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The Effect of *L*-Arginine, a Nitric Oxide Synthase Substrate, on Retinal Cell Proliferation in the Postnatal Rat

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Key Words
Nitric oxide · Proliferation · Immunocytochemistry · Western blot analysis · Rat · Retina

Abstract
The effects of *L*-arginine, a nitric oxide synthase (NOS) substrate, on cell proliferation in the developing postnatal rat retina were studied by immunocytochemistry using anti-bromodeoxyuridine (BrdU) antiserum, and by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL). Densitometric analysis by immunoblotting confirmed that neuronal NOS expression significantly increased in the *L*-arginine-treated retinas in comparison with the control retinas at postnatal day (P) 5 to P10. In the retinas of the control and *L*-arginine-treated rats, BrdU-labeled cells were only seen in the neuroblastic layer of the retinas up to P7. BrdU-labeled cells were significantly more numerous in the retinas of *L*-arginine-treated rats than in control retinas at P5 in the central retina and at P5 and P7 in the peripheral retina. In addition, TUNEL-positive apoptotic cells were more numerous in the retinas of *L*-arginine-treated rats at P5 and P7. Our results suggest that NO might play an important role in retinal maturation through regulating proliferative phases in the early stages of rat postnatal development.

Introduction

Nitric oxide (NO) is a free radical that has been implicated in physiological functions ranging from dilatation of blood vessels to neuronal development and synaptic activity in the central nervous system [Garthwaite, 1991; Bredt and Snyder, 1992; Schuman et al., 1994; Goldstein et al., 1996; Hölscher, 1997]. It has been suggested that NO is involved in brain development [for a recent review, see Contestabile, 2000]. In the developing visual system, NO plays a role in activity-dependent synaptic plasticity in establishing the retinogeniculate pathway [Cramer et al., 1998]. These views are further supported by recent reports that ipsilateral retinocollicular projections are delayed in neuronal development in NO synthase (NOS) knock-out mice [Wu et al., 2000], and that NO released from target neurons in the superior colliculus and lateral

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geniculate body serves as a retrograde signal influencing the growth of retinal afferents [Vercelli et al., 2000].

In the mammalian retina, most retinal neurons containing NOS are presumed to be amacrine cells, although there is considerable variation [Perez et al., 1995; Oh et al., 1998; Kim et al., 1999; Chun et al., 1999]. In the rat retina, studies using NADPH-diaphorase (NADPH-d) histochemistry [Mitrofanis, 1989] and immunocytochemistry using anti-NOS antisera [Patel et al., 1997; Kim et al., 2000] have shown that putative NO-synthesizing cells first appear at P3 or P5 and that these cells are fully developed by the second postnatal week, before the eyes open. In addition, synaptogenesis of the inner plexiform layer (IPL) in the rat retina is fully developed during the second postnatal week [Horsburgh and Sefton, 1987]. Based on these observations, it has been suggested that NO may play an important role in the synaptogenesis of the developing retina. Recently, Goureau et al. [1999] have shown that inhibition of endogenous NOS by addition of NG-monomethyl-*L*-arginine, a NOS inhibitor, prevents the developmental decrease of rod cell numbers in chick retinal cell cultures. Therefore, NO is also thought to be involved in the cell turnover aspects of retinal development.

NO is synthesized from *L*-arginine by three isoforms of NOS: neuronal NOS (nNOS), inducible NOS, and endothelial NOS [Bredt and Snyder, 1994]. Immunocytochemical evidence indicates that *L*-arginine is localized predominantly in glial cells [Aoki et al., 1991; Pow, 1994; Pow and Crook, 1997]. Glial cells have the ability to take up *L*-arginine [Schmidlin and Wiesinger, 1994; Cossenza and Carvalho, 2000] and release it by activation of non-NMDA glutamate receptors [Grima et al., 1997, 1998]. Thus, it has been thought that *L*-arginine released by glial cells is taken up by the neurons to be used as a precursor for the synthesis of NO.

In this study, we investigated the effects of *L*-arginine, a NOS substrate [Deguchi and Yoshioka, 1982], on retinal cell proliferation in the developing rat retina, using immunocytochemistry with anti-bromodeoxyuridine (BrdU) antiserum and by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL).

Materials and Methods

Animals

Ten litters of Sprague-Dawley rat pups were used. The first day after birth was taken as postnatal day (P) 0. The animals were maintained on a daily cycle of 12 h dim light and 12 h darkness. The animals were treated according to the regulations of the Catholic Ethics Committee of the Catholic University of Korea, Seoul, South Korea, which conform to all National Institute of Health guidelines.

