The Modulation of Mitochondrial Nitric-oxide Synthase Activity in Rat Brain Development*

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Different mitochondrial nitric-oxide synthase (mtNOS) isoforms have been described in rat and mouse tissues, such as liver, thymus, skeletal muscle, and more recently, heart and brain. The modulation of these variants by thyroid status, hypoxia, or gene deficiency opens a broad spectrum of mtNOS-dependent tissue-specific functions. In this study, a new mtNOS variant is described in rat brain with an Mr of 144 kDa and mainly localized in the inner mitochondrial membrane. During rat brain maturation, the expression and activity of mtNOS were maximal at the late embryonic stages and early postnatal days followed by a decreased expression in the adult stage (100 ± 9 versus 19 ± 2 pmol of [3H]citrulline/min/mg of protein, respectively). This temporal pattern was opposite to that of the cytosolic 157-kDa nNOS protein. Mitochondrial redox changes followed the variations in mtNOS activity: mtNOS-dependent production of hydrogen peroxide was maximal in newborns and decreased markedly in the adult stage, thus reflecting the production and utilization of mitochondrial matrix nitric oxide. Moreover, the activity of brain Mn-superoxide dismutase followed a developmental pattern similar to that of mtNOS. Cerebellar granular cells isolated from newborn rats and with high mtNOS activity exhibited maximal proliferation rates, which were decreased by modifying the levels of either hydrogen peroxide or nitric oxide. Altogether, these findings support the notion that a coordinated modulation of mtNOS and Mn-superoxide dismutase contributes to establish the rat brain redox status and participate in the normal physiology of brain development.

In recent years, the occurrence of nitric-oxide synthase (NOS) variants located in mitochondria (mtNOS) has been reported: an iNOS protein was detected in rat liver and thymus and pig heart (1–3); eNOS was found in rat skeletal muscle and liver (4, 5), and nNOS was described in rat skeletal muscle (6) and mouse heart (7). Thus, the mtNOS family appears to cover a broad spectrum of structurally and immunologically different proteins that could result from transcriptional or translational modifications that allow them to be targeted to mitochondria. However, differences between mtNOS and the classic NOS isoforms were reported: liver mtNOS exhibits an amino acid sequence similar to iNOS (8, 9), albeit with a distinctive acylation pattern (8); mtNOS activity invariably depends on Ca2+ and calmodulin, even when it may be immunologically related to iNOS (1, 6); and a distinct kinetic pattern has been observed for mtNOS (1, 8).

mtNOS seems to possess functions adapted to tissue-specific needs. In support of this notion, mtNOS activities in liver and skeletal muscle are modulated by the thyroid status (6), and those in brain and liver are modulated by hypoxia (5). Moreover, the level of mtNOS expression is modified by the activity or deficiency of specific genes, such as dystrophin in the heart (7). The regulation of mtNOS provides a new insight into the physiological significance of NO and mitochondria in cell biology. It is well known that NO binds to cytochrome oxidase with high affinity, modulates O2 uptake (10, 11), and increases the mitochondrial production of superoxide anion (O2•−), and depending on mitochondrial matrix Mn-superoxide dismutase levels, of hydrogen peroxide (H2O2) (10, 12). Accordingly, activation of mtNOS could be followed by an increase in the rate of mitochondrial H2O2 production rate (13). H2O2, generated in this manner, is involved in the regulation of different cellular processes: in brain, it could participate in cell signaling networks during early synaptogenesis and plasticity (14); furthermore, following mtNOS activation, an exacerbated oxidant production may play a role in apoptotic signaling (15).

Characteristically, nNOS and splice variants of the nNOS gene with distinctive subcellular localizations are differentially expressed during the embryonic life (16) and are related to the normal development of the brain (17). These studies were aimed at analyzing the occurrence of an NOS activity localized in brain mitochondria, its developmental profile, and its influence on redox metabolism. The physiological significance of these studies was strengthened by an experimental approach aimed at providing a relationship between mitochondrial production of NO and H2O2 and neuron proliferation.

