

*Short Communication*

# Alterations in Dendritic Morphology of Hippocampal Neurons in Adult Rats After Neonatal Administration of *N*-Omega-Nitro-L-Arginine

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**KEY WORDS** nitric oxide; Golgi-Cox stain; schizophrenia; pyramidal neurons; hippocampus; prefrontal cortex; neurodevelopment

**ABSTRACT** The dendritic length and dendritic-spine density of the pyramidal neurons of the prefrontal cortex and the CA1 hippocampus of rats using the nonselective nitric oxide synthase inhibitor *N*-omega-nitro-L-arginine (L-NNA) at different postnatal day (P) periods of the brain development (P1–P3, P4–P6, and P7–P9) were assessed using Golgi–Cox staining after puberty (P60). At P4–P6, the L-NNA treatment produced a significant decrease of the dendritic length and dendritic-spine density of the pyramidal cells of the CA1 hippocampus. In addition, the dendritic length of the pyramidal neurons of the CA1 hippocampus decreased because of the L-NNA treatment at P1–P3. These data suggest that during a specific step in the development of the brain, the nitric oxide levels may play a critical role in the morphological modifications of the pyramidal neurons of the CA1 hippocampus at postpubertal age. **Synapse 61:785–789, 2007.** © 2007 Wiley-Liss, Inc.

Adult rats with low levels of nitric oxide (NO) activity during the neonatal period between P4 and P6 show an increase in locomotor activity in a mildly stressful, novel environment (Mejorada et al., 2006). In contrast, blocking NO synthesis by a nonselective NO-synthase inhibitor, the *N*-omega-nitro-L-arginine (L-NNA) at other neonatal periods, P1–P3, P4–P6, or P7–P9, did not induce changes in the spontaneous locomotor activity after puberty (Black et al., 1999; Mejorada et al., 2006). Data from the neonatal ventral hippocampus lesion suggest that the CA1 region of the hippocampus may have participated in the control of locomotion caused by a novel environment after puberty (Flores et al., 1996, 2005; Lipska et al., 1993) predominantly by its projections to the prefrontal cortex (PFC) and nucleus accumbens (Kelley and Domesick, 1982; Swanson, 1981; Van Groen and Wyss, 1990). We have shown that neonatal ventral hippocampus lesions also produce morphological alterations in the pyramidal cells of the PFC and in the medium spiny neurons of the nucleus accumbens (Flores et al., 2005). Furthermore, several reports suggest

that NO may play a critical role in synaptogenesis and synaptic plasticity with an important role in the development, maintenance, and functional modifications of brain circuits (for reviews see Holscher, 1997). It thus seemed plausible that the emergence after puberty of locomotor hyperresponsiveness to a mildly stressful environment in rats with low levels of NO activity, during the neonatal period between P4 and P6, occurs as a result of dendritic plasticity at the level of the pyramidal neurons of the PFC and/or hippocampus after puberty. Accordingly, in our study we investigated whether the blocking of NO synthesis at different periods during brain development (P1–P3, P4–P6, and P7–P9) affects the dendritic length and

