Nitric oxide synthase inhibition during synaptic maturation decreases synapsin I immunoreactivity in rat brain

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Abstract

During the development of the brain, nitric oxide and synapsins, the latter being phosphoproteins associated to presynaptic membrane vesicles, are abundant in presynaptic terminals and play important and similar roles in neurotransmitter release, morphogenesis, synaptogenesis, and synaptic plasticity. These mechanisms are fundamental for neuronal development and plasticity and constitute important factors for the formation of neuroanatomical structures. Neural nitric oxide synthase (nNOS), synapsin I, and nNOS adapter protein (CAPON) constitute a ternary complex necessary for the formation of synaptic connections. NO absence may affect the presence and/or activity of synapsins during brain development. To understand the role of NO in synaptogenesis, we studied the effects of NO inhibition on synapsin I expression at a postnatal stage. Rat pups were treated with a competitive NOS antagonist, N-nitro-L-arginine methyl ester, from postnatal days 3 to 23. Control pups received an equivalent volume of saline solution. Histochemical and immunohistochemical techniques for NADPH-d and synapsin I, respectively, were carried out. NOS inhibition elicited a significant reduction in synapsin I immunoreactive density and NADPH-d activity in the brain in the analyzed areas—prefrontal cortex, hippocampus, and dorsal thalamus. These data show that the alterations originated by NO and synapsin deficiencies produce a diminution in synaptic density. Thus, functions that depend on the formation of synaptic connections such as learning and memory could be affected by NO deficiency.
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Nitric oxide (NO, nitrogen monoxide), a biological messenger molecule with multiple functions in immune, cardiovascular, and nervous systems, is generated by the enzyme nitric oxide synthase (NOS) by means of three distinct isoenzymes: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) [1–6]. NO and synapsins, the latter being phosphoproteins associated with presynaptic membrane vesicles, are abundant in neuronal terminals and participate in similar functions in the nervous system such as the regulation of neurotransmitter release [7–12], synaptic plasticity [13], neurite outgrowth and extension [14–17], and synaptogenesis, neuronal proliferation, and maturation [18–29], and play a role in synaptic vesicle docking/fusion reactions [30,31]. The development of the mechanisms of neurotransmitter release is an important component for the formation of functional synaptic connections. These processes are fundamental for neuronal development and plasticity and are important factors in the formation of neuroanatomical structures [32–36]. Theoretically, the lack of NO would produce important changes in the development of the CNS. However, knockout nNOS mice do not show patent abnormalities in both neuroanatomical and sensorimotor function studies [37,38], suggesting that other NOS isoforms in the brain, like iNOS and eNOS, may generate NO and compensate for the absence of nNOS [37,39]. In vitro studies have shown that nNOS inhibition affects basic aspects of neuronal viability and function [40]. The inhibition of nNOS during critical periods of brain development originates alterations in the development of retinal pathways [41–45]. Kalb and Agostini [18] reported that this inhibition

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causes the absence of protein expression, such as Cat-305, on the neuronal surface present in mature motoneurons of the spinal cord. This inhibition also affects functions such as learning and memory [46]. The delivery of nNOS to discrete sites of neurons is mediated by adapter proteins [47]. For instance, nNOS is linked to postsynaptic densities by the cytoskeletal protein PSD95/93 [48]. This interaction places nNOS adjacent to other PSD95 ligands, such as the NR2 subunit of the N-methyl-D-aspartate receptor (NMDA) [49], which accounts for the efficient activation of nNOS by NMDA receptor stimulation [50]. Recently, Jaffrey et al. [51] reported that nNOS localization in presynaptic terminals depends on the formation of a ternary complex constituted by nNOS, synapsin I, and a carboxyl terminal PDZ ligand of nNOS protein (CAPON). CAPON contains a C-terminal PDZ domain-binding motif, which interacts with the N-terminal PDZ domain of nNOS. Evidence from binding studies indicates that CAPON is stochiometrically associated with soluble nNOS, indicating that CAPON may serve as a nNOS-targeting protein in neurons [51]. CAPON has an N-terminal phosphoryrosine-binding (PTB) domain whose ligands include Dexras1 (a novel member of the ras family) and the synapsin family of proteins [51,52]. The physiologic activity of Dexras1 is determined by nNOS and CAPON, as Dexras1 activation is selectively diminished in mice bearing a nNOS targeted deletion, whereas Dexras1 activation by nNOS is enhanced in the presence of CAPON [20]. The absence of synapsin I originates modifications in nNOS and CAPON cellular localizations thus affecting the cellular functions of this ternary complex. Additionally nNOS and synapsins are present in presynaptic sites and partially co-localized in cultured hippocampal neurons [53]. Moreover, nNOS+/− mice display defects in dendritic arborization [16], which are similar to the dendritic defects found in synapsin I knockout mice [21]. This evidence suggests a close functional relationship between NO and synapsins allowing the speculation that those deficits in dendritic morphology or neurotransmitter release in synapsin-deficient mice may reflect a decreased access of nNOS to synaptic sites. It is not known whether NO absence may affect the presence and/or activity of synapsins during brain development. To further understand the role of NO in synaptogenesis, we studied the effects of NOS inhibition, during early postnatal stages in rat pups, on synapsin immunoreactive density in cerebral structures related to cognitive functions such as prefrontal cortex, hippocampus, and dorsal thalamus.

