J Neural Transm [GenSect] (1993) 94: 21–30

Journal of Neural Transmission

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Printed in Austria

# Effect of ageing on tissue levels of amino acids involved in the nitric oxide pathway in rat brain

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Accepted June 2, 1993

**Summary.** Nitric oxide (NO) and citrulline are produced from L-arginine by the action of NO synthase after activation of excitatory amino acid receptors. In addition to its role in neurodegeneration, there is convincing evidence that NO is also involved in long-term potentiation, a cellular analog of learning and memory in the mammalian nervous system.

In the present study, concentrations of L-arginine, citrulline, aspartic acid and glutamic acid were determined in various brain regions of young and old rats. The aim was to examine whether changes in brain concentrations of these amino acids might be indicative of a possible decrease in NO production with ageing, in relation with the well-established decline of cognitive function.

Brain aspartic acid, citrulline and L-arginine concentrations were found to be lower in old rats compared to young animals, although the decrease did not always reach statistical significance. In contrast, no change in glutamic acid levels was found. In all brain structures of young and old rats, concentrations of L-arginine were higher than the concentration for NO synthase to function at maximum velocity in the rat brain. Therefore, the decrease in citrulline concentrations found in some brain regions of old rats might be seen, at least partly, as a reflection of a lower production of NO with ageing, although further work is clearly needed to ascertain a decrease in rat brain NO synthase activity with age.

**Keywords:** Nitric oxide, ageing, citrulline, arginine, rat brain.

## Introduction

Nitric oxide (NO) is a neuronal constituent with functions very much like those of a neurotransmitter (Bredt and Snyder, 1992). NO is synthesized from the semi-essential amino acid L-arginine by the cytosolic enzyme NO synthase (NOS) (Knowles et al., 1989). L-Citrulline is the co-product of the reaction. The mechanism of NO formation is not entirely understood. In macrophages,

Stuehr et al. (1991) have established that $N^{\omega}$-hydroxy-L-arginine is formed as an intermediate through an NADPH-dependent hydroxylation of L-arginine. In the brain, NO is formed in a cycle similar to the urea cycle. In the NO-producing cycle, however, a part of the urea cycle is missing, i.e. the formation of citrulline from ornithine and carbamyl phosphate (Garthwaite, 1991). The precursor of NO, L-arginine, is resynthetized from citrulline and aspartate. However, although brain NO and citrulline seem to be preponderantly formed through the action of NO synthase, other mechanisms of formation of both NO and citrulline from either L-arginine or other precursors have also been suggested (Schmidt et al., 1989).

NO is released within the central nervous system in response to activation of the NMDA receptors by glutamate (or aspartate) (Garthwaite et al., 1988). This activation causes a raise in the influx of $Ca^{2+}$ ions into postsynaptic structures, which in turn activates NOS and results in the formation of citrulline and NO from arginine (Bredt and Snyder, 1990). NO is a highly toxic, extremely labile, free radical. NO rapidly reacts with the superoxide radical ($O_2^-$) to from the peroxynitrite anion ($ONO_2^-$) in high yield. Peroxynitrite anion is stable enough at physiological pH to diffuse to critical cellular targets before becoming protonated and decomposing to form the powerful and cytotoxic oxidants hydroxyl radical and nitrogen dioxide (Beckman, 1991). In agreement with a contributory role of NO in neurodegeneration, injection of low doses of the competitive NOS inhibitor $N^G$-nitro-L-arginine to mice after occlusion of the middle cerebral artery has been shown to significantly reduce the volume of cortical infarct (Nowicki et al., 1991). Similar data were obtained by Buisson et al. (1992) in a rat model of focal cerebral ischaemia with another NOS inhibitor. However, the conditions in which NO behaves as a neurotoxin remain to be accurately defined.

In addition to its probable involvement in neurodegeneration occurring in some pathological conditions, NO is also thought to play a role in long-term potentiation (Böhme et al., 1991; O'Dell et al., 1991; Schuman and Madison, 1991), a phenomenon of synaptic plasticity which results from NMDA receptor activation and is considered a cellular analog of learning and memory in the mammalian nervous system. Thus, rats treated with a NOS inhibitor were shown to be impaired in learning a spatial learning task (Chapman et al., 1992). Spatial information processing has been shown to be impaired in aged rats (Gage et al., 1984; Rapp et al., 1987; Cervini et al., 1992), and glutamic and aspartic acid levels have been found to be lower in most brain structures of old rats compared to young animals (Strolin Benedetti et al., 1990 a, b, 1991). The aim of the present work was to examine whether the concentrations of arginine, citrulline and ornithine in rat brain structures are lower in aged animals, as a possible reflection of a decreased production of NO in ageing. In the same structures, brain glutamic and aspartic acid levels were also measured.