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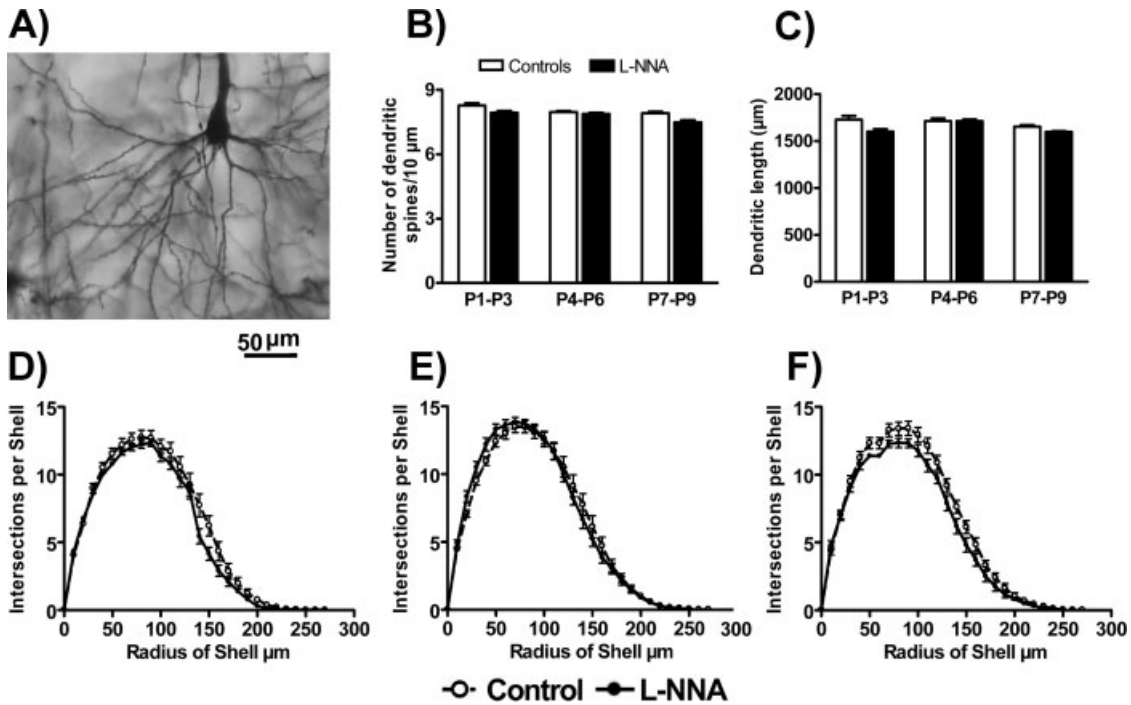


Fig. 1. Analysis of the pyramidal neurons of the Prefrontal cortex in adult animals with neonatal vehicle or L-NNA administration ( $n = 8-9$  animals per group). (A) Photomicrograph showing a representative Golgi-Cox-impregnated pyramidal neuron of the prefrontal cortex from control animals. (B) Spine density. (C) Total dendritic

length. (D) Sholl analysis of intersections per shell of the P1-P3 group. (E) Sholl analysis of intersections per shell of the P4-P6 group. (F) Sholl analysis of intersections per shell of the P7-P9 group.

spine density of pyramidal neurons of Layer 5 of the PFC and the CA1 of the hippocampus at postpubertal age. The results suggest that the presence of NO in a specific period of the development of the nervous system may be critical for these pyramidal cells.

Pregnant Sprague-Dawley rats were obtained at gestational days 14-17 from our animal facility (University of Puebla). Animals were individually housed in a temperature- and humidity-controlled environment in a 12-12 h light-dark cycle with free access to food and water. The day of birth was taken as postnatal day 0 (P0) and litters with a maximum of 10 neonatal rats were formed. The day after birth, male pups were assigned to either a control or treatment group. The treatment group of animals received subcutaneous (s.c.) injections of 10 mg/kg L-NNA (ICN Biomedicals, Aurora, OH) once daily at 0900 h, and the control animals were injected with an equal volume of PBS-vehicle (0.9% NaCl). Six groups of animals were formed. Rats received L-NNA from day P1-P3, P4-P6, and P7-P9 with their corresponding control groups. On P21, animals were weaned and housed in pairs of L-NNA treatment and control rats. All experimental procedures were approved by our local ethics committee at the Universidad Autónoma de Puebla and met governmental guidelines (Mexican Council for Animal Care, Norma Oficial Mexicana NOM-062-ZOO-1999).

At postpubertal (P60) age, the rats were deeply anesthetized with sodium pentobarbital (60 mg/kg, ip) and perfused intracardially with 0.9% saline. The brains were removed and processed with modified Golgi-Cox staining using procedures described by Gibb and Kolb (1998). The brains were first stored in the dark for 14 days in Golgi-Cox solution, then three days in 30% sucrose. Sections of 200-μm thickness in the coronal plane at the level of the PFC and hippocampus were obtained using a vibratome (Camden Instrument, MA752, Leicester, England). Sections were collected on clean, gelatin-coated microscope slides (four sections/slide) and stained with ammonium hydroxide for 30 min. Next, the sections were immersed in Kodak Film Fixer for another 30 min, and then washed with water, dehydrated, cleared, and mounted using a resinous medium.