Administration of L-*Arginine and BrdU*

The pups were injected intraperitoneally, from P0 to P10, with 300 mg/kg per day of *L*-arginine dissolved in normal saline. Control pups received an equivalent volume of saline only. Two hours before they were killed, a single intraperitoneal injection of BrdU (Sigma, St. Louis, Mo., USA; 50 mg/kg body weight in saline with 0.007 *N* NaOH) was administered to pups at several developmental stages (from P1 to P10).

Tissue Preparation

The pups were anesthetized by intraperitoneal injections of 4% chloral hydrate (1 ml/100 g body weight). When unconscious, the animals were killed by an overdose of 4% chloral hydrate. For Western blot analysis, retinal tissues were quickly dissected on an ice-cold plate, frozen on dry ice, and stored at –70°C. For immunocytochemistry, the posterior eyecups were placed in 0.1 *M* phosphate buffer (PB; pH 7.4) containing 4% paraformaldehyde and 0.2% picric acid, for 30 min. Subsequently, the retinas were carefully dissected from the choroid and immersed in the same fixative for a further 2 h at room temperature. After several washes in 0.1 *M* phosphate-buffered saline (PBS; pH 7.4), the retinal tissues were kept in PBS.

Western Blot Analysis

Western blot analysis was performed on the retinal extracts which were homogenized in 10 vol of 20 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 1 m*M* EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.02% sodium azide, 1 m*M* PMSF, and 5 μg/ml leupeptin. Protein concentration in each sample was assayed by the Lowry method [Lowry et al., 1951; Peterson, 1979] in duplicate and the result averaged. Duplicate sets of protein standards containing 0, 1, 3, 5, 10, 20, 40, or 60 μg bovine serum albumin were assayed by the same method and results averaged and graphed to give a linear equation that was used to estimate the protein contents in retinal extracts. Optical density of each sample was measured at 660 nm using a spectrometer (Spectronic 20; Bausch and Lomb, Rochester, N.Y., USA). Aliquots of tissue samples corresponding to 25 μg of total protein were heated at 100°C for 10 min with an equivalent volume of 2 × sample buffer (containing 4% SDS and 10% mercaptoethanol) and loaded onto 10% polyacrylamide gels. The proteins were electrotransferred to a nitrocellulose membrane in Tris-glycine-methanol buffer. The membrane was blocked for 1 h at room temperature in a blocking solution containing 5% nonfat dry milk, 0.05% Tween-20, and PBS (pH 7.4). The membrane was then incubated for 15 h at 4°C with primary antibodies against nNOS (1:7,000, Sigma) in the blocking solution. The membrane was rinsed with 0.05% Tween-20 in PBS for three washes of 10 min and incubated for 1 h at room temperature in a 1:2,000 dilution of peroxidase-conjugated goat anti-rabbit IgG antibody (Vector Laboratories, Burlingame, Calif., USA). The blot was washed for 10 min three times and then processed for analysis using an enhanced chemiluminescence detection kit (Amersham, Arlington Heights, Ill., USA). nNOS protein content was calculated by measuring the pick densitometry area by Eagle Eye™ II Still Video System (Stragene, La Jolla, Calif., USA). The optical densities (mean ± SD) were obtained after five determinations for each band.

Immunostaining

Small pieces of retinal tissue were taken from the central portion near the optic nerve head and the peripheral portion near the ora serrata of the retina. These were washed in PB containing 4.5% sucrose, and then dehydrated in a graded series of alcohol, cleared in propylene oxide, and embedded in Epon 812. The embedded tissue samples were cured for 2 days at 60°C, and then semithin sections were made vertically through the retinas, at a thickness of 1 μm. Semithin sections were collected on 0.5% gelatin-coated slides, and then processed for BrdU-like immunoreactivity. They were etched with alcoholic NaOH for 15 min at room temperature, then washed in distilled water for 5 min and in PBS for another 5 min. The sections were then treated with 0.5% H_2O_2 in absolute methanol for 30 min at room temperature to destroy endogenous peroxidase activity. The sections were incubated for 1 h at room temperature in PBS containing 10% normal goat serum to reduce nonspecific staining. They were then incubated with monoclonal anti-BrdU antisera (Dako Corp., Glostrup, Denmark; diluted 1:100) in PBS containing 3% normal goat serum for 2 h at room temperature. After incubation, the sections were rinsed in four changes of PBS for 5 min, incubated in biotinylated goat antirabbit IgG in PBS for 1 h at room temperature, and then rinsed in PBS. They were then incubated in avidin-biotin-peroxidase complex in PBS for 1 h at room temperature, and rinsed in two changes of PBS and three changes of 0.05 *M* Tris-HCl buffer (TB; pH 7.4) for 5 min at room temperature. Sections were then incubated in 0.01% H_2O_2 plus 0.05% 3,3′-diaminobenzidine tetrahydrochloride in TB for 1–2 min, as determined by the degree of staining. When judged complete, the reaction was stopped with several washes of TB and PBS.