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Materials and Methods

Chemicals and biologicals—3-Amino-1,2,4-triazole, apronitin, t-arginine, bovine serum albumin, calmodulin, catalase, CHAPS, Cu,Zn-superoxide dismutase, cytochrome c, dithiothreitol, EDTA, FAD, FMN, glutathione, HEPES, horseradish peroxidase, hydroxyl peroxide, KCN, leupeptin, NADPH, NAD⁢⁺-monomethyl-t-arginine (l-NMMA), ovomucoid, Pervoll, phenylmethylsulfonyl fluoride, p-hydroxy-phenyl acetic acid, polyclonal anti-serum, tetrahydrobiopterin, Tris, trypsin, xanthine dehydrogenase, xanthine oxidase were from Sigma. Mn⁢(III) tetrakis (4-benzoic acid) porphyrin chloride (Mn(III) TBAP) was from Cayman Chemical (Ann Arbor, MI), and DNase was from Roche Molecular Biochemicals. 7-Acetyl cysteine was a gift from Dr. Daniel Colombari (Poen Labo, Arbor, MI), and DNase was from Roche Molecular Biochemicals. 4% formaldehyde freshly prepared from paraformaldehyde in PBS, pH 7.4, for 2 h at 4°C.

To inhibit oxidative decay of intra-mitochondrial NO, assays were carried out at about 70 μM O₂. All assays were performed at different conditions ranging from 2 to 5 mM H₂O₂. The rate of the reaction was determined as the pseudo-first order reaction (k') constant and expressed as min⁻¹ mg of protein⁻¹. Ubiquinone content in isolated mitochondria was determined by high pressure liquid chromatography (Waters) with UV detection at 275 nm after extraction with cyclohexane:ethanol (5:2) (6 mg of mitochondrial protein/ml and 7 ml of cyclohexane:ethanol (21)).

Western Blotting—Aliquots of 100 μg of protein were separated by electrophoresis on 6% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat milk, 0.1 mM Tris-borate 20 mM Tris-buffered saline solution, pH 7.4, and blocked with 4% non-fat milk. Membranes were incubated with the first antibody (1 h, 1:1000 dilution), washed, and subsequently incubated with a secondary goat anti-mouse or anti-rabbit IgG antibody. The membranes were developed with 0.25% trypsin, 1.2 mM MgSO₄, and 3 mg/ml bovine serum albumin for 15 min at 37°C under gentle shaking. The preparation was then centrifuged for 30 min at 100,000 × g, and the supernatant was separated from the pellet (CHAPS-soluble fraction) and pellet (CHAPS-insoluble fraction). The membranes were then stained and stored at −20°C until use.

Mitochondrial NO Production Activity—Mitochondria were sonicated twice at 40°C with 25 strokes in a 2-mL Eppendorf tube containing 0.25M sucrose, 10 mM HEPES, pH 7.4, containing 2 mM dithiothreitol and 20 mM L-arginine, bovine serum albumin, calmodulin, catalase, CHAPS, Cu,Zn-superoxide dismutase, tetrahydrobiopterin, and 100 mM MnCl₂ were obtained by bubbling NO gas to 99.9% purity (AGA Gas Inc., Maumee, OH) in water degassed with helium at 30 min at room temperature and stocked for a week at 4°C.

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Western Blotting—Cerebellar granule cell cultures were obtained as described previously (23). Five-day-old Sprague-Dawley rats were decapitated, and cerebellar tissue was dissected in Krebs-Ringer solution supplemented with 6 g/l glucose. Meninges were eliminated, and tissue was cut into 1-mm pieces and incubated in saline containing 0.025% trypsin, 1.2 mM MgSO₄, and 3 mg/ml bovine serum albumin for 15 min at 37°C with continuous agitation. The enzymatic digestion was stopped with ovomucoid (trypsin inhibitor), and the tissue was mechanically dissociated with Pasteur pipettes of two different diameters (25 strokes) in saline-containing ovomucoid and 0.01% DNase. The cell suspension was centrifuged at 150 × g for 10 min, and the pellet was resuspended in cell culture medium (Neurobasal, Invitrogen). Cells were incubated on 96 multiwells (300,000 cells/well) coated with poly-l-lysine (molecular weight, 300,000) in serum-free medium supplemented with B27 (Invitrogen).