The Golgi-impregnated pyramidal neurons of Layer 5 of the PFC and of the CA1 of the hippocampus (Cg1 and prelimbic cortex, plate 7-9 and CA1 of the hippocampus, plate 37-42 of Paxinos and Watson, 1986) were readily identified by their characteristic triangular soma-shape, apical dendrites extending towards the pial surface, and numerous dendritic spines. The criteria used to select neurons for reconstruction was essentially as described previously (Flores et al., 2005; Martinez-Tellez et al., 2005; Silva-Gomez et al., 2003). Five neurons in each hemisphere (10 neurons

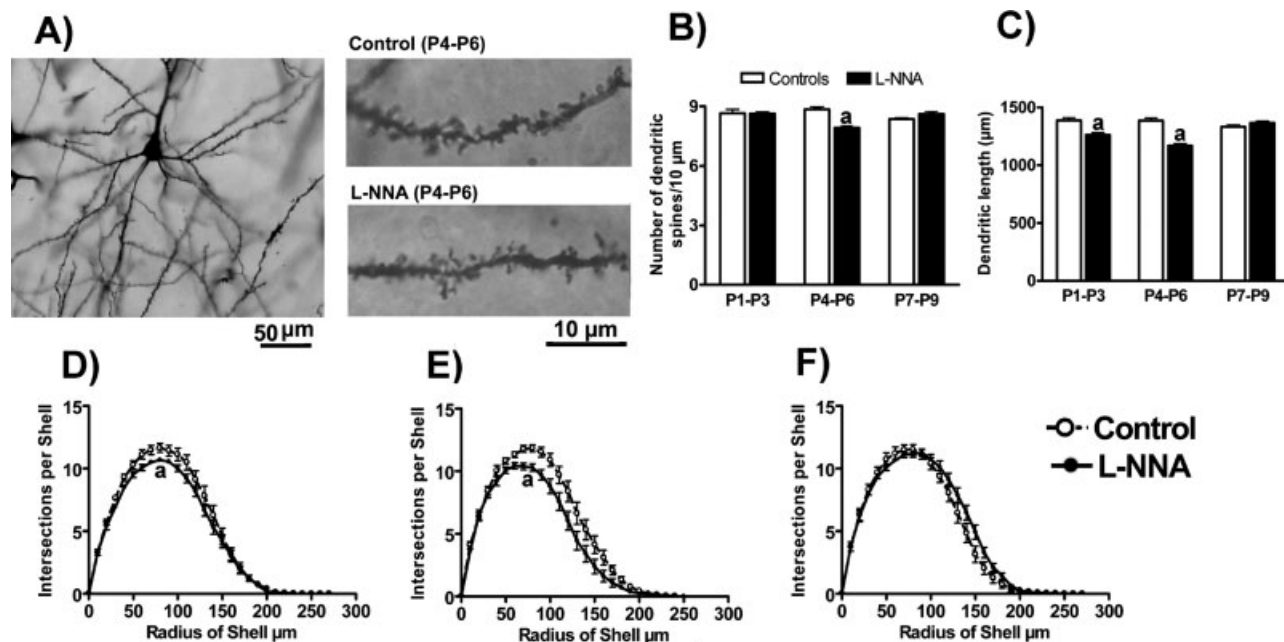


Fig. 2. Analysis of the pyramidal neurons of the hippocampus in adult animals with neonatal vehicle or L-NNA administration ( $n = 8-9$  animals per group). (A) Photomicrograph showing a representative Golgi-Cox-impregnated pyramidal neuron of the hippocampus from control animals and dendritic spine density of the neonatal vehicle or L-NNA administration at P4-P6. (B) Spine density. Rats with neonatal L-NNA administration at P4-P6 exhibit a decrease in the dendritic spines. (C) Total dendritic length. Rats

with neonatal L-NNA administration at P1-P3 and P4 and P6 exhibit a decrease in the total dendritic length. (D) Sholl analysis of intersections per shell of the P1-P3 group. The number of intersections per concentric ring are decreased in the animals with neonatal L-NNA administration. (E) Sholl analysis of intersections per shell of the P4-P6 group. The neonatal L-NNA use caused a decrease in the number of intersections per concentric ring. (F) Sholl analysis of intersections per shell of the P7-P9 group. <sup>a</sup> $P < 0.01$ .