TUNEL Technique

Apoptotic cell death was determined by a modified TUNEL technique [Li et al., 1997] according to the manufacturer's instructions (in situ cell detection kit, Boehringer Mannheim, Mannheim, Germany). Small pieces of retinal tissue were cut out from the central portion near the optic nerve head and the peripheral portion near the ora serrata of the retina, and then dehydrated in a graded series of alcohol, and embedded in wax. Vertical sections (5 μm thick) were cut from the wax-embedded retinas, dewaxed with absolute ethanol at 53°C, and hydrated through a graded series of ethanol. To increase permeability, the sections were incubated in 0.2% Triton X-100 for 5 min and rinsed in distilled water. Endogenous peroxidase activity was blocked by placing the sections in 3% H_2O_2 in distilled water for 10 min. The sections were equilibrated in terminal transferase-labeling buffer (Boehringer Mannheim) and the tailing reaction was performed with terminal deoxynucleotide transferase (0.3 e.u./μl) and biotinylated deoxyuridine triphosphate (1 nmol/μl) in a humidified chamber for 1 h at 37°C. The reaction was terminated by incubation in a solution of 0.3 *M* NaCl and 0.03 *M* sodium citrate. After being rinsed in distilled water, the sections were blocked using 2% BSA in PBS (pH 7.4) for 30 min at room temperature, followed by several rinses in PBS. To visualize incorporated biotinylated dUTP, the sections were processed with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame) in PBS for 30 min at room temperature and the peroxidase reaction was performed with 3,3′-diaminobenzidine tetrahydrochloride in TB as substrates. Sections incubated at 60°C for 1 h served as positive controls, and staining of negative controls was performed in the absence of terminal transferase (data not shown).

Quantitative Analysis

The effects of *L*-arginine on retinal development were quantitatively evaluated by counting the numbers of BrdU-positive and TUNEL-positive cells per 1 mm length (length of the retinal section) in vertical sections of control and experimental retinas. BrdU-positive cells were counted separately in the central and peripheral regions, whereas TUNEL-positive cells were counted in the whole length from the central regions to the peripheral regions, since TUNEL-positive cells were infrequent. Images of each section were captured using a 40× objective and a 10× eyepiece and then processed with an image analysis system (BMI-PLUS; Bummi Universe Co., Ansan, Korea). For each experimental condition, 20 sections obtained from different retinas were analyzed. Values are given as means ± SD. Analysis of variance was used for statistical evaluation followed by Student's t test, with $p < 0.05$ assumed for statistical significance.

Results

In this study, we examined the effect of *L*-arginine on retinal maturation, by counting the number of BrdU-positive and TUNEL-positive cells in the developing retina of postnatal rats.

The nNOS-immunoreactive band of 155 kD was present in the extracts of both control and *L*-arginine-treated rat retinas, but its intensity was much stronger in the treated retina than in the control retina during the experimental periods (fig. 1A). Densitometric analysis of the 155-kD band was performed to estimate relative protein levels. In the control retinas, the density of nNOS proteins gradually increased and showed a peak value at P10 (fig. 1B). In the *L*-arginine-treated ischemic retinas, the density was significantly greater than that in the non-treated retinas during the whole experimental time. At P5, the increase in density was 1.8-fold greater than in the nontreated retinas, and then gradually decreased up to P10. At P10, the increase in density was 1.2-fold greater than in the nontreated retinas (fig. 1B).

The developmental patterns of the control retina were in good agreement with a previous report by Fletcher and Kalloniatis [1997]. At P1, the rat retina consisted of a ganglion cell layer (GCL), an IPL and a large neuroblastic layer (NBL) (fig. 2A). By P5, the general morphology of the retina was similar to that of earlier stages (fig. 2C). At P7, the morphology was similar to that of P5 with widening of the IPL. The general morphology of the retinas of treated rats at any stage was similar to those of the control rats, indicating that *L*-arginine did not induce any histological changes in the developing rat retina.

