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Short communication

Inhibition of nitric oxide synthase induces increased production of growth-associated protein 43 in the developing retina of the postnatal rat

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Abstract

We investigated the effects of N^G-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, on retinal development in the postnatal rat by immunocytochemistry and immunoblotting using antisera against neuronal nitric oxide synthase (nNOS) or growth-associated protein 43 (GAP-43). An nNOS-immunoreactive band of 155 kDa and a GAP-43-immunoreactive band of 48 kDa were present in the extracts of both control and L-NAME-treated rat retinas. The intensity of the nNOS-immunoreactive band was much weaker in the treated rats, whereas the intensity of the GAP-43-immunoreactive band of 48 kDa was much stronger in the treated rats. Much stronger GAP-43 immunoreactivity was visible in the inner plexiform layer (IPL) of the treated retinas at P10, P14 and P21. Our findings suggest that NO may play an important role in the maturation of the IPL in the developing rat retina.

Theme: Development and regeneration

Topic: Visual system

Keywords: Growth-associated protein 43; Western blot analysis; Immunocytochemistry; Postnatal development; Retina; Rat

The free radical nitric oxide (NO) has been implicated in many physiological functions, ranging from dilatation of blood vessels to neuronal development and synaptic activity in the central nervous system, including the retina [3,6,8]. The involvement of NO in brain development is now widely accepted [4,18,19]. In the mammalian retina, most retinal neurons containing nitric oxide synthase (NOS) are presumed to be amacrine cells, although there is considerable variation (for a review, see Ref. [10]). In the rat retina, putative NO synthesizing cells reach maximum differentiation during the second postnatal week, before the eyes open, as estimated using NADPH-diaphorase (NADPH-d) histochemistry [12] and immunocytochemistry with anti-NOS antisera [11,14]. This situation differs from other amacrine cells containing GABA or glycine, which reaches 80% of adult levels at P7 or P11, respectively [5]. In addition, it has been reported that maturation of the inner plexiform layer (IPL) in the rat retina is fully developed during the second postnatal week [9]. Thus, NO is thought to play an important role in the maturation of the IPL of the retina. To evaluate this hypothesis, we investigated the effects of N^G-nitro-L-arginine methyl ester (L-NAME), a non-selective isoform inhibitor of NOS, on the expression of growth-associated protein GAP-43, an intracellular protein that is highly expressed in developing and regenerating axon growth cones [1], in the developing rat retina.

Ten litters of Sprague–Dawley rat pups were used. The first day after birth was defined as postnatal day 0 (P0). The animals were maintained on a daily cycle of 12 h dim light and 12 h darkness. The pups were injected intraperitoneally each day from P0 to P21 with a total dose of 40 mg/kg per day of L-NAME, dissolved in saline. Control pups received an equivalent volume of saline only. Animals were killed by an overdose of chloral hydrate at P5,

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P7, P10, P14, and P21 days. The eyeballs were enucleated, and the retinas were carefully dissected. For Western blot analysis, retinal tissues taken from normal adult and experimental groups were quickly dissected on an ice-cold plate, frozen on dry ice, and stored at −70 °C. Western blot analysis was performed on the extracts of retinal tissue, which were homogenized in ice-cold extraction buffer (10 mM Tris buffer, pH 7.4; 2 mM EDTA, 1 mM DTT). Aliquots of tissue samples, corresponding to 50 μg of total protein, were run on 10% SDS–PAGE gels and blotted onto nitrocellulose membranes. Immunostaining of blotted proteins was carried out using primary antibodies against nNOS (Sigma, St. Louis, MO; dilution rate 1:7000) or goat polyclonal anti-GAP-43 antibody (Sigma; dilution rate 1:500). The blots were analyzed using enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA). nNOS or GAP-43 protein content was calculated by measuring the peak densitometry area using an Eagle Eye™II Still Video System (Stratagene, La Jolla, CA, USA). The optical densities (means±S.D.) were obtained after five determinations for each band. For GAP-43 immunocytochemistry, the posterior eyecup was immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 30 min. The retina was carefully dissected and further immersion fixed in the same fixative for 2 h at 4 °C. After several washes in PB, small pieces of the retinas were cut out from the region close to the optic nerve head. Vertical 50-μm-thick sections prepared from the retina were immunostained following immunocytochemical procedures. The sections were incubated in the following sequence: 10% normal donkey serum (NDS) in 0.01 M phosphate buffered saline (PBS, pH 7.4) containing 0.5% Triton X-100 for 1 h at room temperature to block nonspecific binding sites; a polyclonal goat anti-GAP-43 antibody (Sigma; diluted at 1:500) in PBS containing 3% NDS and 0.5% Triton X-100 for 10–12 h at 4 °C, biotinylated donkey anti-goat IgG (Vector, Burlingame, CA, USA) in PBS for 2 h at room temperature; ABC solution (Vector) in PBS for 2 h at room temperature, and finally 0.05% 3,3′-diaminobenzidine (DAB) in 0.05 M Tris–HCl buffer (TB, pH 7.4) containing 0.01% H_2O_2 for 10 min. The sections were then rinsed in two changes of PBS and cover-slipped.

