Immunohistochemistry of Neuronal Nitric Oxide Synthase and Protein Nitration in the Striatum of the Aged Rat

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ABSTRACT To ascertain the possible implications of the nitric oxide ($NO^{\bullet}$) producing system in striatal senescence, and by using immunohistochemistry and image-processing approaches, we describe the presence of the enzyme nitric oxide synthase (NOS), the NADPH-diaphorase (NADPH-d) histochemical marker, and nitrotyrosine-derived complexes (N-Tyr) in the striatum of adult and aged rats. The results showed neuronal NOS immunoreactive (nNOS-IR) aspiny medium-sized neurons and nervous fibres in both age groups, with no variation in the percentage of immunoreactive area but a significant decrease in the intensity and in the number of somata with age, which were not related to the observed increase with age of the striatal bundles of the white matter. In addition, NADPH-d activity was detected in neurons with morphology similar to that of the nNOS-IR cells; a decrease in the percentage of area per field and in the number of cells, but an increase in the intensity of staining for the NADPH-d histochemical marker, were detected with age. The number of neuronal NADPH-d somata was higher than for the nNOS-IR ones in both age groups. Moreover, N-Tyr-IR complexes were observed in cells (neurons and glia) and fibres, with a significant increase in the percentage of the area of immunoreaction, related to the increase of white matter, but a decrease in intensity for the aged group. On the other hand, we did not detect the inducible isoform (iNOS) either in adult or in aged rats. Taken together, these results support the contention that NADPH-d staining is not such an unambiguous marker for nNOS, and that increased protein nitration may participate in striatal aging. *Microsc. Res. Tech. 64:304–311, 2004.*

INTRODUCTION

Nitric oxide ($NO^{\bullet}$) is a gaseous molecule involved in a broad number of physiological and pathological processes (Bredt, 1999). In biological synthesis, $NO^{\bullet}$ is catalysed by a family of enzymes called nitric oxide synthases (NOS), among which three main isoforms have been widely characterized in mammals (Stuehr, 1999). Two NOS isoforms are constitutively expressed in cells: neural NOS (nNOS or NOS-I), originally detected in rat neurons, and endothelial NOS (eNOS or NOS-III), firstly described in bovine endothelial cells. In addition, an inducible isoform (iNOS or NOS-II) is expressed in almost all nucleated cells when submitted to the appropriate stimuli.

Also, $NO^{\bullet}$ is a free radical that can react with cellular superoxide ($O_2^{\bullet -}$), leading to the formation of peroxynitrite ($ONOO^-$). Peroxynitrite promotes oxidative damage by reacting in complex ways with different biomolecules (Cassina et al., 2000), including the nitration of peptides and proteins, in the phenyl side chain of tyrosine residues, which may occur under physiological conditions, but levels have been found to increase in diverse pathologies (Greenacre and Ischiropoulos, 2001) and, in particular, in neurons of the aged brain (Shin et al., 2002; Uttenthal et al., 1998).

Aging is a pleiotropic process; that is, aspects of phenotype and the associated patterns of gene expression that are advantageous during early life may become disadvantageous in old age (Kirkwood and Austad, 2000). In this sense, the lifespan of an organism may be linked to the survival of key cell populations, a fact that has been recently reported in mammalian neurons (Cowen, 2002). Thus, at least as demonstrated in the aging of the nervous system, the highly selective nature of neurodegenerative diseases involves particular groups of neurons while others may not be affected (Necchi et al., 2002).

The striatum is a basal ganglion of the brain that is particularly involved in several neurodegenerative processes, most of them being frequently detected in old age; moreover, age-related neurochemical changes have also been confined to specific striatal populations (Strong, 1988). Lesions in the striatum are related to

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motor and muscular alterations, including those associated to Parkinson's and Huntington's diseases (Bergman and Deuschl, 2002; Sieradzan and Mann, 2001); in addition, this nucleus takes part of the reward-association and visual-recognition-memory pathways, mechanisms of neuronal plasticity also impaired with age (Parker and Gaffan, 1998).

Striatal neurons receive a myriad of synaptic inputs from different sources. Massive afferents from all areas of the cortex and thalamus represent the most important supply of excitatory amino acids, whereas the nigrostriatal pathway and intrinsic circuits provide the striatum with dopamine, acetylcholine, GABA, adenosine, and nitric oxide. All these neurotransmitters interact to regulate the efficacy of the synaptic transmissions within this nucleus (Calabresi et al., 2000).