To compare the effects of *L*-arginine on retinal cell proliferation, we used BrdU and anti-BrdU antiserum and counted the number of BrdU-labeled cells over 1-mm

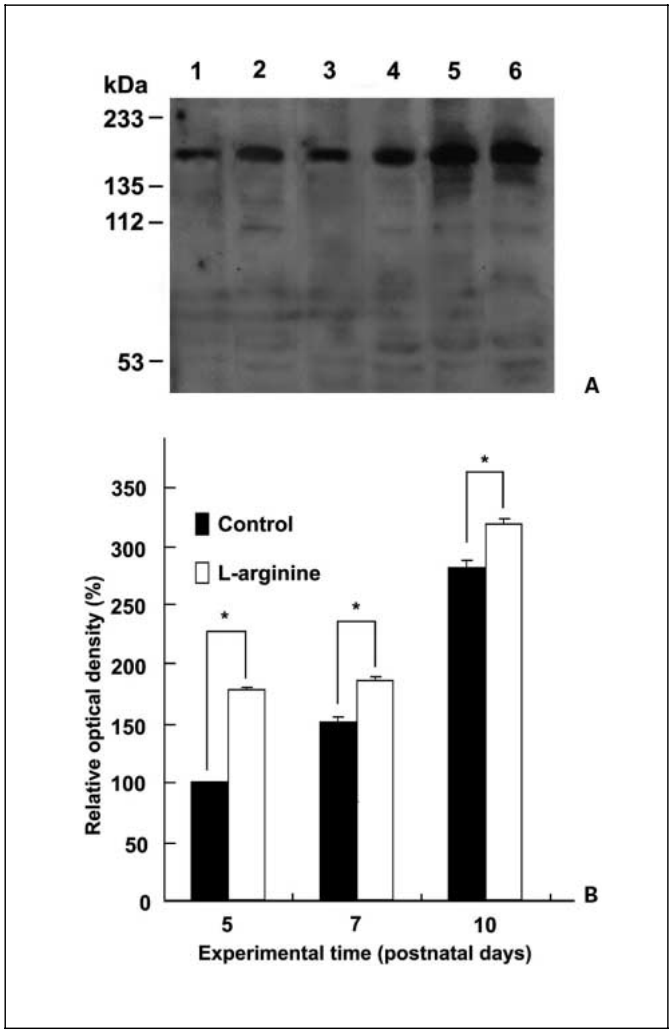

Fig. 1. Immunoblot analysis of nNOS protein levels in the developing retina of control and *L*-arginine-treated rats. A Immunoblot stained for nNOS demonstrating a single band at 155 kD: control (lane 1, P5; lane 3, P7; lane 5, P10) and *L*-arginine treatment (lane 2, P5; lane 4, P7; lane 6, P10). B Densitometric analysis of immunoblots as shown in A. Data represent means ± SD. * $p < 0.05$ (by Student's t test).

lengths of the NBL. BrdU is incorporated into genomic DNA during the S-phase of the cell cycle and can subsequently be detected by means of immunocytochemistry [Millar and Nowakowski, 1988; Del Rio and Soriano, 1989]. BrdU-like immunoreactive cells were only found in the NBL in the retinas of all pups examined in this study (fig. 2, 4). ^{3}H-Thymidine uptake studies in the mammalian retina [Sidman, 1970; Carter-Dawson and LaVail, 1979; Johns et al., 1979; Polley et al., 1986; Zimmerman et al., 1988] have shown that ganglion cells differentiate prenatally and bipolar cells and Müller cells differentiate postnatally. Amacrine cells are highly diverse, with some cells differentiating prenatally and others postnatally. Taken together, it can be inferred that BrdU-labeled cells in the control and *L*-arginine-treated retinas represent a mix of bipolar, amacrine or Müller cells.

In the central retina of the control and *L*-arginine-treated rats (fig. 2, 3) at P1, the numbers of BrdU-labeled cells in the NBL were 351.2 ± 25.7 cells/mm (n = 20) and 366.4 ± 24.3 cells/mm, respectively. In both groups, BrdU-labeled cell counts gradually decreased up to P7. However, by P5, there were 55.6 ± 9.6 BrdU-labeled cells/mm in the retinas of *L*-arginine-treated rats. These were significantly more numerous than in the control retinas, which had 16.1 ± 9.6 cells/mm. In the peripheral retinas (fig. 4, 5) of control rats at P1, there were 380.8 ± 26.5 BrdU-labeled cells/mm. Thereafter, the number of labeled cells gradually decreased to 81.4 ± 3.1 at P7. By P5 and P7, there were 335.4 ± 16.7 and 142.6 ± 7.3 BrdU-labeled cells/mm, respectively, in the retinas of *L*-arginine-treated rats. When compared with the control groups, BrdU cells in retinas of *L*-arginine-treated rats were more numerous at P5 and P7. At P10, no BrdU-labeled cell was visible in the central and peripheral retinas of the control and *L*-arginine-treated rats (data are not shown).

TUNEL-positive cells were also counted (fig. 6, 7). In the control retinas at P1, there were 4.6 ± 5.6 TUNEL-positive cells/mm. Thereafter, the number of TUNEL-positive cells increased gradually, reaching a peak at P10 (36.2 ± 15.6 cells/mm). In the retinas of *L*-arginine-treated rats, there were 8.8 ± 3.8 TUNEL-positive cells/mm at P1. Counts increased gradually up to 50.2 ± 7.0 cells/mm at P7, and then decreased to 35.3 ± 12.9 cells/mm at P10. When compared with the control groups, there were many more TUNEL-positive cells in the retinas of *L*-arginine-treated rats at P5 and P7.