Western Blotting Assay—Cells were seeded on 96 multiwells, and after 2 h, they were incubated with [³H]Thymidine (1 μCi/ml) for 22 h. Subsequently, treatments were performed simultaneously with the addition of 10 μM L-NAME. The cells were harvested by trypsin digestion, and a 2-h incubation with bidistilled water and harvested with a Nunc cell harvester coupled to glass fiber filters (Whatman, GF/C) as described previously (23). Filters were washed seven times with bidistilled water and allowed to dry overnight. Radioactivity was counted in a liquid scintillation β-counter. [³H]Thymidine incorporation was expressed as the ratio (experimental − control)/control.
**RESULTS**

**Nitric-oxide Synthase Activity and Its Localization in Brain Mitochondria**—NOS activity was assessed in rat brain cytosol, synaptosomes, and purified mitochondria (Table I). In samples obtained from adult rats, the activity present in the mitochondrial fraction represented about 10% of that of cytosolic nNOS. The purification of mitochondria from organelles with similar sedimentation properties was confirmed by measuring the ratio between succinate-cytochrome c reductase (mitochondrial marker) and acidic phosphatase (lysosomal marker) activities. The mitochondrial preparations used throughout this study were enriched about 30-fold in mitochondrial markers. In addition, we confirmed the purity and the integrity of mitochondria by electron microscopy.

NOS activity was not affected by pretreatment of intact mitochondria with proteinase K, suggesting that it was not a product of cytosolic contamination and that the protein was localized inside the organelles. Accordingly, after fractionation of submitochondrial particles, NOS activity was enriched by 18-fold in the membrane fraction, which was mainly composed of inner mitochondrial membrane (CHAPS-insoluble fraction) (Table I).

**Fig. 1.** Cofactor dependence of nitric-oxide synthase in brain mitochondria. Black bar represents 100% of activity (18 ± 1 pmol of [3H]-citrulline/min/mg of protein, n = 4) measured in the presence of all substrates and cofactors at concentrations indicated under “Materials and Methods.” Gray bars represent percent of maximal brain mtNOS activity after omitting a particular cofactor from the reaction mixture. BH$_4$, tetrahydrobiopterin.

**Fig. 2.** Immunological characterization of brain mitochondria nitric-oxide synthase activity. A, immunoblots of brain mitochondrial proteins (M) (100 μg) and positive controls (C) (25 μg of protein) obtained from brain cytosol (nNOS), diaphragm homogenate (eNOS), and liver homogenate of septic rats (iNOS). B, effect of antibodies on mitochondrial nitric-oxide synthase activity. The bars express mtNOS activity after 30 min of incubation of mitochondria with different antibodies against C-terminal and N-terminal domains of nNOS (1:1000). C, differential distribution of mitochondrial and cytosolic nNOS in subcellular fractions. Upper panel, Western blot with anti-nNOS (1095–1289); lower panel, blot with anti-cytochrome c antibody; cytochrome c (cyt c) reactivity indicates the presence of mitochondria.

Brain mtNOS activity was dependent on tetrahydrobiopterin, Ca$^{2+}$/calmodulin, and NADPH, and to a lesser extent, on flavins (Fig. 1), as reported previously for nNOS. The mitochondrial NOS activity that represented the $\alpha$-G-monomethyl-L-arginine-sensitive activity was also inhibited by the NOS inhibitors NG-nitro-L-arginine, 7-nitroindazol, and N-iminoethyl-L-ornithine and by the flavoprotein inhibitor, diphenylene iodonium. Preliminary kinetics studies yielded a similar apparent $K_m$ for mtNOS and nNOS (2–12 μM).