In the striatum, the $NO^{\bullet}$-producing (nitrergic) cells are interneurons, which are evenly distributed and represent the 1–2% of all striatal neurones (Kawaguchi et al., 1995). $NO^{\bullet}$ appears to have at least two functions in the striatum: (1) the control of the local blood flow in response to cortical or pallidal inputs (Snyder and Bredt, 1991), and (2) the modulation of the release of other neuroactive substances (Manzoni et al., 1992). Therefore, as previously reported (Kawaguchi et al., 1995), the appropriate activity of NOS-expressing cells may be a key point in the control of the striatal function. In fact, the use of electrophysiological approaches revealed that a selective inhibition of nNOS influences the discharging of striatal neurons (Sardo et al., 2002). Moreover, recent studies have shown an age-related variation in the density of nNOS and NADPH-diaphorase (NADPH-d, which has been considered a selective histochemical marker for NOS in fixed tissues) containing neurons of this basal nucleus of the rat (Cha et al., 2000; Kamisaki et al., 1998; Necchi et al., 2002).

In this light, the aim of the present study is to describe the immunohistochemical location and quantification by image processing of nNOS, iNOS, NADPH-d, and nitrotyrosine-derived complexes (N-Tyr) in the striatum of adult and aged rats in order to ascertain the possible morphofunctional changes of the $NO^{\bullet}$/NOS system during cerebral senescence.

MATERIALS AND METHODS

Animals

The study was performed on five adult (4-month-old, 230–260 g) and five aged (26-month-old, 390–430 g) male albino Wistar rats, kept under standard conditions of light and temperature and given 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).

Tissue Preparation

Deeply anaesthetized animals (15 mg/100 g B.W., i.p.; Ketolar, Parke Davis, Madrid, Spain) were perfused through the left ventricle with 20–50 ml of carbogenated 0.01 M phosphate-buffered saline (PBS; pH 7.4), and then with 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The brains were removed and then postfixed for a further 4 h in the same fixative at room temperature. Samples were then cryoprotected by immersion overnight at 4°C in 0.1 M PB containing 30% sucrose. After this, they were embedded in O.C.T medium and frozen in 2-methylbutane pre-chilled in liquid nitrogen. Serial sections (40 μm) were cut using a cryostat (2800 Frigocut E, Reichert-Jung Vienna, Austria).

Immunocytochemistry

Free-floating sections were incubated for 4 h in PBS containing 0.1% Triton X-100, and then in: nNOS, 1:3,000 (this antiserum was a gift from Dr. V. Riveros Moreno of Welcome Research Laboratories, Beckenham, UK; Riveros Moreno et al., 1995); iNOS, 1:2,500 (Huh et al., 1998); or in nitrotyrosine, 1:300 (Uttenthal et al., 1998) antisera diluted in PBS containing 0.2% Triton X-100, overnight at 4°C. After several rinses in PBS, the sections were incubated with biotinylated goat antirabbit IgG, 1:100 (Vector Laboratories, Burlingame, CA), followed by peroxidase-linked ABC. The peroxidase activity was demonstrated following the nickel-enhanced diamino-benzidine assay (Shu et al., 1988). Control procedures were carried out on adjacent sections of the same tissues. No immunolabelling was detected when the primary antibody was either omitted or replaced with an equivalent concentration either of preimmune serum or normal rabbit serum. Sections were then mounted on slides, dehydrated, and covered using DPX.

Histochemistry

NADPH-diaphorase (NADPH-d) histochemical staining was used as a complementary method for the indirect visualization of NOS by light microscopy. Free-floating sections were incubated for 4 h in PBS containing 0.1% Triton X-100. After several washes in 0.1 M Tris-HCl pH 7.4 buffer, they were incubated in the dark, for 45 min at 37°C, in 0.1 M Tris-HCl, pH 7.4, containing 1 mM β-NADPH and 2 mM NBT (in 70% dimethylformamide). The sections were then washed twice with 0.1 M Tris-HCl, pH 7.4, quickly dehydrated in a graded ethanol series, cleared and mounted in DPX (Fluka, Madrid, Spain).