Discussion

Our results clearly demonstrate that treatment with *L*-arginine increased the number of BrdU-labeled and TUNEL-positive cells in the developing retina. The exact mechanisms responsible remain unknown. In the present study, nNOS was upregulated in the *L*-arginine-treated retinas, suggesting that NO might be overproduced. NO is generated by the oxidation of arginine, a reaction catalyzed by the NOS [Deguchi and Yoshioka, 1982; Palmer et al., 1988], which utilizes reduced NADPH as a cofac-

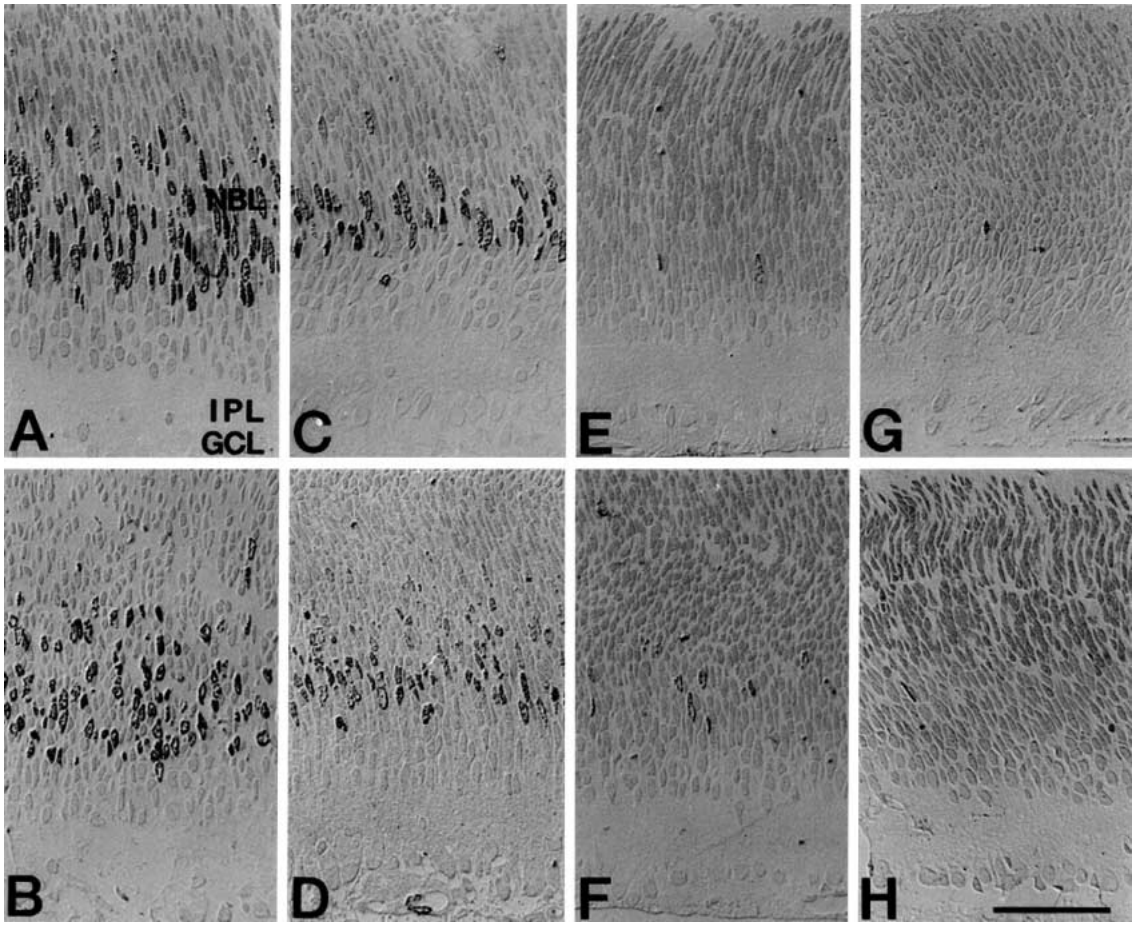

Fig. 2. Light micrographs of the central retinas of the control (A–D) and *L*-arginine-treated (E–H) rats at P1 (A, E), P3 (B, F), P5 (C, G), and P7 (D, H). BrdU-immunoreactive cells are exclusively seen in the NBL. Note that more labeled cells are seen at P5 in the retina of *L*-arginine-treated rat. Scale bar, 50 μm.

tor. The generated NO acts on cells by activating soluble guanylate cyclase, thus increasing the levels of cyclic GMP which then mediates the effects on the cell [Moncada et al., 1991]. NOS has been identified as an enzyme capable of producing an NADPH-d reaction [Hope et al., 1991], and the histochemical demonstration of NADPH-d has been suggested to reflect the presence of NOS [Grozdanovic et al., 1992; Valtschanoff et al., 1993]. Thus, exogenously applied *L*-arginine might induce the upregulation of NOS. This assumption could be further corroborated by the fact that treatment with *L*-arginine in retinal explants induces NADPH-d reaction in retinal neurons from P1.