**Immunological Characterization of Brain mtNOS—Cytosolic nNOS (157 kDa) was not observed in mitochondria; instead, a band of 144 kDa was detected by two anti-nNOS antibodies directed to the C-terminal domain (anti-nNOS 1095–1289) but not by antibodies directed to the N-terminal segment (anti-nNOS 1–181) (Fig. 2A). In addition, the 144-kDa protein did not cross-react with anti-

| Subcellular level | Specific Activity (pmol [3H]-citrulline/min/mg of protein) |
|-------------------|---------------------------------------------------------|
| Cytosol           | 180 ± 16                                                |
| Synaptosomes      | 54 ± 4                                                  |
| Mitochondria      | 17 ± 1                                                  |
| Submitochondrial level |                                       |
| Soluble           | 2 ± 0.1                                                 |
| Membranes         | 60 ± 4                                                  |
| CHAPS-soluble fraction | 43 ± 4                                   |
| CHAPS-insoluble fraction | 252 ± 19                              |
eNOS or anti-iNOS antibodies (Fig. 2A). The antibody against the NADPH-binding site of nNOS (anti-nNOS 1095–1289) abolished NOS activity in mitochondria, whereas neither anti-nNOS 1409–1429 nor anti-nNOS 1–181 did, thus confirming the identity of the band (Fig. 2B). The synaptosomal fraction, nerve end terminals containing synaptic mitochondria, exhibited a double band of nNOS (157 kDa) and mtNOS (144 kDa) proteins (Fig. 2C).

**Ultrastructural Mitochondrial Nitric-oxide Synthase Detection**—The specific detection of mtNOS by anti-nNOS 1409–1429 was used to assess by means of immuno-electron microscopy the localization of mtNOS at the ultrastructural level. In these conditions, colloidal gold particles were clearly detected in mitochondria (Fig. 3A). Moreover, the background levels were low, and samples processed with normal rabbit serum as a primary antibody did not show any labeling, thus confirming the specificity of the reaction. A more detailed study of the ultrastructural localization of the enzyme in mitochondria using a higher dilution of the first serum showed that labeling was mainly located in the inner face of the mitochondrial inner membrane, and to a lesser extent, in the mitochondrial matrix (Fig. 3B).

**Developmental Modulation of Rat Brain Mitochondrial Nitric-oxide Synthase**—The up-regulation of a 144-kDa nNOS variant in rat synaptosomes was reported to occur early after birth (17). In this study, the expression (Fig. 4) and activity (Fig. 5) of mtNOS in the brain of embryos and neonatal animals in parallel to cytosolic nNOS were addressed as follows. (a) Cytosolic NOS activity and expression were faintly detectable by embryonic day 15 and 19 and increased after birth to peak around the second and third postnatal weeks (Fig. 4A) (as reported previously (17)). (b) mtNOS activity showed a different developmental pattern: its expression and activity were elevated throughout the late embryonic period (Fig. 4B; E15–E19) up to the first postnatal week (Fig. 4B; P0–P6) and markedly decreased beyond this age. Thus, at P0, mtNOS protein expression and activity were about 6-fold higher than the corresponding adult values. The modulation of mtNOS activity was attributed to changes in protein expression, as inferred from the respective ratios (Fig. 5). Furthermore, mt-NOS followed a similar developmental pattern in cerebellum, although elevated activity persisted until postnatal day 15 (Fig. 5, inset). (c) Studies on the synaptosomal fraction permitted us to observe the co-modulation of both mtNOS and nNOS: in this preparation, mtNOS from synaptic mitochondria was apparently unique during the first 4 days; beyond this time...
point, both enzymes could be detected (Fig. 4C).

The Nitric Oxide-dependent Production of H$_2$O$_2$ in Brain Mitochondria—In different rat tissues, NO induces an increase of mitochondrial O$_2^{-}$ and H$_2$O$_2$ production rates by mechanisms involving inhibition of electron transfer at the bc$_1$ segment (10) and oxidation of membrane-bound ubiquinol (Reaction 1) (12, 13, 24) followed by ubisemiquinone autoxidation (Reaction 2).

\[
\text{UQH}^+ + \cdot \text{NO} \rightarrow \text{UQ}^+ + \text{NO}^- \\
\text{REACTION 1}
\]

\[
\text{UQ}^+ + \text{O}_2 \rightarrow \text{UQ} + \text{O}_2^-
\]