Image Processing and Statistical Treatment

The immunoreactive or NADPH-d positive structures, and white-matter bundles (WM), were quantified by computerized-assisted image analysis using ImageJ (an NIH image analysis and processing software downloaded free from http://rsbweb.nih.gov/ij/) connected to a light microscope (Olympus, Hamburg, Germany). For nNOS, one random 1.56 mm^2 field (image 10×) on each section, and five sections (from rostral to caudal striatum) for each rat, were digitally captured and analysed after background substration (minimal particular size 10 pixels); for N-Tyr, five random 0.1 mm^2 fields (images 40×) on each section, and five sections (also from rostral to caudal striatum) for each rat, were processed after background subtraction (minimal particle size 70 pixels). The field areas were chosen both according to the extension of the striatum and to the immunoreactive intensity in order to avoid repeated measurements. As a means of avoiding the usual biased segmentation of the stained structures, the immunoreactive area and the staining intensity, from black (0) to white (255), of each field was calculated as a function of the optical density following Sternberger's method (Sternberger and Sternberger,

TABLE 1. Area of white-matter bundles and coefficient of variation[1]

	Area (percentage of field)	Coefficient of variation (%)
nNOS inverted images		
Adult	16.13 ± 2.52	54.06
Aged	46.47 ± 8.07*	60.12
NADPH-d inverted images		
Adult	62.95 ± 2.40	13.22
Aged	84.27 ± 2.75*	11.29

[1]Area: mean ± SEM.
*$P \leq 0.01$, aged vs. adult.

1986). The line segment corresponding to immunostaining was mathematically extrapolated (with Origin 5.0, a data analysis and technical graphics software) to the axis representing the percentage of area per field.

The number of nNOS-IR, or NADPH-d-positive, somata was determined by direct visual counting from the same images. N-Tyr-IR somata were not quantified because most of them showed a slight immunoreactivity for which visual counting is highly prone to error (see below), even though Sternberger's quantitative method eliminates the need to discriminate immunostained structures for the background, while calculating only the percentage of immunoreactive area (Sternberger and Sternberger, 1986).

The area of white-matter bundles was quantified using either NADPHd or nNOS inverted images and following the method described above. As shown in Table 1, different area values were found, depending on the images analysed, even though a statistical difference was found between adult and aged groups in both NADPHd and nNOS approaches. Because the lower coefficients of variation (standard deviation × 100/mean, reflecting the best measure approach) resulted from the NADPHd images, these data were used for slope comparisons of regression-line analysis (see below).

The statistical treatment was performed with Statgraphics Plus 5.1 software. Because the data did not follow a normal distribution, as reflected by the Kolmogorov-Smirnov test, the degree of statistical significance was established by applying the non-parametric Mann-Whitney test. The regression lines were compared using the *Comparison of Regression Lines Analysis Dialog Box* (Statgraphics Plus 5.1), which automatically constructs the necessary indicator variables for comparing two or more simple regression models. Previously, data corresponding to area or neuronal number were log transformed to avoid comparisons between absolute variations. Slope comparatives were made in absolute values.

RESULTS

nNOS

In the striatum of both adult and aged rats, nNOS-immunoreactive (nNOS-IR) neurons and nerve fibres were detected distributed around the characteristic white-matter striatal bundles (these bundles being immuno-negative but increasing in area with age) (Fig. 1A,B). The nNOS-IR neurons showed a similar morphology in both experimental groups, most corresponding to aspiny medium-sized cells, as revealed at higher magnifications (Fig. 1C,D). In addition, image processing indicated no variation of the nNOS-IR percentage of area in the striatal sections with age, even though an increase in the WM area was detected (Table 2); a lack of statistical difference between the WM and nNOS area slopes was found (see Fig. 4A), indicating a correlation between them. The average optical density was significantly higher (indicating a lower nNOS-IR intensity), but the number of nNOS-IR neurons was significantly lower in the old animals (Table 2). When the increase of WM area was compared to the nNOS neuronal number decrease, the significant difference reflects a lack of correlation between the two parameters (see Fig. 4C).

iNOS

No iNOS immunoreactive structures were detected in either age group.

NADPH-d

In the striatum, the NADPH-d histochemical reaction product (formazan salts) was detected in neurons showing a morphology resembling that of nNOS-IR cells, i.e., medium-size aspiny cells in both age-groups (Fig. 2C,D). The intensity of the formazan salts was apparently higher in the sections from the old group, according to the photomicrographs (Fig. 2A–D), this not being corroborated by image analysis, even with a non-significant decrease in optical density (Table 2). On the other hand, a significant decrease in the number of NADPH-d positive neurons in this basal ganglion was detected with age, together with a slight decrease in the area percentage (Table 2). These changes (reflected in the corresponding slopes; see Fig. 4A,C) statistically differed from those detected in the WM, indicating the lack of correlation with the WM increase.

