NO-Synthase Activity and Generation of Reactive Oxygen Species in the Brain in Old Rats: Association with Individual Behavior

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NO synthase activity and the generation of reactive oxygen species (ROS) were measured in different brain regions of old rats separated in an "emotional resonance" test into two groups: passive rats (those preferring a dark space) and active ones (those preferring a lighted space). In both groups, NO synthase activity and ROS generation were at the highest level in the cerebellum. In the tested brain regions of active rats, NO synthase activity was lower and ROS generation more strongly marked than in the respective regions of passive rats. Interregional positive linear correlations were discovered both for NO synthase activity and for ROS generation. When the two groups were considered together, negative correlations were detected between NO synthase activity and ROS generation.

Key Words: *nitric oxide; NO synthase; brain; individual behavior*

Mounting interest in the function of nitric oxide (NO) in the central nervous system has stimulated studies of NO synthase (NOS) as the major (and, possibly, the only) source of NO in the brain. Interest in the study of NOS in various brain structures and regions is piqued by the widely held view that NO plays a part in synaptic plasticity and memory mechanisms [5,12]. Regional differences in the rates of NO generation may be presumed to play a role in shaping strategies of individual behavior.

In addition to NO, another product of free-radical nature, superoxide, is generated in the reaction of L-arginine oxidation catalyzed by brain NOS [14]. The interaction of these two products gives rise to highly reactive radicals (including peroxynitrite) which are believed to be the major agents of NO neurotoxicity [9,10]. This suggests that NOS activity is closely associated with the generation of reactive oxygen species (ROS) and plays an important part in establishing a steady-state level of free-radical processes in the brain and in modifying them under various circumstances.

In this study we examined NOS activity and ROS generation in different parts of the brain in two groups of old rats with different types of individual behavior.

MATERIALS AND METHODS

Fifty male Wistar rats aged 23 months, maintained under standard conditions in the vivarium with free access to food and water, were each subjected to a single "emotional resonance" test [1]. For this test, the rat was placed in a spacious illuminated compartment of the experimental chamber, which also contained a small dark compartment. Each time it entered the dark compartment, the rat heard the cry

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TABLE 1. NO Synthase Activity (nmol/min/g wet tissue) in Different Parts of the Brain in Old Rats ($M \pm m$)

Brain part	Group		
	1 (n=12)	2 (n=6)	1+2 (n=18)
Cerebellum	66.5±6.5+	59.5±8.3	64.2±5.1
Left cerebral cortex	54.4±7.8+	35.8±7.0*	48.2±5.9*
Right cerebral cortex	54.0±9.1	39.8±7.2*	49.3±6.6*
Subcortical structures	65.4±5.7+	54.7±7.6	63.8±5.5

Note. *p<0.05 in comparison with the cerebellum in the same group. Here and in Table 2: +p<0.05 in comparison with group 2.

of another rat ("victim") subjected to unavoidable painful stimulation with electric current. The cry ceased as soon as the test rat returned to the illuminated compartment. During the test, which lasted 300 sec, the number of passages between the compartments and the time spent in each were recorded.

From the 50 rats 18 were selected on the basis of the test results: 12 passive rats (group 1) preferring the dark space (number of passages = 1 or 2; time spent in the lighted compartment <50 sec), and 6 active rats (group 2) preferring the lighted space (3-5 passages and >150 sec, respectively). Two weeks later the rats were decapitated and their brains were rapidly removed, washed in glacial isotonic NaCl solution, and frozen in liquid nitrogen. From the frozen brain the cerebellum, cortex of the right and left hemispheres, and subcortical structures were dissected out and homogenized with 2 volumes of buffer containing 50 mM HEPES, pH 7.4, and 1 mM $CaCl_2$. Samples of these homogenates were used to determine NOS activity, while the remaining homogenates were centrifuged at 3000 g for 10 min and the supernatants used for the evaluation of ROS generation.