per animal) were drawn using a camera lucida at a magnification of  $250\times$  (DMLS, Leica Microscope) by a person blind to the treatment conditions. Though the Golgi-Cox procedure has been extensively used to determine dendritic morphology, a caveat of the method is that only a small number of neurons are impregnated with the stain and that some spines hidden behind the dendritic shafts may not be counted. Nevertheless, given the random nature of these events, we consider that the neurons studied here are representative of the total population and that spine-density measurements are relative. For each neuron, the three-dimensional basal-dendritic tree, including all branches, was reconstructed in a two-dimensional plane and the dendritic tracing was quantified by Sholl analysis (Sholl, 1953). A transparent grid with concentric rings, equivalent to  $10\text{-}\mu\text{m}$  spacing, was placed over the dendritic drawing and the number of branches intersecting each ring was used to estimate the total dendritic length (TDL) (Flores et al., 2005). The density of dendritic spines was estimated by drawing at least  $10\text{-}\mu\text{m}$  long segments from the terminal tips at high power ( $1000\times$ ) and counting the number of spines.

Data from the total dendritic length and the spine densities were analyzed using a two-way ANOVA, followed by Newman-Keuls tests for posthoc comparisons, with L-NNA treatment and age of treatment as

independent factors. The analysis of intersections per radius of shell was analyzed by a Wilcoxon matched-pair test.  $P < 0.05$  was considered significant.

The effects of L-NNA, a nonselective NOS blocker of the accumulation of NO in the PFC and hippocampus of the adult animals at three different ages, P1-P3, P4-P6, and P7-P9, are illustrated in Figures 1 and 2. The dendritic branching and density of dendritic spines on neurons of the PFC were measured by Golgi-Cox stain between neonatal L-NNA administration and control rats. The spine density and total dendritic length obtained were similar to our previous reports (Flores et al., 2005; Martinez-Tellez et al., 2005; Silva-Gomez et al., 2003). The Golgi-Cox impregnation procedure clearly filled the dendritic shafts and spines of layer-5 pyramidal neurons of the PFC (Fig. 1A). A two-way ANOVA for the analysis of the density of the dendritic spines revealed that L-NNA was significant ( $F_{1,48} = 19.9$ ,  $P < 0.001$ ), as was application age ( $F_{2,48} = 13.7$ ,  $P < 0.001$ ), and without interaction of L-NNA  $\times$  application age ( $F_{2,48} = 2.4$ ,  $P = 0.09$ ) (Fig. 1B). As measured by Sholl analysis, a two-way ANOVA for the total dendritic length showed there was a significant effect of the neonatal administration of the L-NNA ( $F_{1,48} = 8.46$ ,  $P < 0.01$ ), as was application age ( $F_{2,48} = 6.06$ ,  $P < 0.01$ ), and without interaction of neonatal L-NNA  $\times$  application age ( $F_{2,48} = 3.14$ ,  $P = 0.05$ ) (Fig. 1C). The analysis of intersection per radius of shell also showed

that the neonatal L-NNA did not produce changes in the dendritic arborization when compared with the corresponding control group (Figs. 1D–1F).

The dendritic shafts and spines of the CA1 pyramidal-neurons of the hippocampus after the Golgi-Cox-stain impregnation procedure are illustrated in Figure 2A. A two-way ANOVA for the analysis of the density of the dendritic spines revealed that L-NNA was significant ( $F_{1,46} = 9.43$ ,  $P < 0.01$ ), as was application age ( $F_{2,46} = 3.95$ ,  $P = 0.02$ ), and with interaction of L-NNA  $\times$  application age ( $F_{2,46} = 21.8$ ,  $P < 0.0001$ ) (Fig. 2B). A posthoc test revealed that the dendritic spine density was significantly lower in the groups of rats with neonatal L-NNA at P4–P6 compared with their corresponding control group ( $P < 0.001$ ) (Fig. 2B). A two-way ANOVA for the total dendritic length showed there was a significant effect of the neonatal administration of the L-NNA ( $F_{1,48} = 40.5$ ,  $P < 0.001$ ), as was application age ( $F_{2,48} = 7.08$ ,  $P < 0.01$ ), and with the interaction of neonatal L-NNA  $\times$  application age ( $F_{2,48} = 20.84$ ,  $P < 0.001$ ). The posthoc test revealed that total dendritic length was significantly lower in the pyramidal neurons from animals with neonatal L-NNA at P1–P3 and P4–P6 ( $P < 0.001$ ) compared with their corresponding control groups (Fig. 2C). Finally, the analysis of intersection per radius of shell also showed that the neonatal L-NNA at P1–P3 and P4–P6 rats showed significantly less dendritic arborization ( $P < 0.01$ ) when compared with the control animals (Figs. 2D and 2E).