Protein Nitrotyrosilation

Nitrotyrosine immunoreactive (N-Tyr-IR) cells and fibres were detected in the striatum of adult rats (Fig. 3A), and the related sections of the aged animals showed N-Tyr-IR both in neuronal bodies (and associated nearest projections) (Fig. 3B) and in glial cells (Fig. 3C). Most of the N-Tyr-IR somatas showed a slight immunoreactivity, making visual counting highly prone to error; therefore, this quantitative parameter was not included in the study. Data from image analysis also indicated a significant increase with age in the percentage of area immunostained, but a decrease in the intensity of immunoreaction (i.e., a significant increase in the grey level value; Table 2). Because no statistical difference was found between the slopes of WM and N-Tyr area changes with age, a positive concomitant and correlative increase with age may be interpreted (Fig. 4B).

DISCUSSION

The striatum, the main component of the basal ganglia, becomes impaired with age (Strong, 1988). Because $NO^{\bullet}$ has been widely involved in the oxidative injury that takes place during the aging process, and also in neurodegenerative diseases, the results presented here attempt to elucidate the alterations and possible morpho-functional implications of the $NO^{\bullet}$/NOS system in this

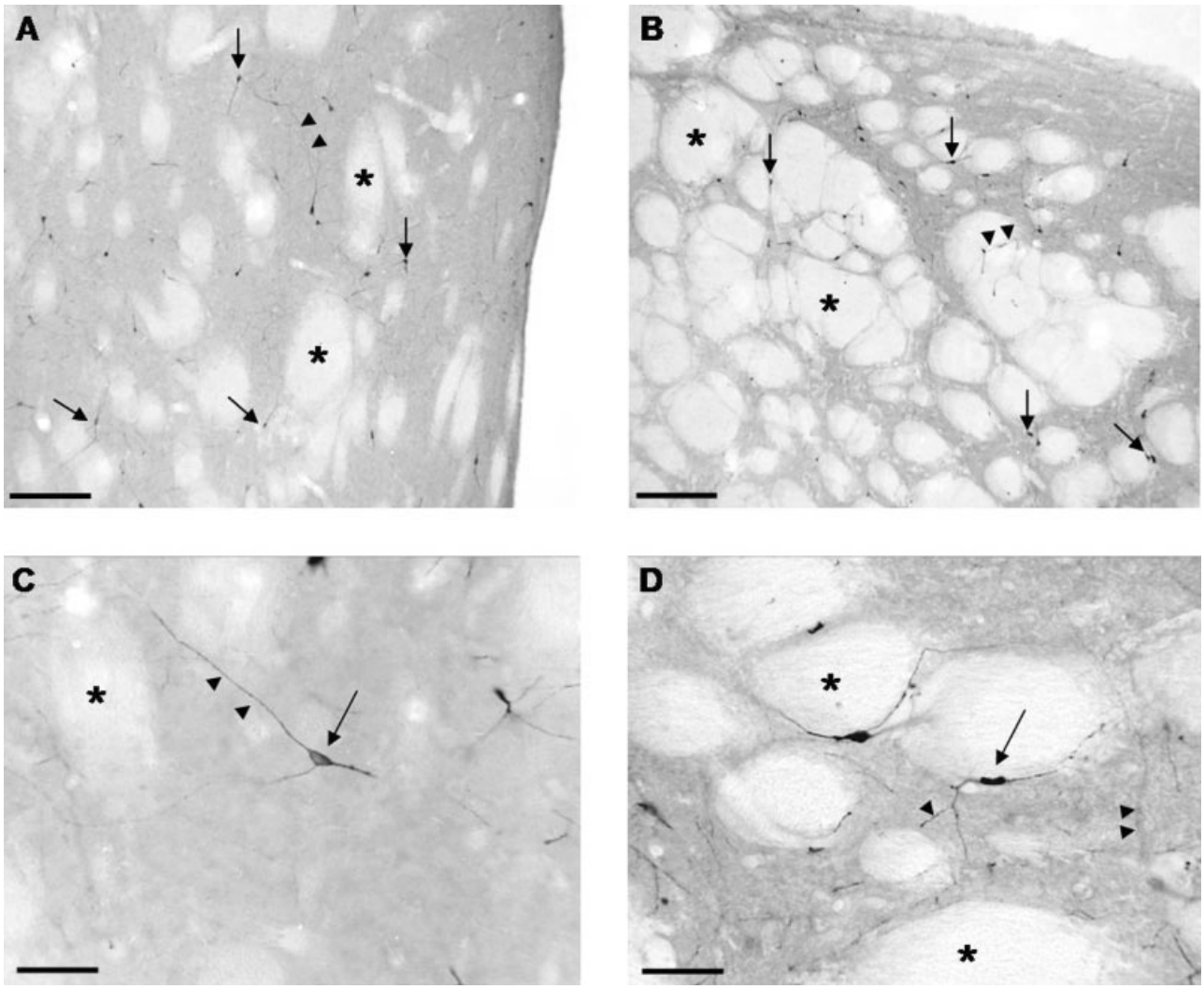

Fig. 1. Micrographs of nNOS immunoreactivity in sections of rat striatum. **A,B:** nNOS immunoreactivity in neurons (arrows) and nerve fibres (arrowheads) distributed around the white-matter striatal bundles (asterisks) (these bundles being immuno-negative) in the striatum of both adult (A) and aged rats (B). **C,D:** Higher magnification showing immunoreactive neurons with a similar cell-body morphology in both experimental groups. Scale bars = (A,B) 200 μm, (C,D) 50 μm.