NO has been suggested to have a role in neural cell proliferation during early stages of brain development. Treatment using inhibitors of NO synthesis prevents the segregation of retinal inputs into sublaminae in the lateral geni-

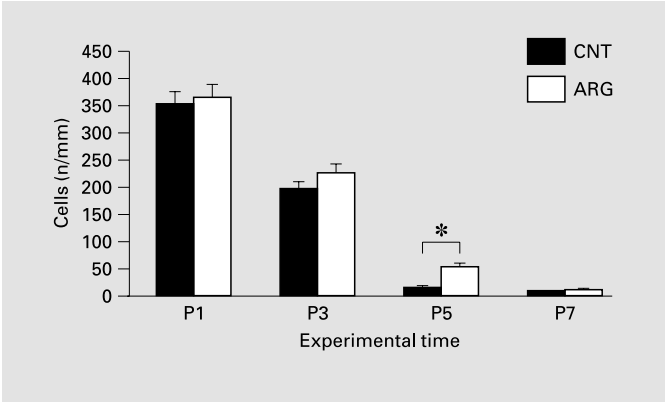

Fig. 3. Figure showing the number of BrdU-labeled cells in the NBL of the central retinas of the control (CNT) and *L*-arginine-treated (ARG) rats at different developmental stages. At P5, there are significantly more labeled cells in the retinas of treated rats than those of control rats. * $p < 0.05$.

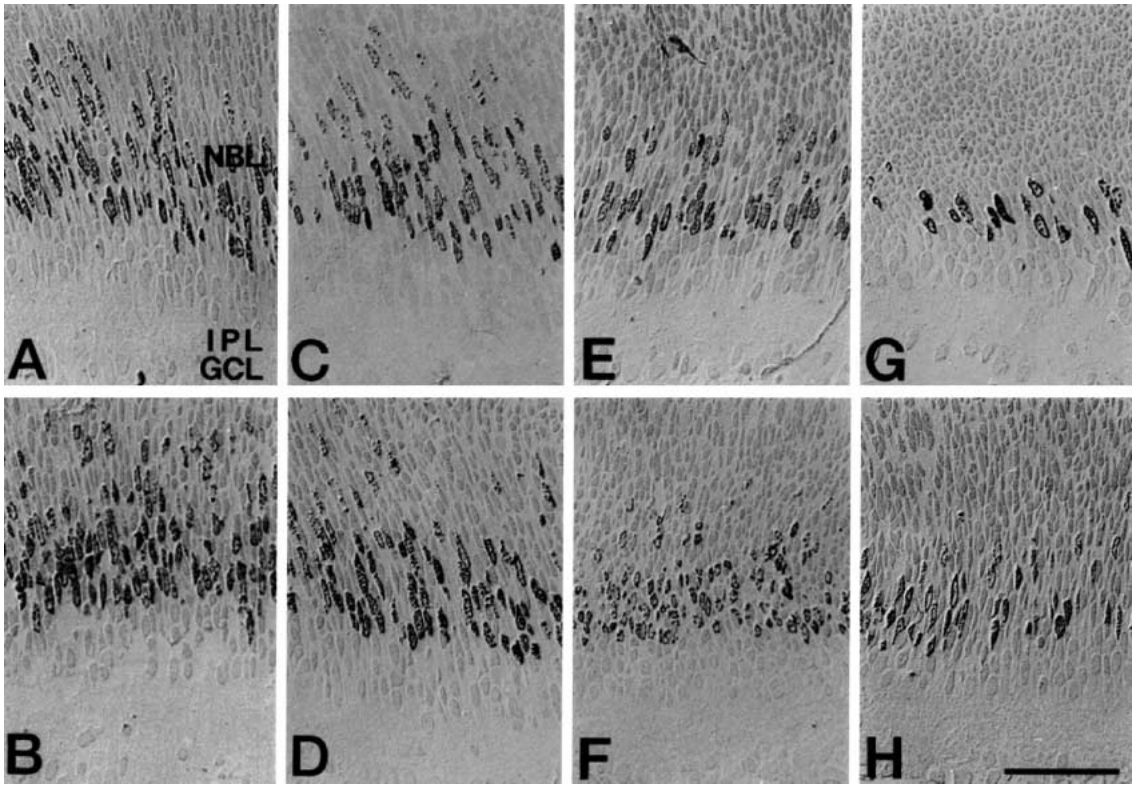

Fig. 4. Light micrographs of the peripheral retinas of the control (A–D) and *L*-arginine-treated (E–H) rats at P1 (A, E), P3 (B, F), P5 (C, G), and P7 (D, H). BrdU-immunoreactive cells are exclusively seen in the NBL. Note that more labeled cells are seen at P5 and P7 in the retina of *L*-arginine-treated rat. Scale bar, 50 μm.