**Experimental procedures**

All experiments were performed according to NIH guidelines for care and use of laboratory animals and approved by the Project Commission of the Instituto Nacional de Psiquiatría Ramón de la Fuente.

Wistar rat pups (both sexes) were housed together with their mothers and maintained under standard laboratory conditions. Pups were treated (n = 7) from postnatal days 3 to 23 (day of birth being considered as postnatal day 1, pn1) with a daily subcutaneous injection of 60 mg/kg N-nitro-L-arginine methyl ester (L-NAME, Sigma) in isotonic saline (10–50 µl). Control pups (n = 7) received an equivalent volume of saline. The pups were weighted and observed daily prior to treatment administration.

We carried out an NADPH-d histochemistry and a synapsin I a/b immunohistochemistry in L-NAME- and saline-treated 24-day-old rats. Previously animals were anesthetized with sodium pentobarbital 40 mg/kg i.p. and perfusion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). Brains were removed, post-fixed 12–16 h in the same fixative, and washed in 30% sucrose buffer until used. Serial 50 µm coronal sections of the brain were obtained in a freezing microtome. Alternate slices, corresponding to bregma −4.16 mm [54], were chosen for the procedures (Fig. 2A). Control and treated sections were processed simultaneously for each technique.

**NADPH-d histochemistry**

Floating sections from both control and treated animals were permeabilized in 0.1% Triton X-100 in PB for 5 min, NADPH-d activity was revealed by incubation in PB containing 0.1 mg/ml nitro blue tetrazolium and 1 mg β-NADPH for 45 min at 37 °C, subsequently washed in PB, dried, cleared, and mounted as described in detail elsewhere [24,55].

**Synapsin I immunohistochemistry**

The avidin–peroxidase method (kit PK-6105, Vector, USA) was performed in floating sections incubated in 0.03% hydrogen peroxide (H₂O₂, 30 min), washed in 0.1 M PB saline (PBS), and incubated (1 h) in PBS with 0.3% Triton X-100 (PBS-T), 1% bovine albumin (BA), and 1% normal rabbit serum (RS). They were then incubated with a primary polyclonal antibody against synapsin I a/b protein (Santa Cruz, USA), diluted 1:600 in PBS-T, 1% RS for 3 days at 4 °C, followed by a rinse in PBS and incubation in biotinylated rabbit anti-goat IgG (Vector, diluted 1:200) for 2 h. After further washing in PBS, the sections were incubated in avidin–biotin–peroxidase complex for 1 h. The peroxidase activity was revealed by reacting the sections with a mixture of 10 µg 3,3-diaminobenzidine in 26 ml Tris–HCl buffered saline (5 µM, pH 7.6), 12 µl H₂O₂ 30%, and 300 µl nickel sulfate
30%. Subsequently, the sections were washed in PBS, dried, cleared, and mounted.

Quantitative tissue analysis for NADPH-d histochemistry and immunohistochemistry

The optical density of both immunoreactivity and histochemistry staining in prefrontal cortex, hippocampal subregions, and dorsal thalamus sections was analyzed by a computer-based image-analysis system as an average of gray values. Sections were analyzed under a Leitz Laborlux 12 microscope and photographed using a SPOT 2 camera (Diagnostic Instruments), captured and digitized in a PC computer. Measurements were taken from coronal sections of the right and left hemispheres of selected regions (described in Fig. 2A), in two alternate sections (one for NADPH-d and one for synapsin I) for each animal (n = 14). Optical density of positive areas was quantified using a Scion Image 4.0.2 analysis program and standardized between white (0) and black (255). For each cerebral region, the mean of the optical densities in the control group was taken as 100%. In the case of NADPH-d histochemistry, we also measured the optical density in randomly chosen soma neurons in the same regions to quantify changes in staining intensity. The optical density of the same number of control and treated neurons was measured with the above-mentioned software. The total number of cells processed in the different regions was: 121 in prefrontal cortex, 174 in hippocampus, and 246 in ventral lateral geniculate nuclei of dorsal thalamus.

Statistical analysis

Weight differences between control and treated animals were assessed by means of a two-way ANOVA for repeated measures followed by the Tukey test.