## Materials and methods

Two groups, each of 8 male Wistar rats (Iffa Credo, France) aged 22 and 3 months, respectively, were used. The animals were fasted overnight and killed by decapitation. Brains were removed immediately and the different areas dissected on an ice-cooled steel plate as described in Strolin Benedetti et al. (1991). After dissection, the brain areas were immediately frozen on dry ice, weighed and then homogenized in a solution of cooled (4 °C) methanol/water (80/20, v/v) containing L-homoserine as internal standard, in the proportion 1 g tissue/10 ml solution using an ultrasonic desintegrator (Soniprep 150).

### *Procedure used for determination of amino acids concentrations*

The procedure was essentially identical to that described in Strolin Benedetti et al. (1990 a) with minor modifications. Briefly, the homogenates were centrifuged at 4 °C (10 min, 12,500 g) and the supernatants were separated and diluted with cooled distilled water (1/10 v/v) for all areas. Analysis of the amino acids was carried out by HPLC with fluorimetric detection after derivatization with o-phthaldialdehyde (OPA) /2-mercaptoethanol as described by Jones and Gilligan (1983), with some modifications. Twenty µl of the solution obtained after dilution of supernatants with water, which contains 21 pmol/µl of internal standard, were reacted with 20 µl of OPA solution (Pierce) using a LABNET modular Spectra-Physics (SP) liquid chromatography system equipped with an automatic system for pre-column primary amino acid derivatization; 10 µl of the reaction mixture were injected directly into the analytical column. The HPLC system consisted of a SP 8800 Pump, a SP 8880 Autosampler and a SP 4270 Computing Integrator equipped with a LABNET Data Capture Module. A Jasco model 821-FP fluorescence detector set up at 340/455 nm was used. The chromatography was performed by a gradient elution on a 3 µm particle size Hypersil ODS column (100 × 4.6 mm I.D.) at a flow rate of 1 ml/min. Solvent A: 0.1 M sodium acetate pH 7.2/methanol/tetrahydrofuran (90/9.5/0.5); solvent B: methanol. For all the amino acids, except ornithine, the gradient was: Solvent A 100% to 95% in two min, 95% to 82% in 23 min and 82% to 65% in 10 min. For ornithine the gradient was: Solvent A 100% to 95% in 2 min, 95% to 82% in 23 min, 82% to 65% in 10 min, 65% to 50% in 10 min and 50% to 30% in 5 min. The limit of sensitivity was 1 pmole for aspartic acid, glutamic acid and arginine, i.e. 0.02 µmol/g tissue. The limit of sensitivity was 0.3 and 5 pmol for citrulline and ornithine, respectively, i.e. 0.006 and 0.1 µmol/g tissue.

## Results

The elution profile of standard OPA-derivatized arginine, citrulline and L-homoserine is presented in Fig.1 A.The elution profile of the accumbens of an old rat and that of the same region spiked with 24 and 4 nmoles of standard arginine and citrulline are presented in Figs.1 B and C, respectively. Construction of standard curves for arginine and citrulline has shown that the peak area ratio citrulline/internal standard is linear between 0.313 and 2.50 pmol injected and that the peak area ratio arginine/internal standard is linear between 1.875 and 15 pmol injected.

In all brain regions the peak of ornithine, the retention time of which was 50 min, was inferior to the limit of detection. Concentrations of the other four amino acids in the different brain areas of old and young rats are presented in Table 1. In the same table are indicated also the values of the concentration ratio arginine/citrulline. Aspartic acid concentrations significantly decreased