NOS activity was measured by a method which recorded mononitrosyl complexes of NO with diethyldithiocarbamate and bivalent iron [3]. For the evaluation of ROS generation, the total emission of luminol-dependent chemiluminescence induced by H_2O_2 [4,15] was recorded with a KhLM-3M chemiluminometer (Russia) in glass cuvettes at 20°C; 50 µl of 0.34 mM luminol and 20 µl of the supernatant diluted 400-fold were added to 1 ml of buffer (pH 7.4) containing 20 mM KH_2PO_4 and 115 mM KCl, and the reaction was initiated by adding H_2O_2 (final concentration 0.48 mM). All chemical reagents were from Sigma. Statistical analysis was performed on a computer using the standard STATGRAPHICS software package.

RESULTS

The two groups of rats differed significantly in both test parameters (p<0.01): the number of passages between the chamber's compartments was 1.8±0.3 in group 1 and 3.8±0.4 in group 2, while the time spent in the lighted compartment was 21.0±9.7 and 246.1±15.9 sec, respectively.

Table 1 demonstrates regional differences in NOS activity in the brain of old rats. In both groups, NOS activity was highest in the cerebellum and subcortical structures; in the cerebral cortex, it was significantly higher in group 1 and in the combined group (1+2). As a rule, NOS activity in the different brain regions of group 2 (active rats preferring the lighted space) was lower than in those of group 1 (passive rats preferring the dark space).

TABLE 2. Generation of Reactive Oxygen Species, as Assessed by H_2O_2–Induced Luminol–Dependent Chemiluminescence (arb. units), in Different Parts of the Brain in Old Rats ($M \pm m$)

Brain part	Group		
	1 (n=12)	2 (n=6)	1+2 (n=18)
Cerebellum	216.3±9.9+	246.5±16.2	226.3±8.9
Left cerebral cortex	148.8±4.7+	176.2±6.0	157.9±4.8
Right cerebral cortex	153.8±6.4+	170.2±2.0	159.3±4.6
Subcortical structures	174.1±6.3+	194.2±9.0	180.8±5.5

Note. Statistically significant differences by Student's t test: in group 1: cerebellum – left cortex and cerebelllum – right cortex (p<0.0001); cerebellum – subcortical structures, left cortex – subcortical structures, and right cortex – subcortical structures (p<0.05); in group 2: cerebellum – left cortex and cerebellum – right cortex (p<0.003); cerebellum – subcortical structures (p<0.02); right cortex – subcortical structures (p<0.03); in group 1+2: cerebellum – left cortex, cerebellum – right cortex, and cerebellum – subcortical structures (p<0.0001); left cortex – subcortical structures and right cortex – cerebellum (p<0.005).

The results of evaluating ROS generation are presented in Table 2. ROS generation was maximal in the cerebellum and minimal in the cerebral cortices. In group 2, it was significantly greater than in group 1 in all brain regions studied.

Thus, NOS activity and ROS generation in the cerebellum were higher than in the cerebral cortices in both groups. In our study, however, differences in NOS activity between the cerebellum and other parts of the brain were less marked than those reported by Forstermann *et al.* [7], who used an indirect indicator of NOS activity, namely activation of guanylate cyclase. We employed a direct method of estimating NOS activity in dense brain homogenates, which yields activity values closer to those occurring *in vivo*. Analysis of variance (ANOVA) showed no significant association of NOS activity with type of behavior in any of the brain regions ($F<2.35$, $p>0.15$), but did show such an association for ROS generation (cerebellum: $F=5.17$, $p<0.03$; left cortex: $F=11.89$, $p<0.004$; right cortex: $F=3.30$, $p<0.09$; subcortical structures: $F=3.34$, $p<0.09$).

In the combined group (1+2), the following interregional positive linear correlations were found for NOS activity: cerebellum - left cortex, cerebellum - right cortex, cerebellum - subcortical structures, left cortex - right cortex, left cortex - subcortical structures, right cortex - subcortical structures ($r=0.73$-0.93, $p<0.001$-0.0001). Such correlations were also detected in group 1 ($r=0.69$-0.94, $p<0.02$-0.0001) and group 2 ($r=0.82$-0.98, $p<0.05$-0.0006).