The nNOS-immunoreactive band of 155 kDa was present in the extracts of both control and L-NAME-treated rat retinas, but its intensity was much weaker in the treated rats than in the controls during experimental periods (Fig. 1A). In the control retinas, the density of nNOS protein levels gradually increased up to P21. In the L-NAME-treated retinas, the density of nNOS protein levels also increased, but with a degree of increment lower than that

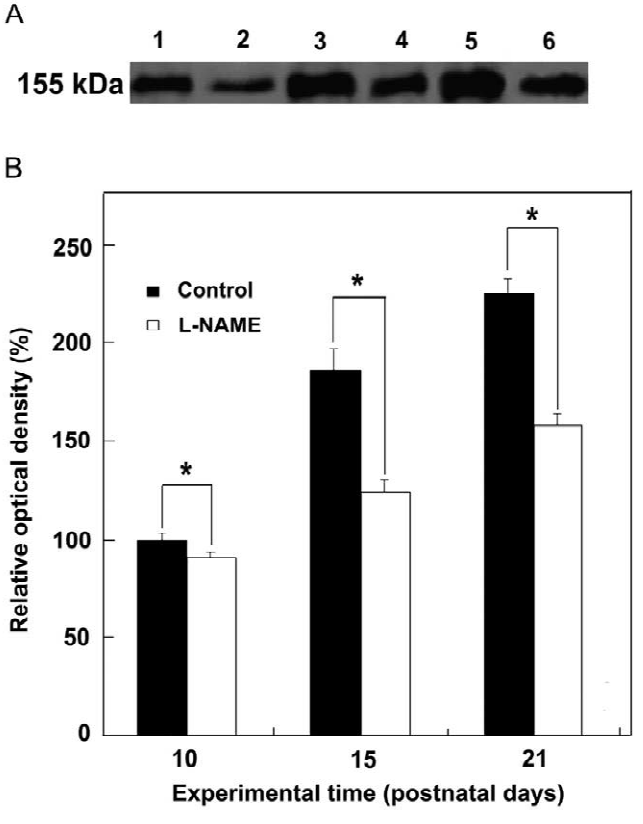

Fig. 1. Immunoblot analysis of nNOS-43 protein levels in the developing retina of control and L-NAME-treated rats. (A) Immunoblot stained for nNOS demonstrating a single band at 155 kDa: control (lane 1, P10; lane 3, P14; lane 5, P21) and L-NAME treatment (lane 2, P10; lane 4, P14; lane 6, P21). (B) Densitometric analysis of immunoblots as shown in A. Data represent means±S.D. *$P<0.05$ (by Student's *t*-test).

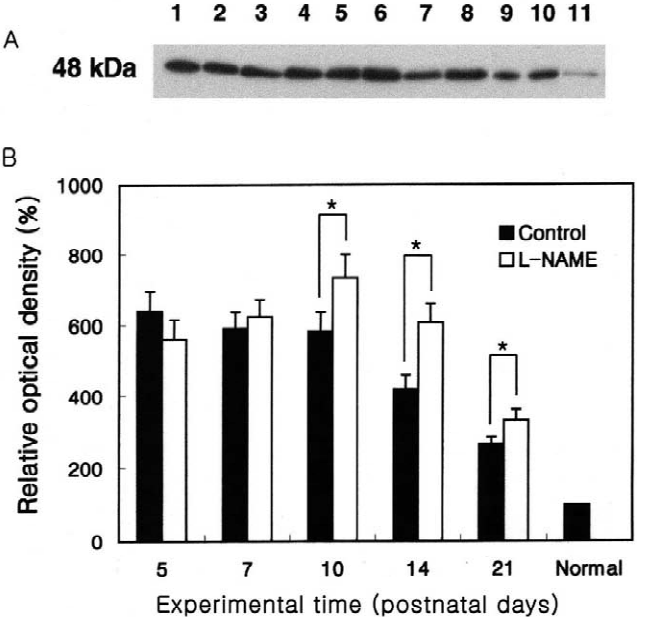

Fig. 2. Immunoblot analysis of GAP-43 protein levels in the developing retina of control and L-NAME-treated rats. (A) Immunoblot stained for GAP-43 demonstrating a single band at 24 kDa for all time points tested: control (lane 1, P5; lane 3, P7; lane 5, P10; lane 7, P14; lane 9, P21; lane 11, adult) and L-NAME treatment (lane 2, P5; lane 4, P7; lane 6, P10; lane 8, P14; lane 10, P21). (B) Densitometric analysis of immunoblots as shown in A. Data represent means±S.D. *$P<0.05$ (by Student's *t*-test).