culate nucleus during early postnatal development [Cramer et al., 1996]. Application of an NOS inhibitor to cultures of slices of the developing rat cerebellum causes an apparent impairment of the intrinsic tendency to slow down cell division in the external granular layer [Tanaka et al., 1994]. In the developing rat, inhibition of NO synthesis results in delayed maturation of retinofugal connections [Vercelli et al., 2000]. Inhibition of NOS induces increased production of growth-associated protein 43, a membrane-associated protein which plays an intrinsic neuronal development and plasticity [Benowitz and Routtenberg, 1997], in the developing retina of the postnatal rat [Oh et al., 2002]. Oh et al. [2002] suggested that the increased levels of growth-associated protein 43 might be attributed to delayed maturation of the IPL caused by a decrease of NO production in the N^G-nitro-*L*-arginine methyl ester-treated developing rat retina. Furthermore, Guimarães et al. [2001] have shown that treatment with *L*-arginine in retinal explants induces NADPH-d-labeling in the cells located both in the GCL and IPL from P1, and

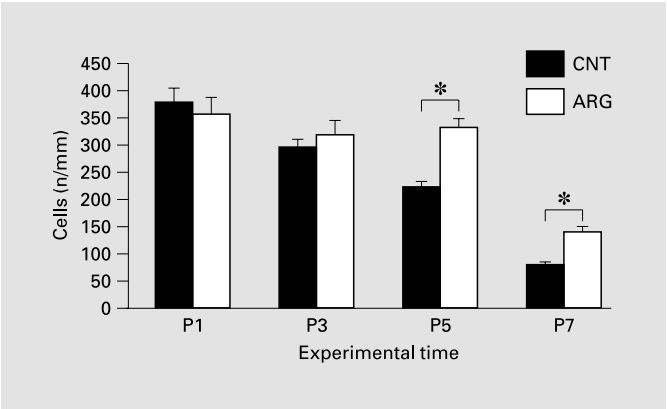

Fig. 5. Figure showing the number of BrdU-labeled cells in the NBL of the peripheral retina of the control (CNT) and *L*-arginine-treated (ARG) rats at different developmental stages. At P5 and P7, there were significantly more labeled cells in the retinas of treated rats than in those of control rats. * $p < 0.05$ (Student's t test).