Interregional linear correlations in the combined group were also found for ROS generation: cerebellum - left cortex, cerebellum - right cortex, cerebellum - subcortical structures, left cortex - right cortex, left cortex - subcortical structures, right cortex - subcortical structures ($r=0.50$-0.70, $p<0.04$-0.002). These correlations were all detected in group 1 as well ($r=0.75$-0.95, $p<0.05$-0.0001), but only three of them in group 2 (apparently because the number of animals was too small): left cortex - right cortex, left cortex - subcortical structures, and right cortex - subcortical structures ($r=0.86$-0.98, $p<0.03$-0.004). The presence of interregional correlations in NOS activity and ROS generation indicates that NO and oxygen radicals are formed in a coordinated manner and suggests the existence of a mechanism for such coordination.

In the combined group (1+2), negative linear correlations (both statistically significant and trends) between NOS activity and ROS generation were discovered for the cerebellum ($r=-0.40$, $p<0.1$), left cortex ($r=-0.52$, $p<0.03$), right cortex ($r=-0.51$, $p<0.03$), and subcortical structures ($r=-0.33$, $p<0.2$).

Although studies of NO reactions *in vitro* [6,9,10,13,14] and of radical formation associated with activation of N-methyl-D-aspartate receptors [8,11] have provided evidence suggesting enhancement of ROS generation with rising NOS activity, the presence of a negative association between NOS activity and ROS generation indicates that the direct relationship between these two parameters established *in vitro* does not hold *in vivo* (at least in the brain of old animals). There is probably a regulatory system which controls and coordinates NO and ROS generation.

The "emotional resonance" test, which is often utilized for separating rats according to behavioral responses [1,2], involves factors meaningful for these animals, such as the choice between a large illuminated space and a small dark space and signals of pain from another rat ("victim"). This test reveals characteristics of individual behavior correlating with other behavioral variables, for example learning capacity [2] and activity in an open field [1]. The present study demonstrated behavior-related differences in NOS activity and ROS generation between active rats preferring a lighted space and passive ones preferring darkness: the former animals were found to show lower NOS activity and more intensive ROS generation in the brain than the latter. In contrast to our earlier study [1], where differences in free-radical processes in the brain were detected between rats with different types of behavior in the "emotional resonance" test, the present study failed to reveal clear-cut differences in the interhemispheric asymmetry of NOS activity or ROS generation. The results we have obtained should help in developing neurochemical patterns for different types of individual behavior.

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Recording of NADPH Oxidation as a Means of Estimating NO Synthase Activity

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A novel approach to the measurement of NO synthase activity in brain tissue is described. In the NO synthase-catalyzed reaction, NADPH undergoes stoichiometric oxidation and, using a known specific inhibitor of this oxidation, NO synthase activity can be estimated by recording the oxidation rate. In the proposed approach, NADPH oxidation is recorded fluorimetrically, and the rate of this reaction in the presence of Nω-nitro-L-arginine is subtracted from its initial rate in the presence of L-arginine. This approach can be used to develop a simple, sensitive, and specific method for estimating NO synthase activity in brain structures of small animals.

Key Words: *nitric oxide; NO synthase; NADPH; brain*

The keen interest shown in the role played by nitric oxide (NO) in the central nervous system [3,10] has stimulated the development of methods for estimating the activity of the NO-synthesizing enzyme, NO synthase (NOS). Because the direct methods of NOS activity determination are complicated and not always reliable, the method resorted to most frequently to demonstrate the roles of NO and NOS *in vivo* involves inhibition of this enzyme by L-arginine analogs; indeed, it can be asserted with confidence that most of the data relating to NO functioning in the body have been obtained in this way. Unfortunately, such data are not open to a single interpretation, for it is not known which enzymes of arginine metabolism other than NOS can alter their activity in the presence of these inhibitors. Of the biochemical tests available for measuring NOS activity in brain tissue, the following four are most commonly employed: estimation of L-citrulline synthesis from radiolabeled L-arginine [4,9,11]; chemiluminescent determination of NO after its chemical reduction [2,12]; stimulation of soluble fibroblast guanylate cyclase as a measure of NO generation [5,7]; and spectrophotometric recording of oxyhemoglobin oxidation to methemoglobin in the presence of NO [7,8]. Generally, these tests are very time-consuming and/or insufficiently specific and for this reason are best suited for measuring the activity of purified NOS. EPR spectroscopy, with which NO generation in brain tissue can be fol-

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