Data regarding quantification of the optic density from the histochemical and immunohistochemical experiments are presented as means ± SEM. Statistical comparisons of these data between L-NAME-treated and control groups were performed by means of the paired Student’s t test, the optic density of neurons was evaluated by means of the Mann–Whitney U test. Statistical significance was considered for p < 0.05.

Results

Pups treated postnatally with L-NAME on days 3–23 presented a moderate weight diminution with age as compared to control animals, [two-way ANOVA for treatment $F_{(1,20)} = 28.9$, $p < 0.001$, for postnatal age $F_{(4,80)} = 449.9$, $P < 0.001$, and for the interaction between both factors $F_{(8,80)} = 8.66$, $P < 0.001$]. The diminution became statistically significant from P12 onwards, being the decrease in body weight of a 15% in average at the end of the L-NAME treatment (P23) (Fig. 1). No other evident macroanatomic or motor activity alterations were noticed.

NADPH-d histochemistry

Inhibition of nNOS activity in brain sections of the treated group resulted in a reduction in the number of NADPH-d positive neurons and as well as in a diminution in the labeling intensity when compared to control group (100%). Prefrontal cortex presented a staining intensity 42.83 ± 5.03% (i.e., a decrease of 57.17% of the control value) (Figs. 2B and 3A and B) and a soma optical density ($n = 121$) equal to 56.17 ± 1.96% (Fig. 2C). The hippocampus exhibited also a low staining intensity of 43.39 ± 2.74% (Figs. 2B and 3C and D). The optical density of neuronal bodies in the hippocampus ($n = 174$) was only measured in 4 of the 7 treated pups, because labeled neurons were completely absent in this region in the 3 remaining animals. The mean optical density for these animals was 43.89 ± 1.32% (Figs. 2C and 3D). The dorsal thalamus presented an optical density of 46.28 ± 4.65% (Figs. 2B and 3E and F) and the mean value obtained for the somas in the ventral lateral geniculate nuclei ($n = 246$) was 42.20 ± 1.32% (Figs. 2C and 3G and H).

Synapsin immunohistochemistry

A significant reduction in synapsin I immunoreactivity was found in the three selected brain areas of the
treated group (Table 1). Prefrontal cortex showed an optical density 65.15 ± 2.57% of the control value (100%) (Figs. 2D and 4A and B). The hippocampus was quantified in subregions (Fig. 2A), and all the values in parentheses are given with respect to control values as follows: polymorphic zone of the dentate gyrus (65.52 ± 2.57%), hilus (64.87 ± 7.32%); and stratum lucidum in areas CA3a (61.28 ± 5.98%) and CA3b (58.89 ± 6.94%); stratum oriens in areas CA3a (66.59 ± 4.61%) and CA3b (67.18 ± 6.88%). Other hippocampal regions where synapsin density is normally low also exhibited a significant reduction in immunoreactivity when compared to control values as follows: stratum oriens in area CA2 (71.53 ± 10.31%), granular cell layer area of dentate gyrus (69.61 ± 7.86%), stratum lacunosum-moleculare (62.67 ± 4.86%), and inner layer of dentate gyrus (61.08 ± 5.05%) (Figs. 2E and 4C, D, G, and H). Finally, synapsin immunoreactivity in the dorsal thalamus was 66.61 ± 4.76% of control value (Figs. 2F and 4E and F).

**Discussion**

Several groups have used the labeling of synapsins as an index of synaptic number and density that may be related to the gain or loss of functions [25,56,57]. The strength of this assumption is based on the following evidence. Synapsins represent one of the most abundant families of synaptic proteins in the brain [58–60]. They are expressed only in neurons and are specifically localized in the presynaptic compartment of the synapsis [61–65]. During the development of the nervous system, the appearance of the synapsin I protein in the neuropil correlates temporarily and topographically with synaptogenesis [56,62,66–69]. Studies of synapsin expression have revealed that an increase in synapsin I and synapsin II levels parallels the establishment of synaptic contacts in different brain areas both in situ and in cultured neurons [56,66,70]. Injections of synapsin I or synapsin II into spinal neurons of Xenopus embryos result in the acceleration of both morphological and functional...
development of neuromuscular synapses [71–73]. In synapsin I knockout mice, there is a decrease in the size of synaptic terminals and in the number of synaptic vesicles per terminal [21]. Thus, Melloni et al. [56] suggested that examination of synapsin I immunoreactivity in the brain may be used as a sensitive indicator of the establishment/maintenance and/or the loss/recovery of functional synapses in the CNS. Based on the aforementioned studies, we utilized synapsins as markers of synaptic density. Similar to synapsins, NO participation in synaptogenesis [20,21,27,29], regulation of neurotransmitter release [7–11,31], and synaptic plasticity [13] has been demonstrated. This functional similarity suggests a close relationship between NO and synapsins. Specificity of NO reactions with its neuronal target is partially determined by nNOS availability and precise cellular localization [47–51]. The discovery of the CAPON protein by Jeffrey et al. [51], which binds nNOS and synapsins at the presynaptic site to form a triad, provides evidence for the functional relationship between these proteins. The lack of synapsin I originates modifications in nNOS and CAPON cellular localization, thus affecting the cellular functions of this ternary complex. We were interested in elucidating whether NOS