TABLE 2. Quantitative data from image analysis[1]

	Area (percentage of field)	Optical density (mean gray level)	Neuronal number/field
nNOS			
Adult	0.53 ± 0.05	142.8 ± 2.1	32.5 ± 1.9
Aged	0.53 ± 0.08	155.3 ± 2.5**	20.7 ± 3.1**
NADPH-d			
Adult	1.45 ± 0.09	163.2 ± 2.6	38.2 ± 1.5
Aged	1.38 ± 0.08	160.0 ± 2.6	33.6 ± 1.9*
N-Tyr			
Adult	1.16 ± 0.07	143.8 ± 0.9	NQ
Aged	1.71 ± 0.12**	145.9 ± 0.7	NQ
White matter			
Adult	62.95 ± 2.40	NA	NA
Aged	84.27 ± 2.75**	NA	NA

[1]Aged vs. adult. NQ: not quantified; NA: not applicable. Values represent the mean ± SEM.
*$P \leq 0.05$.
**$P \leq 0.01$.

basal ganglion, using the aged rat as an experimental model. The ultimate aim is to add new results to the debate concerning the role of $NO^{\bullet}$ in cerebral aging, taking into consideration in the same experimental approach, iNOS and nTyr immunolocation.

The results show no variation in the percentage of area of the nNOS-IR structures, but a significant decrease with age in the average intensity and in the number of neuronal nitrergic somata. This lower neuronal number could be related to the increase in WM area with age, but when comparing the two parameters using a regression line analysis, the significant difference supports the fact that both parameters were not correlated.

It is worth noting that a decrease in neuronal nNOS content but an increase of nNOS-IR fibres in the aged rats possibly takes place in order to compensate for the