\text{REACTION 2}

The production of H$_2$O$_2$, by both P2–4 neonatal and adult brain mitochondria showed a biphasic response to 'NO (Fig. 6). The ascending and descending slopes of the curves are determined by the relative ratio of the two competing reactions (Reactions 3 and 4) that drive mitochondrial matrix O$_2^{-}$ to either H$_2$O$_2$ (catalyzed by Mn-superoxide dismutase; Reaction 3; $k_3 = 2 \times 10^9$ M$^{-1}$ s$^{-1}$) or ONOO$^-$ (by reacting with 'NO; Reaction 4; $k_4 = 1.9 \times 10^{10}$ M$^{-1}$ s$^{-1}$) (12).

\[
\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_3
\]

\text{REACTION 3}

\[
\text{O}_2^- + \cdot \text{NO} \rightarrow \text{ONOO}^-
\]

\text{REACTION 4}

Comparable maximal H$_2$O$_2$ production rates (0.4–0.5 nmol/min/mg of protein) were achieved at 0.1 $\mu$M 'NO in adults and at 0.2 $\mu$M 'NO in neonates (Fig. 6). In addition, total ubiquinone was ~20% lower in mitochondria from neonates than in those from adults. The ubiquinone-reducing activities of complexes I–III and complexes II–III as well as the activity of complex IV

Fig. 5. Nitric-oxide synthase-specific activities during rat brain development. The nitric-oxide synthase-specific activities during development were measured in the brain cytosolic and mitochondrial fractions described in the legend for Fig. 4 and according to "Materials and Methods." Inset, mitochondrial nitric-oxide synthase activity during postnatal maturation of rat cerebellum. The arrows indicate the moment of birth.

Fig. 6. Effects of NO on the H$_2$O$_2$ production rate of rat brain mitochondria. The reaction mixture contained 0.1 mg of mitochondrial protein/ml from either P2–4 neonates (○) or adults (●) in buffer as described under "Materials and Methods." The assay temperature was 30 °C. Data represent the mean of three experiments.
were ~45–50% lower in neonates than those in adult animals (Table II). These data may explain the 2-fold higher NO concentration required in neonatal mitochondria to elicit an H2O2 production rate similar to that in adult mitochondria.

The mtNOS-dependent Production of H2O2 during Development—Supplementation of brain mitochondria from P2–4 animals with L-arginine, the universal substrate of NOS, elicited a selective and marked increase in the rate of H2O2 generation obtained by supplementation with exogenous NO (Fig. 6) or antimycin A (Fig. 7), a complex III inhibitor that stimulates mitochondrial production of O2− and H2O2. The L-arginine-mediated increase was prevented (~80% lower value) by a molar excess of the NOS inhibitor L-NMMA, thus indicating that the activation of mtNOS was necessary for mediating the effect of L-arginine. Conversely, H2O2 production by mitochondria isolated from P15 or adult animals was not significantly affected by L-arginine supplementation, although these mitochondria retained the response to antimycin A (Fig. 7). Considering the temporal expression (Fig. 4) and activity (Fig. 5) of mtNOS, it may be surmised that (a) mitochondrial H2O2 production parallels the developmental modulation of mtNOS and (b) H2O2 production by mitochondria from adult animals is more susceptible to NO regulation, but the limited expression of mtNOS renders H2O2 production by these mitochondria less sensitive to L-arginine activation.

Co-modulation of mtNOS and Mn-Superoxide Dismutase during Brain Development—At low physiological NO concentrations, O2− is preferentially dismutated to H2O2 by Mn-superoxide dismutase, whereas at high levels, O2− is channeled toward the formation of ONOO− (12). Because the matrix levels of Mn-superoxide dismutase are essential for determining the fate of O2−, a study on superoxide dismutase activity through the period of high mtNOS expression was undertaken. Remarkably, the developmental time course of Mn-superoxide dismutase activity (the mitochondrial isoform) (Fig. 8A) paralleled that of mtNOS (Fig. 5), whereas cytosolic Cu,Zn-superoxide dismutase activity increased with age to reach constant levels (Fig. 8A).

