be membrane-associated [16]. In fact, in our preliminary study on nNOS and iNOS in the aging cerebral cortex [43] we investigated their expression by Western blot, taking into account only the cytosolic fraction of the brain homogenates; in those homogenates, we found an overexpression of both soluble isoenzymes in 26-month-old versus 2-month-old rats. Today, it is clear that nNOS and iNOS are approximately 40% membrane bound [6]. Consequently, in the present research, we have evaluated the total amount of these proteins (cytosolic and membrane associated); this procedure also enabled us to detect a greater number of nitrated proteins than in our previous report [43].

The issue of animal age has been extensively discussed in relation to the malfunction of the NO pathway [30,45]. In this sense, we have now studied not only young (2-month-old) and very old (30-month old) animals but also adult ones (4-month old). Consequently, the NO pathway evaluation in the three age groups has allowed identifying the changes that are really a consequence of the aging process.

The regional distribution of the two constitutive isoforms, nNOS and eNOS, indicates that both are expressed mainly in the cerebellum and decorticated brain, respectively. Thus, in the cerebellum, where in agreement with other authors [9,15] we found the highest levels of nNOS expression, this isoenzyme has been related to learning and memory processes as well as motor activity [37]. With regard to eNOS, the highest level detected in the decorticated brain could reflect the participation of NO in regulating the blood–brain barrier, in a region whose nuclei are involved in functions such as reproduction and sexual behavior [13,33] or central cardiovascular regulation [19].

Our results in relation to the age of the animal showed that the expression of the constitutive isoforms undergoes changes only in the cerebral cortex and cerebellum; particularly, nNOS and eNOS decreased with aging in both regions and nNOS increased in the cerebral cortex of adult animals. In the cerebellum, NO is related to memory and cognitive processes that may be impaired with aging [26]. Furthermore, it has been demonstrated that the infusion of NO in the cerebellum could facilitate the learning process

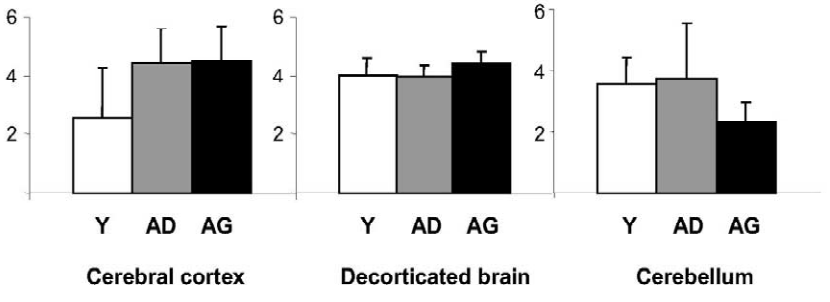

Fig. 8. Densitometric quantifications of bulk nitrotyrosine modified protein expression in the CNS of young (Y), adult (A) and aged (AG) rats. Three independent Western blots for each CNS region (cerebral cortex, decorticated brain and cerebellum) were carried out to evaluate nitrotyrosine immunoreactivity. Results are average values of three experimental animals.

[38]. The reductions in the nNOs and eNOS with aging detected in the cerebral cortex and cerebellum may support the involvement of the NO system in the aging memory and cognitive changes described above, although further experimental analysis should be done to prove this hypothesis.

With respect to the decorticated brain, our study has not detected age-related changes in the expression of the constitutive isoenzymes. It is possible that the numerous functional zones, which include this wide and heterogeneous region, exhibit opposite changes that may compensate one another. Cha et al. [11] has also reported regional discrepancies with aging in the number of immunoreactive NOS neurons in basal ganglia; thus, while this number fell in the striatum and substantia innominata, it rose in the subthalamic nucleus. Consequently, other complementary in situ studies, including thalamic and hypothalamic nuclei, that allow the delineation of a precise NOS-expression map throughout this region, may be required. In fact, we have already detected heterogeneous immunoreactive histological age-related changes in nuclei of these zones using densitometric image-analysis methods (in preparation).

The distribution of the inducible isoenzyme that, although showing age-related changes in the cerebellum, was barely detectable in the three CNS regions analyzed. It is also noticeable that iNOS is the only isoenzyme for which age-related variations are correlated with NO production, and thus iNOS should be the main agent responsible for the changes in NO release with age. These results agree in part with Ferrini et al. [13], who found increases in iNOS expression and NOS activity but no changes in nNOS expression in rat hypothalamus during aging. Vernet et al. [44] found similar results not only in the hypothalamus but also in other brain regions.

The NOS activity, determined by NO_x quantification, indicates that, although the levels found in all age groups in cerebral cortex and decorticated brain were similar, values were significantly higher in the cerebellum of adult and aged animals. This age-related rise in NO_x in the cerebellum was presumably due to iNOS, as discussed above. In this sense, Yu et al. [47] found a fall in nNOS activity but not in nNOS protein expression in the cerebellum of 24-month-old rats. This lesser activity of nNOS could be offset by iNOS, given that we found greater expression of this isoenzyme with age. Moreover, this hypothesis may be reinforced by our data showing an age-related decline in the expression of the constitutive isoforms, especially eNOS.

Nitrative stress is involved in the neurodegenerative disorders [41] through the formation of $ONOO^-$, which nitrates tyrosine residues of proteins to form the stable compound 3-nitrotyrosine [3,4]. It has been suggested that protein nitration hastens protein degradation [17], leading to the hypothesis that physiological aging may also be affected by protein nitration, as a consequence of oxidative stress [26].

The results of the present work indicate that, although bulk protein nitration detected with anti-nitrotyrosine antibody was similar among the three CNS regions, and significant increases were found only in the cerebral cortex of adult animals, the band pattern of nitrated proteins differed markedly among the three regions. Thus, the decorticated brain was the region with the greatest number of nitrated proteins (88, 73, 50.4, 42, 34.7 kDa). Such a high number of susceptible proteins to nitration may have resulted from the diversity of the functional zones within this region, apart from the wide and generalized distribution of the three NOS isoforms, particularly vascular eNOS that release NO to all tissular territories, allowing an easy accessibility of this molecule to more cellular proteins.

The cerebellum, with the most elevated NO_x production, was conversely the region with the least number of nitrated proteins (50.4, 34.7 kDa), although the total degree of nitration was similar to that of the other CNS regions studied. This fact could be due to a lesser level of proteins susceptible to nitration or (less likely) to an increase in the degree of denitrase activity [21].

The cerebral cortex was the only region that showed age-related changes in the number and level of nitrated proteins. Thus, in the adult and aged rats, we detected four nitrated proteins (88, 50.4, 42 and 34.7 kDa); however, in the young ones, only the 88 and 34.7 kDa bands appeared, and both of these were less immunoreactive than in adult and aged animals.

In conclusion, our study shows that in the brain areas analyzed, the NO system undergoes different changes over the course of life. Nevertheless, in non-pathological aging those changes do not imply a strong modification in bulk NO production, but if such alterations occur, as we have detected in cerebellum, the isoenzyme responsible appears to be iNOS.

Acknowledgements

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