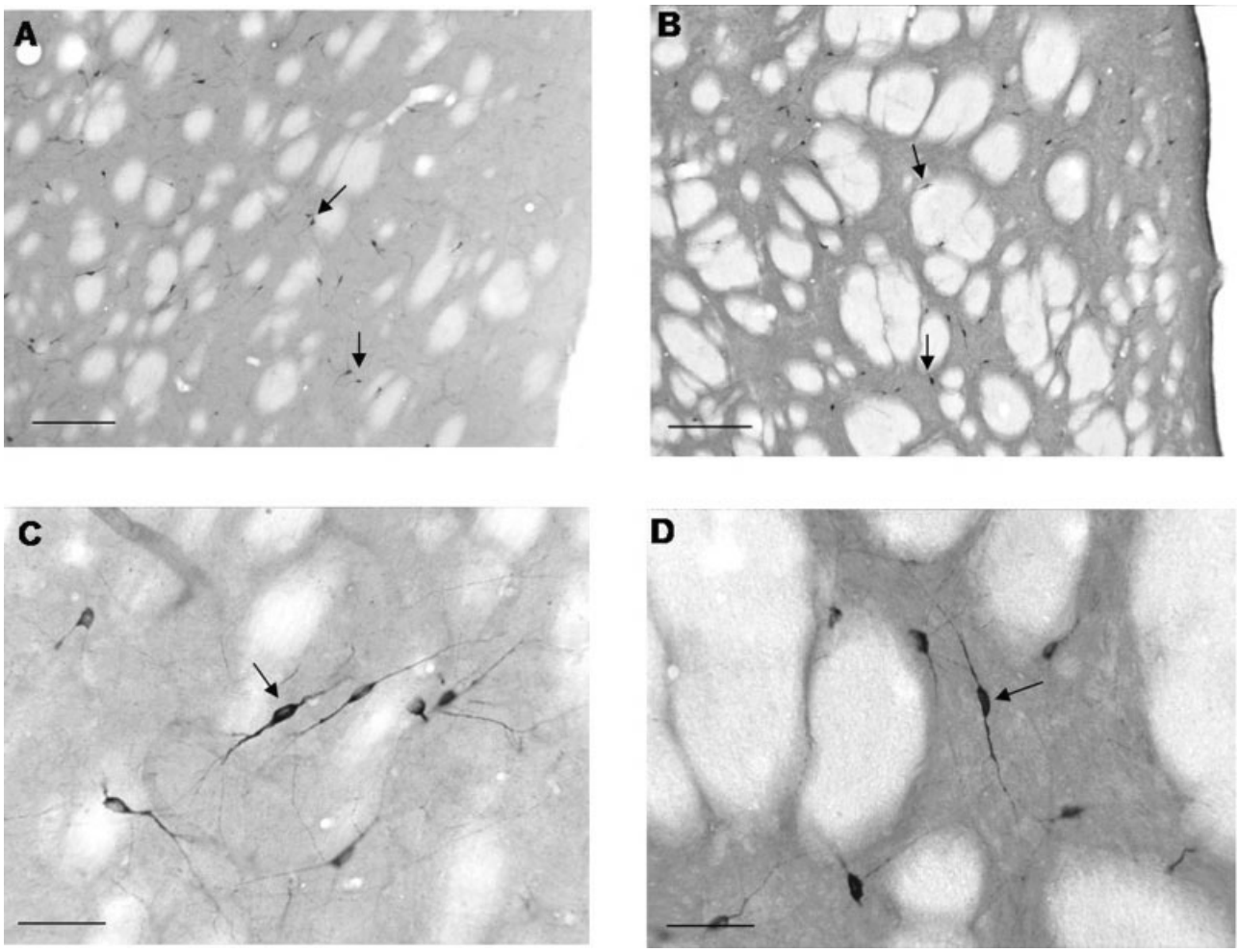

Fig. 2. NADPH-diaphorase staining. **A,B:** Micrographs showing the presence of a slightly more intense staining in the striatal nucleus of the aged animals (B) with respect to the adults (A). **C,D:** Higher magnifications showing similar morphology of medium-size aspiny neurons (arrows) in an adult (C) or aged (D) rat. Scale bars = (A,B) 200 μm;, (C,D) 50 μm.

significant decrease in the number of nNOS-IR somata. The determination of whether the immunoreactive fibres contributing to this increase are part of striatal neurons or whether they belong to neurons in which the somata is located outside the striatum require further study. Thus, our results partially agree with other findings indicating a decrease not only in the number of striatal nNOS-IR neurons (Cha et al., 2000; Necchi et al., 2002) but also in the expression pattern of nNOS in the striatum with age (Necchi et al., 2002) and in striatal-associated neurodegenerative diseases (Deckel et al., 2002), even though in the present study we found an increase in the nitrergic plexus. Thus, like others, as we have detected in different regions of the aged brain (Necchi et al., 2002; Siles et al., 2002), there is an alteration in the nNOS striatal expression with age. The affirmation of the correct balance must await new physiological results, but at present it is clear that a basal level of synthesised $NO^{\bullet}$/nNOS influences the release of other striatal neurotransmitters (Manzoni et al., 1992; Ohkuma and Katsura, 2001; Sardo et al., 2002). This is crucial in the mechanisms of striatal cognitive and emotional functions (Jain et al., 2001), given that it may be involved in the impairment of the striatal function with age (Cha et al., 2000; Necchi et al., 2002).

Concerning the histochemical location of NADPH-d, classically related to NOS in fixed tissues, the lack of concordance in the percentage of area and in the neuronal number when related to nNOS supports the idea previously proposed (Beesley, 1995; Blottner et al., 1995; Necchi et al., 2002) that NADPH-d is not such an unambiguous nNOS marker, and that other NADPH-d enzymes may be active under fixation. Thus, the decrease in the percentage of area of the NADPH-d-stained sections with age may not be unequivocally related to NOS dynamics; however, there is a similar reduction in the number of neurons, a fact also reported by others (Yamada et al., 1996).