The activities of Mn- and Cu,Zn-superoxide dismutases correlated with the respective variations in the activities of mtNOS (Fig. 8B) and nNOS (Fig. 8C), respectively. Intramitochondrial formation of ONOO− was not detected during the studied period, as inferred by the absence of protein tyrosine nitration (data not shown). Considering the two alternative reactions of O2− utilization (Reactions 3 and 4 above) (25), these results suggest that the 2-fold increased Mn-superoxide dismutase activity in P2–4 (with respect to that in adults) (Fig. 8A) would support the decay of O2− to H2O2 rather than to ONOO−, thus favoring the net flux of H2O2 into cytosol.

Role of H2O2 in Granular Cerebellar Neuroblast Proliferation—At variance with brain neurons, which exhibit a low proliferation rate after birth, cerebellar granular cells from cerebellum proliferate during a relatively prolonged postnatal period. Therefore, these cells represent a suitable model to study the effects of mtNOS expression and mitochondrial H2O2 in developing cerebellum. At P5, cerebellar tissue exhibited maximal expression and activity of mtNOS, which subsequently declined in adult life (P90) (Fig. 9A). Accordingly, the L-arginine-dependent mitochondrial production rate of H2O2 was 3-fold higher in neonates at P5 than in adult rats at P90.

**TABLE II**

Mitochondrial complex activities and ubiquinone content in brain from newborn and adult rats

| Complexes | I−III | II−III | IV | [UQ9] |
|-----------|-------|--------|----|-------|
| nmoles cyt c/min/mg of protein | k′ min/mg of protein | µg/mg of protein |
| Newborn  | 185 ± 21 | 42 ± 9 | 9 ± 2 | 408 ± 1 |
| Adult    | 337 ± 20 | 81 ± 17 | 18 ± 5 | 522 ± 3 |

**Fig. 7. Effects of brain maturation on mtNOS-dependent production of H2O2.** mtNOS-dependent production rate of H2O2 was assessed in the presence of either 100 µM L-Arg or 100 µM L-arginine plus 1 mM of the nitric-oxide synthase inhibitor L-NMMA (L-Arg + NMMA) or 2 µM of the complex III inhibitor antimycin A (AA). Bars represent mean ± S.E. of five separate experiments. * denotes significantly different from L-Arg and ** denotes significantly different from antimycin A by Student’s t-test (p < 0.01).
H$_2$O$_2$ diffuses outside mitochondria, and its steady-state concentration ([H$_2$O$_2$]$_{ss}$) in cytosol was calculated at different stages (26) according to Equation 1 (where $\frac{d[H_2O_2]}{dt}$ is the rate of L-arginine-dependent H$_2$O$_2$ production, $k_1$ is the second order rate constant for the catalase-catalyzed metabolism of H$_2$O$_2$, and $k_2$ is the second order rate constant for the glutathione peroxidase-driven reaction).

$$[H_2O_2]_{ss} = +\frac{d[H_2O_2]}{dt}h_1[\text{catalase}] + k_2[\text{glutathione peroxidase}] \quad (\text{Eq. 1})$$

The steady-state levels of H$_2$O$_2$ in proliferating cerebellum from neonates were 4-fold higher than those in the quiescent mature cerebellum of adult animals. These results suggest that the differences in neonatal and adult mitochondria are of physiological significance in vivo.

In support of this hypothesis, changes in the H$_2$O$_2$ steady-state levels in immature granular cerebellar neurons isolated from P5 rats significantly affected proliferation rate in terms of $[^3H]$thymidine incorporation (Fig. 9B). In these experimental conditions, primary granular cells grow in culture by 24 h in an enriched medium with Dulbecco’s modified Eagle’s medium/B27 and in the absence of serum supplementation or growth factors (23). Cells are rounded, and few of them express tetanus toxin fragment C, a specific marker of postmitotic neuronal phenotype (23). In agreement with the different redox states of neonates and adults, the magnitude of spontaneous proliferation of the primary cerebellar neurons at P5 was maximal. $[^3H]$Thymidine incorporation was abated in conditions that decreased [H$_2$O$_2$]$_{ss}$ or the mtNOS-sensitive production of H$_2$O$_2$, such as addition of relatively low amounts of catalase, the NOS inhibitor L-NMMA, GSH, or N-acetylcysteine (Fig. 9B). These treatments may approach [H$_2$O$_2$]$_{ss}$ values similar to those in the adult (Fig. 9A; $0.7 \times 10^{-10}$ M) and thus, a substantially decreased proliferation rate. Moreover, the [H$_2$O$_2$]$_{ss}$ values in P5 and the adult state ($2 \times 10^{-10}$ and $0.7 \times 10^{-10}$ M, respectively) suggest that regulation of proliferation rate occurs within a narrow range of H$_2$O$_2$ steady-state levels.