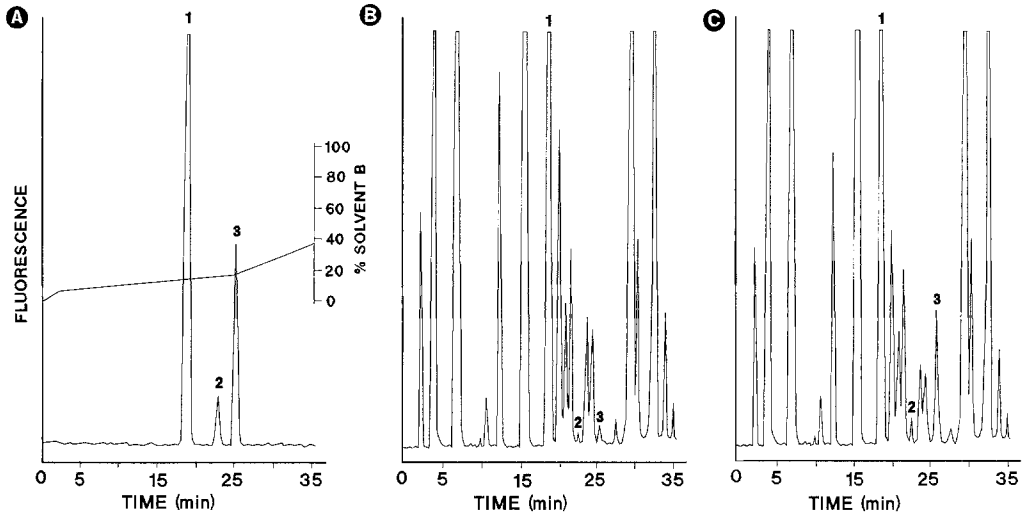


**Fig. 1.** Elution profile of OPA-derivatized L-homoserine (1), citrulline (2) and arginine (3) from a standard mixture [A] (peak 1, 2 and 3 represents 105, 5 and 30 pmoles, respectively), from one accumbens of old rat [B] (added with 84 nmoles of L-homoserine), and from one accumbens of old rat spiked with 84, 4, and 24 nmoles of L-homoserine, citrulline and arginine, respectively

with age in 6 out of the 9 brain regions studied (substantia nigra, midbrain, hippocampus, pons medulla, cerebellum and frontal cortex). Glutamic acid concentrations showed a slight tendency to decrease with age but in no region statistical significance was reached. Arginine and citrulline concentrations significantly decreased with age in 4 and 3 out of the 9 brain regions studied, respectively (substantia nigra, accumbens, pons medulla, and cerebellum for arginine, accumbens, pons medulla and frontal cortex for citrulline). Pons medulla was found to be the brain region with the highest number of amino acids (3 out of the 4 measured) significantly decreased with age. In all brain regions of young rats arginine concentrations were two to four times higher than citrulline concentrations, with the exception of hypothalamus and substantia nigra, where arginine concentrations were more than 5 and 8 times higher than citrulline concentrations, respectively. The concentration ratio arginine/citrulline significantly increased with age in pons-medulla and in frontal cortex.

## Discussion

The changes in concentrations of aspartic acid observed with age in the present study are in reasonable agreement with those noted in two previous studies where male Wistar rats of the same breeder were used (Strolin Benedetti et al., 1990a, 1991). In contrast, no age-related statistically significant decrease in glutamic acid concentrations was observed in the present work, in which rats were not injected with 3-mercaptopropionic acid prior to decapitation, as was done in the two previous studies where GABA levels had to be measured. The