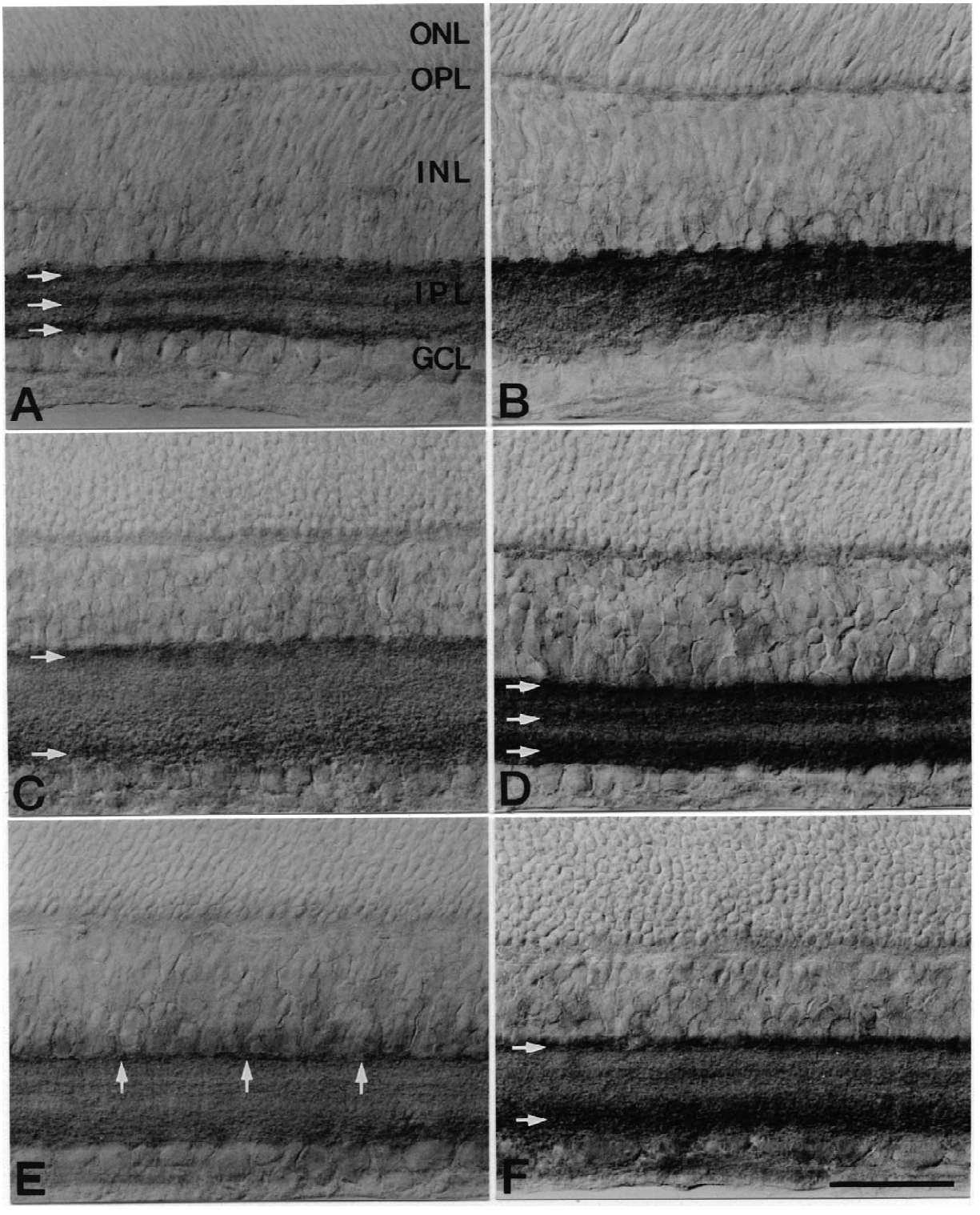

Fig. 3. Photomicrographs taken from 50-μm-thick vibratome sections processed to demonstrate GAP-43 immunoreactivity in the retinas of controls (A, C and E) and L-NAME-treated rats (B, D and F). GAP-43 immunoreactivity is much stronger following L-NAME treatments. (A, B) Retinas taken at day P10. In A, weakly labeled profiles are seen in three strata (arrows), while there is strong immunoreactivity in the whole inner plexiform layer (IPL) in B. (C, D) Retinas taken at P14. In C, weak labeling is visible in two strata (arrows). In D, strongly labeled profiles form three distinct strata (arrows). (E, F) Retinas taken at P21. In the control retina (E), labeled profiles are visible as puncta in the IPL close to the inner nuclear layer (INL). In the L-NAME treated rat retina, labeled profiles form two distinct strata (arrows). ONL, outer nuclear layer; OPL, outer plexiform layer; GCL, ganglion cell layer, Calibration bar: 25 μm.