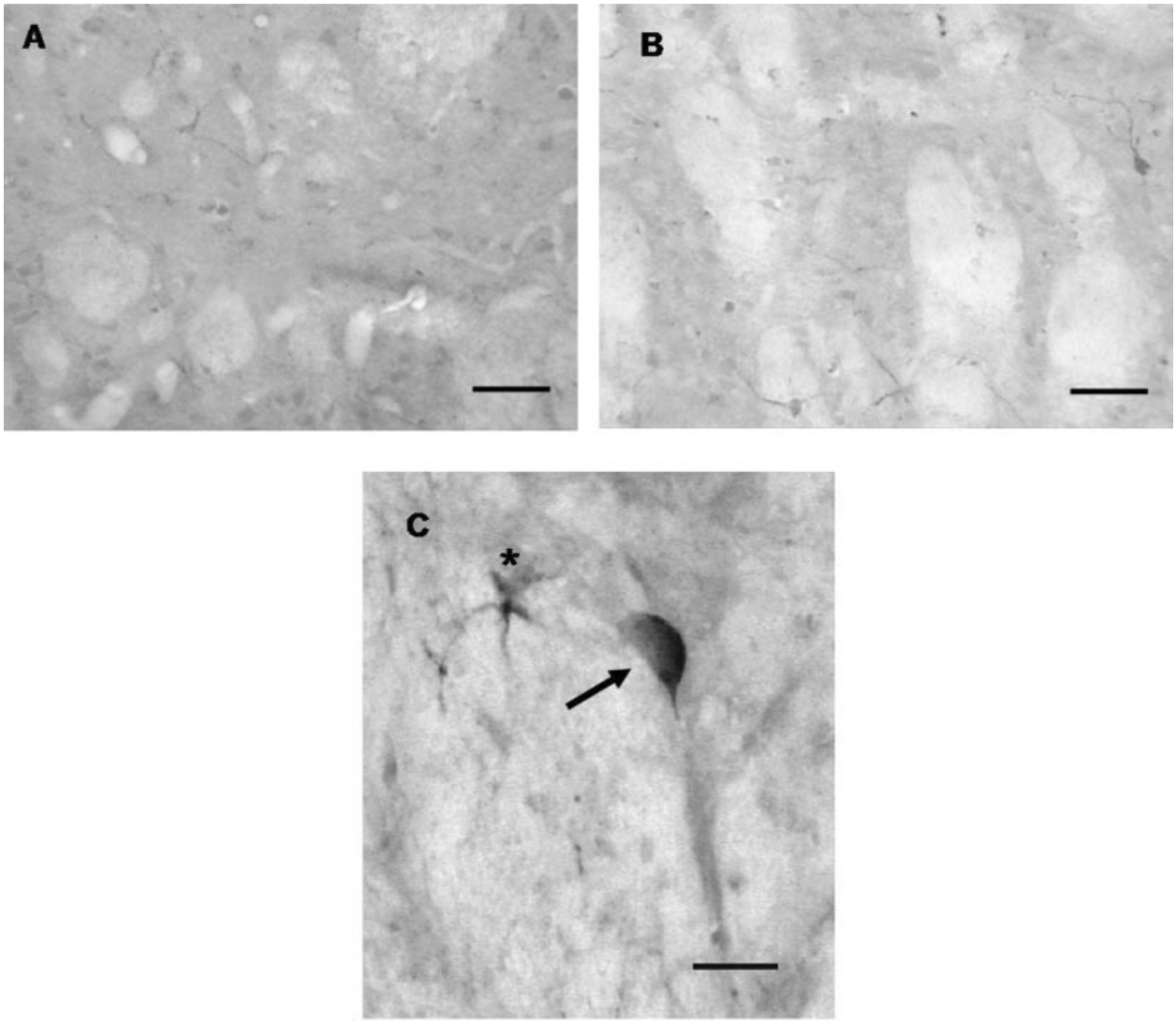

Fig. 3. N-Tyr immunostaining in sections of rat striatum. **A,B:** N-Tyr immunoreactivity neurons and nerve fibres in the striatum of both adult (A) and aged rats (B). **C:** Higher magnification showing immunopositive neurons (arrow) and glial cells (asterisk) in the striatum of the aged animals. Scale bars = (A,B) 50 μm; (C) 20 μm.

The study of the distribution of iNOS-IR structures showed that iNOS immunoreactivity is not present in the striatum of both adult and aged animals; in this sense, iNOS mRNA has been described as practically undetectable in the aged-rat striatum (La Porta and Comolli, 1999). Accordingly, we have detected very low iNOS protein expression in the decorticated brain of the aged rats (Siles et al., 2002); probably this isoform only changes its expression with age in specific vulnerable brain areas (Uttenthal et al., 1998) or after pathological conditions that take place in the cerebral cortex of rats submitted to in vitro glucose and oxygen deprivation (Moro et al., 1998), as well as in the glia and neurons of the substantia nigra of patients with Parkinson's disease (Knott et al., 2000).