**Table 1.** Amino acid levels (μmol/g wet tissue) in different brain regions from young and old Wistar rats

| Brain regions | Aspartic acid | Glutamic acid | Citrulline | Arginine | Arginine / Citrulline |
|---|---|---|---|---|---|
| S. nigra | 2.52 ± 0.08 | 6.32 ± 0.23 | 0.024 ± 0.003 | 0.177 ± 0.008 | 8.971 ± 1.613 |
| | 2.28 ± 0.08 | 5.70 ± 0.25 | 0.018 ± 0.004 | 0.146 ± 0.007 | 10.119 ± 1.462 |
| | (90%)* | (90%) | (75%) | (82%)* | |
| Striatum | 1.53 ± 0.07 | 9.67 ± 0.34 | 0.054 ± 0.004 | 0.108 ± 0.006 | 2.043 ± 0.119 |
| | 1.38 ± 0.03 | 9.01 ± 0.18 | 0.043 ± 0.003 | 0.104 ± 0.003 | 2556 ± 0.217 |
| | (90%) | (93%) | (80%) | (96%) | |
| Accumbens | 2.84 ± 0.15 a | 10.42 ± 0.28 a | 0.038 ± 0.001 a | 0.081 ± 0.003 a | 2.128 ± 0.118 a |
| | 2.70 ± 0.08 | 10.08 ± 0.31 | 0.028 ± 0.001 | 0.066 ± 0.004 | 2.347 ± 0.168 |
| | (95%) | (97%) | (74%)** | (81%)** | |
| Midbrain | 3.06 ± 0.06 | 12.86 ± 0.32 | 0.026 ± 0.002 | 0.094 ± 0.004 | 3.637 ± 0.149 |
| | 2.73 ± 0.06 | 11.98 ± 0.31 | 0.020 ± 0.002 | 0.083 ± 0.004 | 4.554 ± 0.706 |
| | (89%)** | (93%) | (77%) | (88%) | |
| Hippocampus | 2.15 ± 0.10 | 13.24 ± 0.29 | 0.059 ± 0.005 | 0.143 ± 0.011 | 2.466 ± 0.226 |
| | 1.90 ± 0.03 | 12.97 ± 0.15 | 0.047 ± 0.005 | 0.131 ± 0.014 | 2.816 ± 0.157 |
| | (88%)* | (98%) | (80%) | (92%) | |
| Pons-medulla | 2.98 ± 0.09 | 8.51 ± 0.27 | 0.039 ± 0.002 | 0.156 ± 0.004 | 4.001 ± 0.158 |
| | 2.64 ± 0.05 a | 8.15 ± 0.20 a | 0.023 ± 0.003 a | 0.142 ± 0.004 a | 6.818 ± 0.856 a** |
| | (89%)** | (96%) | (60%)** | (91%)* | |
| Hypothalamus | 2.50 ± 0.05 | 9.18 ± 0.16 | 0.034 ± 0.004 | 0.180 ± 0.016 | 5.701 ± 0.578 |
| | 2.34 ± 0.05 | 9.01 ± 0.17 | 0.028 ± 0.003 | 0.154 ± 0.012 | 6.037 ± 0.889 |
| | (94%) | (98%) | (82%) | (86%) | |
| Cerebellum | 2.43 ± 0.03 | 11.11 ± 0.11 | 0.045 ± 0.004 | 0.107 ± 0.003 | 2.524 ± 0.237 |
| | 2.18 ± 0.07 | 11.30 ± 0.31 | 0.036 ± 0.001 | 0.082 ± 0.004 | 2.251 ± 0.111 |
| | (90%)** | (102%) | (80%) | (78%)** | |
| Frontal cortex | 2.70 ± 0.06 | 12.64 ± 0.13 | 0.034 ± 0.003 | 0.094 ± 0.007 | 2.887 ± 0.279 |
| | 2.43 ± 0.05 | 12.18 ± 0.20 | 0.022 ± 0.002 | 0.084 ± 0.003 | 4.060 ± 0.392* |
| | (90%)** | (96%) | (65%)* | (89%) | |

in ( ): % of variation; upper data: young rats; data are expressed as mean ± SEM from 8 or 7 (a) animals; * $p < 0.05$, ** $p < 0.01$: Mann-Whitney U-test

lack of significant decrease in glutamic acid concentrations with age in the absence of 3-mercaptopropionic acid injection is at first glance surprising and remains unexplained.

Arginine concentrations found in this work are similar to those reported by Ida and Kuriyama (1983). These authors used animals of the same strain, sex and age as the young rats of the present work and found arginine concentrations ranging from 0.145 to 0.198 μmol/g wet tissue in different brain regions. The concentrations of arginine and citrulline determined in the present study are in keeping with the data reported by Zanchin et al. (1979), as also with those of Sasaoka et al. (1976), who reported arginine and citrulline concentrations of 0.13 and 0.05 μmol/g wet tissue in whole brain of male weanling Wistar rats, respectively. Patacchioli et al. (1990) reported unusually high levels of arginine (3.4 μmol/g fresh tissue) in whole brain of adult Wistar rats, at variance with the values they found in different brain regions. In whole brain of 3-month-old Sprague-Dawley rats arginine and citrulline concentrations of 0.075 and 0.036 μmol/g fresh tissue were found (Bayer and McMurray, 1967), in reasonable agreement with the values determined in Wistar rats.