of the control retina. Densitometric analysis of these nNOS immunoreactive proteins was performed on the control and L-NAME-treated retinas at P10, P14 and P21 (Fig. 1B). Densitometric analysis revealed that nNOS protein levels were significantly decreased in the L-NAME-treated retinas compared with those of the control retinas at P10, P14 and P21 (Fig. 1B). The GAP-43-immunoreactive band of 48 kDa was present in the extracts of both control and L-NAME-treated rat retinas, but its intensity was much stronger in the treated rats than in the controls during experimental periods (Fig. 2A). In the control retinas, the density of GAP-43 protein levels showed a peak value at P5 and then decreased gradually to 50% of the day P5 levels at P21. In the adult retina, only a weak band was detectable. However, in the L-NAME-treated retinas the density of GAP-43 protein levels increased, reached a peak value at P10 and then had decreased gradually up to P21. Densitometric analysis of these GAP-43 immunoreactive proteins was performed on the control and L-NAME-treated retinas at each developmental stage (Fig. 2B). Densitometric analysis revealed that GAP-43 protein levels were significantly increased in the L-NAME-treated retinas compared with those of the control retinas at P10, P14 and P21 (Fig. 2B). Between P5 and P7, there were no significant changes in the GAP-43 protein levels. At P10, P14 and P21, GAP-43 protein levels had increased to about 125%, 144% and 125% of the control levels, respectively.

In the control retinas, GAP-43 immunoreactivity was observed in some profiles in the IPL from day P5. The staining intensity decreased with increased age. At P10, GAP-43 immunoreactivity was seen in the profiles, which formed three distinct strata in the IPL (Fig. 3A): IPL close to the inner nuclear layer (INL), the middle of the IPL, and IPL close to the ganglion cell layer (GCL). At P14, GAP-43 labeled profiles were seen in the IPL close to the INL and close to the GCL (Fig. 3C). At P21, labeled profiles were observed mainly as puncta in the IPL close to the INL, but sparsely distributed labeled puncta were also seen in the IPL close to the GCL (Fig. 3E). In the L-NAME-treated retinas, GAP-43 immunoreactivity was much stronger than that of the controls from P10 to P21. At P10, strong immunoreactivity was observed in the whole IPL (Fig. 3B). From P14 onward, labeling intensity gradually decreased. At P14, strong GAP-43 immunoreactive profiles formed three distinct strata in the IPL (Fig. 3D). At P21, strong GAP-43 immunoreactive profiles were seen in two strata of the IPL close to the INL and the GCL, respectively (Fig. 3F).

Our results clearly demonstrate that GAP-43 is increased in the L-NAME-treated retina. GAP-43 is a membrane-associated protein, found in the axonal growth cones of sprouting central nervous system axons and plays a critical role in guiding the axonal outgrowth and in modulating the new synaptic formation (for a review, see Ref. [1]). During neuronal growth in early development, GAP-43 is expressed at high levels but is normally found at lower levels in adults [16]. In addition, recent studies have demonstrated that the increased GAP-43 is observed in regions of the adult rat brain after ischemia [15,17], in primary sensory neurons after peripheral nerve injury [7], and in retinal ganglion cells after optic nerve transection [2]. This suggests that GAP-43 might be involved in axonal growth, fiber sprouting and synaptic reorganization of delayed neuronal cell death. The exact mechanisms responsible for the upregulation of GAP-43 in the L-NAME-treated developing rat retina remains unclear. In the present study, the expression of NOS is reduced in developing rat retinas by treatment with the L-NAME. A delay in the development of retinocollicular and retinogeniculate pathways has been reported in rats and mice treated with NOS inhibitors [13,18], and in the eNOS and nNOS gene knockout mice [13,19]. In the present study, the three strata formed by labeled processes in the IPL of the L-NAME-treated retina at P14 correspond to the three strata in the control retina at P10; the two strata in the treated retina at P21 correspond to the two strata in the control retina at P14. These results clearly demonstrate that maturation of the IPL is delayed in the treated retina. Taken together, the increased levels of GAP-43 might be attributed to delayed maturation of the IPL caused by decrease of NO production in the L-NAME-treated developing rat retina. Thus, decreased NO activity is apparently associated with the increased production of GAP-43, indicating that NO plays an important role in the maturation of the IPL in the developing rat retina.

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