The major finding of this study is that blocking of NO synthesis by the nonselective NO-synthase inhibitor L-NNA at different neonatal days did not produce the same changes in the morphology of the pyramidal neurons in the studied regions. The pyramidal neurons of the hippocampus from the neonatal L-NNA administration at P4–P6 show morphological changes in the form of reduced length and branching and decreased density of the dendritic spines after puberty. The administration of the L-NNA at P1–P3 only causes a decrease in the dendritic length and arborization of the hippocampus pyramidal neurons. Interestingly, we did not find differences in the adult animals that received the L-NNA at P7–P9. At the level of the PFC, the administration of the L-NNA did not cause differences in any of the analyzed groups after puberty. These changes may be associated with increased locomotor activity in a mildly stressful novel environment, reported in the adult animals with neonatal L-NNA administration at P4–P6 (Mejorada et al., 2006). Moreover, these data may be related to the NO levels recently reported by our group (Mejorada et al., 2006). Rats with neonatal L-NNA administration at P4–P6 showed a clear decrease in the NO activity in the brain, whereas the animals with neonatal L-NNA administration at P1–P3 only exhibited a trend toward a decreasing NO

activity in the same brain regions (Mejorada et al., 2006). In addition, recent report (Chung et al., 2004) suggests that the pyramidal cells of the hippocampus shown a transient expression of nNOS at P3–P7. Our present data are in agreement with our hypothesis that a decrease of the NO between P4 and P6 may modify structures related to the mesolimbic-dopaminergic system such as the hippocampus (Mejorada et al., 2006). Additionally, several reports suggest that neonatal ventral hippocampus lesions lead to increase postpubertal locomotor responses to a novel environment, amphetamine, and apomorphine (Flores et al., 1996, 2005; Lipska et al., 1993) with increased excitability of the nucleus accumbens neurons after VTA stimulation (Goto and O'Donnell, 2002). Furthermore, Black et al. (1999) using the same NO-synthase inhibitor L-NNA showed that adult animals with neonatal administration of L-NNA at P3–P5 exhibit changes in the amphetamine-induced locomotion.

Regardless of the mechanisms responsible for the dendritic changes, our data are consistent with the idea that NO regulates the dendritic spines and branching in the pyramidal neurons of the hippocampus (Audesirk et al., 2003; Nikonenko et al., 2003; Seress et al., 2002). The stimulatory effect of NO under dendritic arborization of the hippocampus neurons (Audesirk et al., 2003) is absent in the rats with neonatal L-NNA administration at P4–P6 and results in a decrease in the variables analyzed in our work. Interestingly, rats with neonatal L-NNA administration at P7–P9 also showed a decrease in NO levels (Mejorada et al., 2006) though these animals did not exhibit changes in the dendritic arborization and dendritic spines. As we concluded in our previous report (Mejorada et al., 2006), during a specific step in the development of the brain (P4–P6 of the rat) the NO levels may play a critical role in the maturation of some structures that control the dopamine-related behaviors after puberty. Alternatively, the decrease in the length and spine density of the dendrites of the pyramidal neurons of the hippocampus reported here may also be in part produced by stress reported in this animal model (Mejorada et al., 2006).

Interestingly, several reports have shown reduced dendritic arborization and spine number and size in postmortem brains of schizophrenics (Broadbelt et al., 2002; Glantz and Lewis, 2000; Rosoklija et al., 2000). In addition, several reports have demonstrated alterations in the NO activity and its metabolites in schizophrenia (Brzustowicz et al., 2004; Suzuki et al., 2003; Xu et al., 2005). For example, association between nNOS and proteins related to the *N*-methyl-*D*-aspartate receptor complex are altered in schizophrenia and lead to changes consistent with *N*-methyl-*D*-aspartate receptor hypofunctioning in the prefrontal-cortex hypothesis of this disease (Brzustowicz et al., 2004; Xu et al., 2005). Whether the similarities in neuronal

changes of the hippocampus suggest common substrates for the behavioral abnormalities in postpubertal rats with neonatal L-NNA administration at P4–P6 and human schizophrenia is a moot point. Nevertheless, our data add further evidence of the utility of neonatal L-NNA administration at P4–P6 animals as a model for studying neurodevelopmental aspects of schizophrenia.

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