In the present work, a significant decrease in arginine and citrulline concentrations with age was found in several brain regions, with a concomitant decrease of both amino acids in accumbens and pons medulla (Table 1). The decrease in arginine might, at least partly, reflect the age-related decrease in aspartic acid levels, as arginine is resynthesized from citrulline and aspartate in the brain. Both arginine and aspartic acid concentrations lowered with age in all brain structures examined (Table 1), although the decrease did not always reach statistical significance. It is interesting to note that inborn defects of the two enzymes, arginosuccinase and arginosuccinate synthetase, involved in the transformation of citrulline into arginine are known to cause severe mental retardation. The decrease in arginine concentrations might also result from an increased formation of ornithine with age. Sasaoka et al. (1976) reported ornithine concentrations of 0.040 μmol/g tissue in rat whole brain. However, under our experimental conditions, ornithine concentrations were lower than the limit of detection (0.1 μmol/g tissue) in all the brain regions examined.

Using an indirect bioassay to estimate NOS activity in different rat brain regions, Förstermann et al. (1990) found the highest activity in cerebellum and the lowest activity in pons-medulla, while NOS activity in hypothalamus, midbrain, striatum, hippocampus and frontal cortex was about 3–6 times lower than that of cerebellum. NOS was purified to homogeneity from rat cerebellum (Bredt and Snyder, 1990; Schmidt et al., 1991), and a $K_m$ value of 1.5–2.2 μM was determined with arginine as substrate. Using crude synaptosomal cytosol preparations from rat forebrain, Knowles et al. (1989) found a $K_m$ value of 6 μM for the production of NO from L-arginine, with maximum reaction velocity being reached at substrate concentrations of about 50 μM. In the different brain regions of the young and old rats, arginine concentrations were found to range from ca. 66 μM (accumbens of old rats, Table 1) to ca. 180 μM (hypo-

thalamus of young rats), suggesting that, in both old and young rats, the formation of citrulline from arginine should occur under conditions of substrate saturation. In some brain regions of old rats (accumbens, pons medulla and frontal cortex) citrulline levels were significantly lower than in young animals. Thus, at least in these brain regions, NOS activity appears to decrease with ageing, without it being possible to determine whether the change in NOS activity would result from neuronal loss causing a decrease in the enzyme concentrations and/or from modifications of the enzyme properties.

In the rat brain, NOS activity seems to be exclusively localized in neurons (Bredt et al., 1990). Brain enzymes, such as choline acetyltransferase and glutamic acid decarboxylase (Lai et al., 1981), monoamine oxidase type A (Strolin Benedetti and Keane, 1980), tyrosine hydroxylase (David et al., 1989), which are markers of neuronal cells, have been shown to decrease in rats with ageing. In contrast, the activity of the brain enzymes: glutamine synthetase (Cao Danh et al., 1985) and monoamine oxidase-B (Strolin Benedetti and Keane, 1980), which are preponderantly present in glial cells, has been found to be enhanced in old rats compared to young controls. Furthermore, the activity of rat brain manganese-containing superoxide dismutase, an isoenzyme that seems to be at least largely present in glial cells (Saggu et al., 1989), was also shown to increase with ageing (Cao Danh et al., 1983). There is evidence for neuronal NADPH diaphorase being a NOS (Bredt et al., 1991; Hope et al., 1991; Dawson et al., 1991). Based on the analogy of their respective amino acid sequence and the presence of recognition sites for the same cofactors, NOS and NADPH-cytochrome P-450 reductase have been shown to display close homology (Bredt et al., 1992). Like NOS, cerebral NADPH-cytochrome P-450 reductase appears to be localized in neurons (Ghersi-Egea et al., 1989; Ravindranath et al., 1990). The activity of this enzyme has been shown to decline with age in several rat tissues (Schmucke and Wang, 1983; Santa Maria and Machado, 1986). Since neuronal enzyme activities are generally found to be lower in aged animals and humans, a decrease in NOS activity could be expected with ageing. Although, additional experiments are clearly needed to conclusively establish whether NOS activity is modified with ageing, a significant decrease in citrulline concentrations was found in some brain areas of old rats, while arginine concentrations remained high enough for NOS activity to occur at maximum velocity. These results suggest that brain NOS activity may be lower in aged rats compared to young animals, in good accordance with the possible role of NO in synaptic plasticity and the well-established age-related decline of cognitive function in laboratory animals and humans.

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Received January 22, 1993