ELSEVIER

Neuroscience Letters 267 (1999) 193–196

Neuroscience Letters

Immunocytochemical localization of nitric oxide synthase in the mammalian retina

In-Beom Kim[a], Eun-Jin Lee[a], Keun-Young Kim[a], Won-Kyu Ju[a], Su-Ja Oh[a], Choun-Ki Joo[b], Myung-Hoon Chun[a,*]

[a]*Department of Anatomy, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Socho-gu, Seoul 137–701, South Korea*
[b]*Department of Ophthalmology, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Socho-gu, Seoul 137–701, South Korea*

Received 2 March 1999; received in revised form 19 April 1999; accepted 20 April 1999

Abstract

The localization of nitric oxide synthase (NOS) was investigated by immunocytochemistry and immunoblotting using an antiserum against neuronal NOS in the rat, mouse, guinea pig, rabbit and cat retinae. Western blot analysis of retinal tissue extracts showed that the NOS-immunoreactive band of 155 kDa was present in all species. In the rat, mouse, guinea pig and rabbit retinae, two types of amacrine cells and a class of displaced amacrine cells were consistently NOS-labeled. In the cat retina, unlike other mammals, one type of amacrine cells and two types of displaced amacrine cells showed NOS immunoreactivity. NOS immunoreactivity was further found in some bipolar cells of the rat and guinea pig, some interplexiform cells of the mouse, some photoreceptor cells of the rabbit and some Müller cells of the cat.

Keywords: Nitric Oxide Synthase; Retina; Rat; Mouse; Guinea Pig; Rabbit; Cat

Nitric oxide (NO) is synthesized from L-arginine by nitric oxide synthase (NOS) [1]. NO generated in the central nervous system including the retina diffuses across cell membrane rapidly and activates soluble guanylate cyclase (sGC), thus increasing the levels of cyclic GMP (cGMP) [2–4]. It has been known that nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) is colocalized with NOS in several neuronal systems [3,5], and that NADPH-d reaction is not seen in the retina of mice devoid of neuronal NOS gene [6]. Therefore, NADPH-d histochemistry has been used to identify NO-synthesizing cells. However, recent studies have shown that the match between NADPH-d reaction and NOS immunoreactivity in the retina is not complete [5,7,8]. Particularly in the rat retina, only one amacrine cell class was labeled by NADPH-d [9], whereas three cell types were labeled by NOS immunoreactivity [8]. Such discrepancy might be due to the fact that NADPH-d staining pattern is variable according to the fixation conditions such as the time of fixation and the nature of fixative [10]. In the present study, we examined mammalian retinal cells showing NOS immunoreactivity using an antisera specifically directed to a C-terminal fragment of the rat brain NOS.

Adult rats, mice, guinea pigs, rabbits and cats were used. The animals were maintained on a daily cycle, 12 h in the dim light and 12 h in the darkness. They were deeply anesthetized by intraperitoneal injection of 4% chloral hydrate (1 ml/100 g body weight). The eyeballs were enucleated and the animals were killed by an overdose of 4% chloral hydrate. The anterior segments of the eyeballs were removed, and the retinae were carefully dissected. For

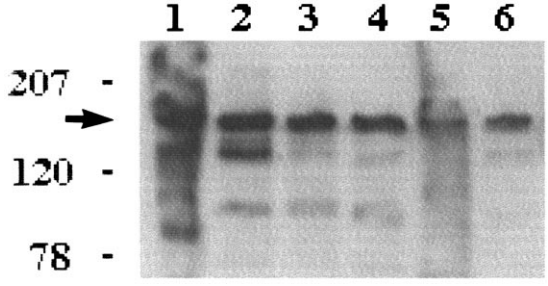

Fig. 1. Western blot analysis of neuronal NOS in the rat (lane 2), mouse (lane 3), guinea pig (lane 4), rabbit (lane 5) and cat (lane 6) retinae. Positions of molecular weight (kDa) makers (Transduction) are indicated on the lane 1.