It may be surmised that during development, mtNOS and the resulting H$_2$O$_2$ production contribute to sustain the neuronal proliferation. Maximal neuronal proliferation is expected to be modulated in vivo within a narrow range of H$_2$O$_2$ concentrations; considering the H$_2$O$_2$ steady-state levels in adult life (non-proliferation) and in P5 (maximal prolifera-
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**Fig. 9.** \( H_2O_2 \) levels and cerebellar neuron proliferation. As shown in A, the expression and activities of mtNOS and their respective mitochondrial \( H_2O_2 \) yields were compared in organelles from quiescent adult and proliferating P5 newborn rat cerebellum as described under “Materials and Methods.” The contribution of mtNOS to cytosolic \( H_2O_2 \) steady-state concentration ([\( H_2O_2 \)]ss) was estimated from the \( t^-\)Arg-dependent mitochondrial \( H_2O_2 \) production rate and the specific activities of cytosolic catalase and glutathione peroxidase (Equation 1 under “Results”). As shown in B, immature granular cerebellar cells were isolated from P5 rats. Two h after seeding in Dulbecco’s modified Eagle’s medium Neurobasal B27 without serum supplementation, [\(^3H\)thymidine was added to cultures, and plates were incubated for 2 h. Drug treatments were performed simultaneously with the addition of [\(^3H\)thymidine. Proliferation was assessed at basal \( H_2O_2 \) concentration (white bar) after decreasing the basal \( H_2O_2 \) level with different compounds (\( t^-\)NMMA, catalase, \( N^-\)acetylcysteine) (gray bars). * denotes \( p < 0.05 \) (Student’s t test) with respect to adult values in panel A and to the basal condition in panel B.

**DISCUSSION**

Submitochondrial fractionation and immuno-electron microscopy analysis indicated that the nitric-oxide synthase variant in rat brain mitochondria described here had a predominant localization at the inner mitochondrial membrane. Differences between brain mtNOS and classic nNOS suggest a variation in the N-terminal domain of the protein (Fig. 2). Moreover, apparent molecular weight, immunological detection, and developmental modulation resemble those of the previously reported mouse synaptosomal 144-kDa nNOS (17). The two variants share a similarly reduced catalytic activity respect to the classic 157-kDa nNOS, although specific activity, \( Ca^{2+} \) dependence, and requirements are similar to all other mitochondrial isoforms.

Taking into consideration the immunological characterization shown in Fig. 2, brain mtNOS appears to be an entity different from rat or mouse liver mtNOS (1, 5). This discrepancy suggests the occurrence of tissue- and species-specific different mitochondrial variants. In this regard, mitochondrial variants of iNOS-like (3) and nNOS-like (7) isoforms in pig and rat heart organelles were described. Moreover, several years ago, an eNOS-like mtNOS in the rat brain was reported (27).

Immunological and functional observations are indicative of a developmental modulation of the brain mtNOS variant. This notion is supported by data showing that brain mtNOS is highly expressed and active in the late stages of fetal development and during the first postnatal days followed by a decreased expression in the adult brain. In agreement with previous reports, cytosolic nNOS was poorly detected in embryos or immediately after birth, and its expression increased sharply after postnatal day P6 (17).

The occurrence of mtNOS is consistent with a fine modulation of critical mitochondrial functions by \( NO \). In the brain, \( NO \) inhibits respiration (11), promotes de-energization of synaptic mitochondria (28), and induces \( Ca^{2+} \) release from the mitochondrial matrix (29). Furthermore, \( NO \) and reactive oxygen species participate in significant neuronal events, such as dendritic growth and arborization (30) and apoptosis (31).