It is well known that excessive $NO^{\bullet}$ plays a major role in the neurotoxicological alterations associated with neurodegenerative disorders (Beal, 1998). Our results concerning the detection of N-Tyr complexes support the fact that $NO^{\bullet}$ production diminishes in the striatum of aged rats, suggesting that its functionality, and therefore the synaptic circuitry, may change with age. Moreover, we found some n-Tyr-IR glial cells in the aged striatum, where the functionality was suspected to be altered and, thus, probably the life expectancy of the striatal neurons (Kamisaki et al., 1998). In this sense, a recent model shows, in the striatum, the relationship between greater peroxynitrite production with aging and its induced neurotoxicological mechanisms (Imam and Ali, 2001).

We may conclude that the $NO^{\bullet}$/NOS system appears to be involved in the morphofunctional alterations associated to the striatum during senescence. In this sense, the increased formation of nitroty-

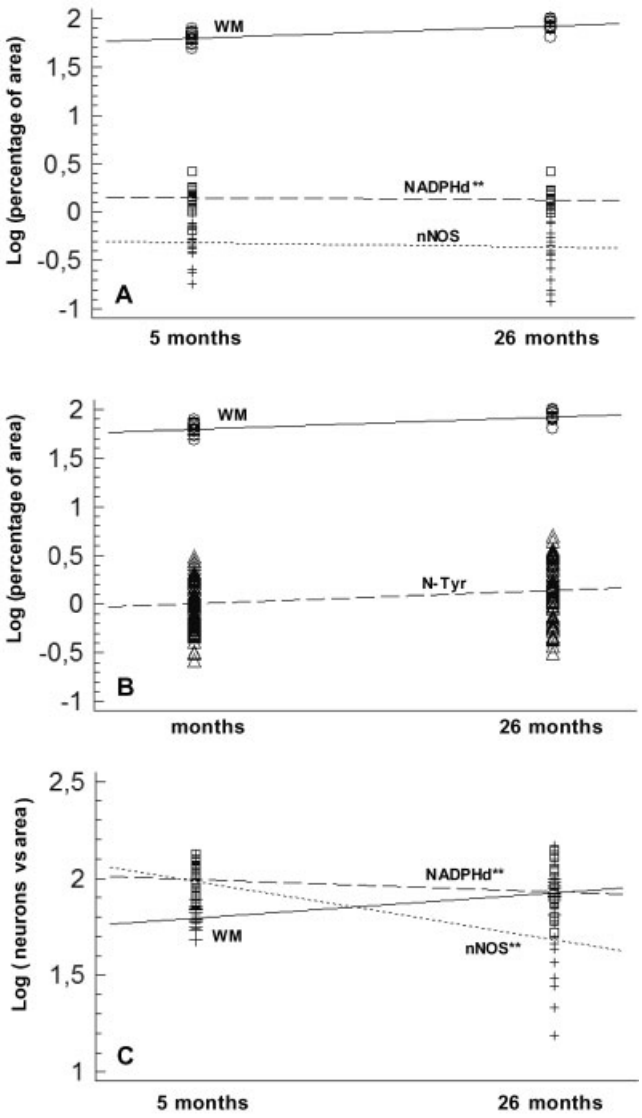

Fig. 4. Comparison of regression lines analysis. **A:** Regression analysis of white matter (WM), NADPHd and nNOS areas against age; NADPHd** indicates a significant ($P \leq 0.01$) difference between WM and NADPHd slopes (in absolute values). **B:** Regression analysis of white matter (WM) and N-Tyr areas against age; when comparing the slopes, note the different magnification used for getting results. **C:** Regression analysis of white-matter area (WM), number of NADPHd positive neurons, and number of nNOS immunoreactive somatas against age; NADPHd** and nNOS** indicates a significant ($P \leq 0.01$) difference versus WM slope (in absolute values).

rosine complexes found in the present study suggests an increase in the overall action of striatal $NO^{\bullet}$. Because there is no variation in the nNOS area, a decrease in the number of nNOS-IR somata, and no iNOS expression, the increased nitration may proceed not by an increase in $NO^{\bullet}$ production but perhaps through a heme/iron-induced protein tyrosine nitration mechanism (Bian et al., 2003) due to the known aging-associated increase in the production of H_2O_2 (Sandhu and Kaur, 2002). This supports the free-radical theory of aging and the involvement of reactive oxygen and nitrogen species, such as nitric oxide, H_2O_2, and its by-products, in cerebral senescence (Drew and Leeuwenburgh, 2002).

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