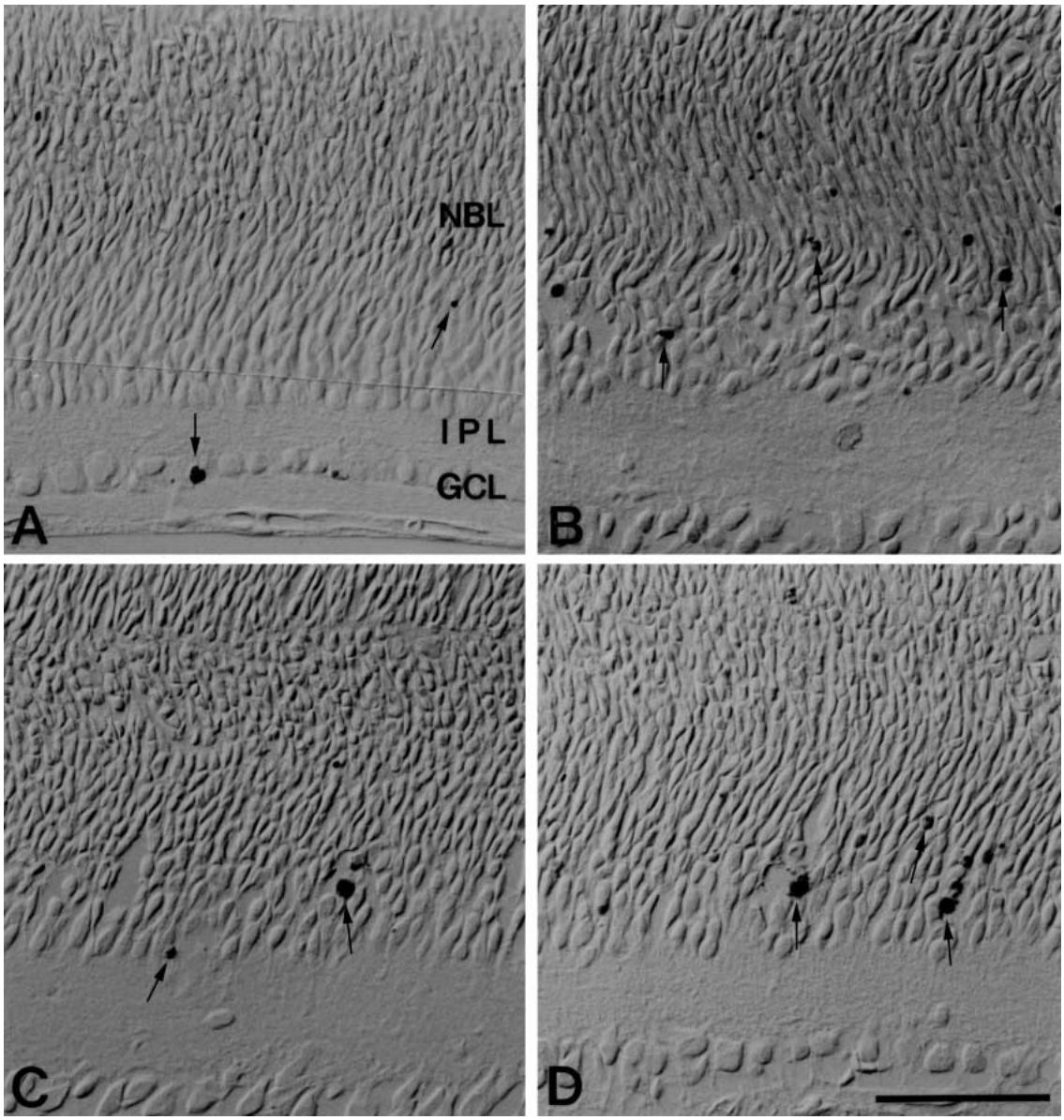

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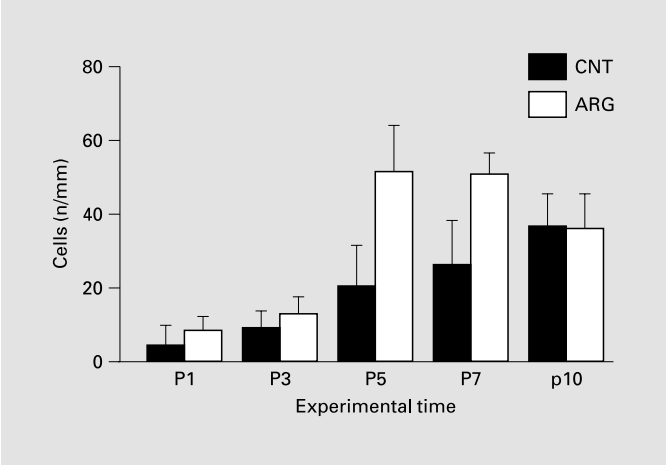
Fig. 6. Light micrographs of the retinas of control (A, C) and *L*-arginine-treated (B, D) rats at P5 (A, B) and P7 (C, D). Examples of TUNEL-positive cells are indicated by arrows. Scale bar, 50 µm.

Fig. 7. The number of TUNEL-positive cells in the retinas of the control (CNT) and *L*-arginine-treated (ARG) rats at different developmental stages. At P5 and P7, the numbers of labeled cells in retinas from treated rats were significantly greater than in those from control rats. * $p < 0.05$ (Student's t test).



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indicated that NO produced by *L*-arginine treatment blocks cell death induced by anisomycin in the NBL. Taken together, overproduction of NO by treatment with *L*-arginine might be a reason for the increase in BrdU-positive cells at P5 and P7. Therefore, NO might act as a controlling agent for proliferation in early postnatal developmental stages.

In the present study, TUNEL-positive cells were more numerous in the retinas of *L*-arginine-treated rats, compared with control rats, at P5 and P7, indicating that more retinal cells die at these stages. During development, cell number homeostasis is regulated by programmed cell death or apoptosis [for a review, see Clarke and Clarke, 1996]. Thus, the increase in cell death might be secondary to the increase in proliferating cells induced by the overproduction of NO. However, the possibility that NO has a proliferative effect on certain cell types, and induces apoptosis in other cell types could not be excluded.

In conclusion, treatment with *L*-arginine induced an increase in retinal cell proliferation at P5 and P7, possibly through the overproduction of NO. Thus, NO might play an important role in regulating the proliferative phase of some neurons in the early developmental stages of the retinas of postnatal rats. However, further detailed studies are clearly needed to elucidate the actual role of NO in these phenomena.

Acknowledgments

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