Most of mitochondrial \( O_2 \) and \( H_2O_2 \) production is a consequence of reactions of \( NO \) with components of the respiratory chain (10, 12). Accordingly, \( l^-arginine is known to stimulate \( H_2O_2 \) release through the activation of mtNOS (13), an observation extended to brain mitochondria (Fig. 7) and dependent on the mtNOS expression level. This study established a link between \( NO \) metabolism and the generation of reactive oxygen species by mitochondria during development: mitochondria with different mtNOS content, such as those from newborn P2–4 and adults, yielded similar \( H_2O_2 \) production rates in the presence of \( NO \) (Fig. 6). Nevertheless, only neonatal organelles containing significant amounts of mtNOS responded to \( l^-arginine stimulation (Fig. 7), thus leading to a temporal increase in \( H_2O_2 \) production. Thus, the similar temporal pattern of brain mtNOS (Fig. 5) and Mn-superoxide dismutase (Fig. 8A) gains further significance: an increased dismutation rate of \( O_2^- \) to \( H_2O_2 \) by mitochondrial matrix Mn-superoxide dismutase (Reaction 3) is expected to outcompete the formation of \( ONOO^- \) (Reaction 4), prolong \( NO \) half-life, and consequently, increase cytosolic \( H_2O_2 \) steady-state levels (32, 33). A similar correlation between \( Cu/Zn \) superoxide dismutase and cytosolic \( NO \) is consistent with previous reports on protection against \( NO \) toxicity in neurons by superoxide dismutase (34).

Considering that at maximal mtNOS expression, the formation of \( ONOO^- \) was not detected, this study supports the following notions: (a) mitochondrial production of \( NO \) is an important factor in the developmental setup of brain \( H_2O_2 \) steady-state concentration in parallel with Mn-superoxide dismutase activity and (b) as a corollary, the accurate temporal up-regulation of mtNOS may represent a redox signaling mechanism rather than a stressful event. This view is supported by recent observations that propose that \( NO \) and \( H_2O_2 \) participate in the development and maturation of the nervous system, particularly through potentiation of \( Ca^{2+} \) signaling by redox changes (31). Moreover, \( NO \) and \( H_2O_2 \) may promote cell cycle arrest and quiescence in different tissues by mechanisms such as activation of p38 MAPK (35), inhibition of activity or expression of cyclin D1,
increase of cyclin D1 ubiquitination (36), activation of phosphatases, dephosphorylation of Rb protein, and activation of p21 and p53 proteins (37). Some of these pathways had been confirmed in nervous tissue (14, 38).

From this perspective, the sequential activation of mitochondrial and cytosolic isoforms of nNOS and superoxide dismutase in the brain might play a role in synaptogenesis and synaptic remodeling that follows proliferation arrest (39). The temporal correlation of mtNOS up-regulation with neuronal network remodeling is consistent with this perspective. In support of this notion, the persistence of mtNOS activity until postnatal day 15 in the cerebellum correlates with the longer period of proliferation and plasticity of this brain region. In this period, mtNOS was significantly expressed and 8-fold more active in immature cerebellum at P5 than in the quiescent adult organ at P90; this effect resulted in increased cerebellar mitochondrial H$_2$O$_2$ production rate and estimated steady-state concentrations (Fig. 9). Taken together, these effects suggest that maximal neuronal proliferation depends on a narrow range of H$_2$O$_2$ concentrations, which is likely to be dependent on mtNOS activity.

The modulation of neuronal-like mtNOS variants is consistent with the diversity of nNOS gene expression (40) adapted to perform tissue-specific functions. For example, brain mtNOS regulates mitochondrial free radical production; cardiac mtNOS modulates mitochondrial parameters in a beat-to-beat fashion, and its increased expression in muscular dystrophy in mouse models contributes to generate cardiac damage (7); and rat skeletal muscle neuronal mtNOS activity varies with the thyroid status, which has a central role in development (6). Likewise, the fine modulation of brain 144-kDa mtNOS and mitochondrial reactive oxygen species during the perinatal period suggests an essential role of NO in the chronological phases of brain maturation and synaptic plasticity.

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