Fig. 3. NADPH-d histochemistry. Photomicrographs of brain coronal sections (50 μm) of 24-day-old rat pups treated with L-NAME from postnatal days 3–23 and controls (saline solution). Sections in A, C, E, and G correspond to the control group and those in B, D, F, and H to the treated group. Prefrontal cortex (A,B); hippocampus (hilus) (C,D); dorsal thalamus (E,F); and magnification of geniculate nucleus area (G,H). Arrows (†) show stained neurons and fibers. Sections in the treated group (right) exhibit a decrease in staining intensity when compared with control sections (left). Notice the complete absence of stained neurons in hippocampus (D) and the reduction in staining intensity of positive somas in the geniculate nucleus (H). Scale bars for A–F = 350 μm; for G–H = 50 μm.
inhibition exerted an effect on synapsin expression at early postnatal stages, since during this period synaptogenesis and brain maturation take place in areas where synapsin [56,66,69,70] and nNOS [22,29] expressions are high. Our results show that NOS inhibition at a postnatal day 1 (P1), originated a high mortality during the first days after birth (P1–P3). We initiated the L-NAME treatment (60 mg/kg/day, sub-dermal and post-natally applied) complete inhibition of NOS catalytic activity [38]. It has been reported that L-NAME inhibition affects NOS isoforms differentially, being the endothelial and inducible forms in a lesser degree than the neuronal NOS, which is largely involved in NADPH-d positive staining, which entails nNOS enzymatic activity [38].

Our results suggest that NOS inhibition may have an effect on synaptic formation, since there is a significant decrease in its density. This decrease might reflect both the effects of the local disruption of NOS activity as well as possible effects secondary to functional alterations due to the inhibition of the different NOS isoforms at a peripheral level.

On the one hand, NO at the synaptic level behaves as a retrograde neurotransmitter to maintain the activity in the presynaptic neuron, although its synthesis occurs in the postsynaptic neuron. On the other hand, NO in the presynaptic neuron facilitates neurotransmitter release [7–11,31]. When the presynaptic neuron is inactive, synapsins are bound to the cytoskeleton by actin (reserve pool), but when the neuron is activated, synapsins are phosphorylated and become part of the release vesicles located in the vicinity of the presynaptic membrane [31,65]. To form the CAPON/synapsin/NO triad, synapsins must be phosphorylated. This triad keeps nNOS available at the presynapse [51]. NO absence not only affects synapsins at the synaptic vesicles, but also modifies the activation at the core complex of other synaptic proteins (n-secl, syntaxin 1a, VAMP 2, and SNAP-25) involved in the docking and fusion of synaptic vesicles [31] and inhibits the calcium-independent neurotransmitter release [10]. This suggests that physiological alterations due to NO absence, which are similar to the ones originated by synapsin deficiency, stem from an unavailability of the enzyme at the synaptic terminal, affecting neurotransmitter release and synaptic plasticity.

NOS chronic inhibition originated a marked decrease in NADPH-d positive staining, which entails nNOS enzymatic activity [38]. It has been reported that L-NAME inhibition affects NOS isoforms differentially, being the endothelial and inducible forms influenced to a lesser degree than the neuronal NOS, which is largely affected [75]. Virgili et al. [40] reported that with this L-NAME treatment (60 mg/kg/day, sub-dermal and post-natally applied) complete inhibition of NOS catalytic activity was attained during its administration, but four days after the end of treatment, the enzyme activity was restored. With this same treatment, we did not find any evident neuromorphological impairment, macroscopic malformation or mortality. Prickaerts et al. [76] reported that a sub-dermal 25 mg/kg/day L-NAME dose, started on postnatal day 1 (P1), originated a high mortality during the first days after birth (P1–P3). We initiated the L-NAME treatment (60 mg/kg/day) on day P3 and, even though the dosage was higher, 100% of our animals survived. It is important to mention that although we did not conduct specific experiments to test motor behavior,
no evident abnormalities were noticed in the treated pups as compared to controls during the daily observations. In line with this perception, there are other works using the same l-NAME inhibition protocol that report no alteration in behavior and motor activity in the treated rats [40]. However, other authors like Pickaerts et al. [76] report on an increase in the motor activity of rat pups tested in the open field as a result of l-NAME treatment.

Our results provide morphological evidence showing that the lack of NO induces a reduction in synaptic density and a synapsin deficiency. Thus, it can be hypothesized that synapsin depletion resulting from NO absence may alter those brain functions that depend on the formation of synaptic connections, such as learning and memory.

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