* Corresponding author. Tel.: +82-2-590-1152; fax: +82-2-536-3110.
E-mail address: mhchun@cmc.cuk.ac.kr (M.-H. Chun)

0304-3940/99/$ - see front matter
PII: S0304-3940(99)00363-8

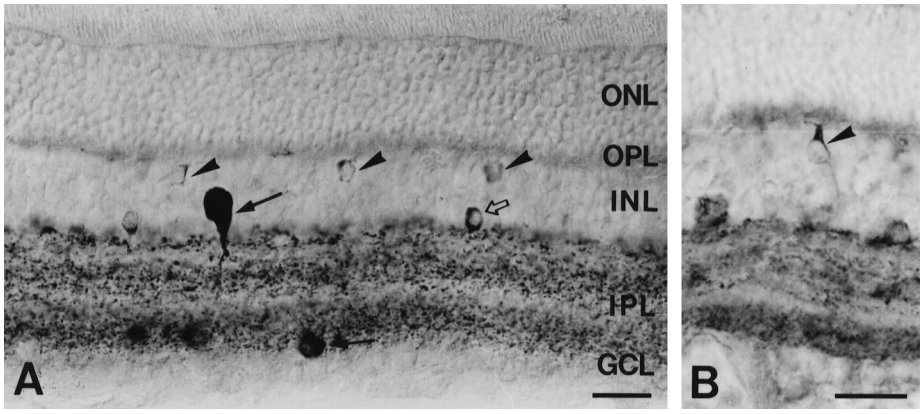

Fig. 2. NOS immunoreactivity of the rat retina. (A) Type 1 (arrow) and type 2 (open arrow) labeled amacrine cells are seen in the inner nuclear layer (INL). Arrowheads point to cell bodies of bipolar cells in the INL. In the ganglion cell layer (GCL), a labeled displaced amacrine cell (arrow) is seen. (B) An arrowhead points to a bipolar cell with a strongly labeled dendrite. ONL, outer nuclear layer; OPL, outer plexiform layer; IPL, inner plexiform layer. Scale bar, 25 μm.

Western blot analysis, retinal tissues were quickly dissected on ice-cold plate, frozen on dry ice, and stored at −70°C. Western blot analysis was performed on the extracts of retinal tissue which was 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 gel and blotted onto nitrocellulose membrane. Immunostaining of blotted proteins was carried out using a rabbit polyclonal anti-NOS antibody (Sigma, St. Louis, MO; dilution rate 1:3000). The anti-bNOS antiserum was developed in rabbit using the synthetic peptide corresponding to the C-terminal fragment of bNOS of rat brain origin (amino acids 1409–1429 with N-terminally added lysine). For NOS immunoreactivity, the retinae were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB) for 2–3 h. Subsequently, they were transferred to 30% sucrose in PB for 24–48 h. They were then frozen in liquid nitrogen, thawed, and rinsed in 0.01 M phosphate buffered saline, pH 7.4 (PBS). Immunostaining was performed by using the avidin-biotin-peroxidase complex method. Fifty-micrometer thick vibratome sections were incubated in 10% normal goat serum (NGS) and 1% Triton X-100 in PBS for 1 h at room temperature in order to block non-specific binding sites, followed by an incubation in the rabbit polyclonal antiserum against bNOS (Sigma; dilution rate 1:3000) in PBS containing 0.5% Triton X-100 for 3 days at 4°C. After washing in PBS for 45 min (3 × 15 min), the retinae were incubated for 12 h in biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA) with 0.5% Triton X-100 at 4°C, rinsed in PBS, subsequently incubated in ABC (Vector) in PBS for 12 h at 4°C. Retinae were rinsed twice in PBS and three times in 0.05 M Tris–HCl buffer, pH 7.4 (TB) for 5 min at room temperature and incubated in 0.05% 3,3′-diaminobenzidine tetrahydrochloride in TB for 10 min. Hydrogen peroxide was added to the incubation medium to make a final concentration of 0.01% H_2O_2, and the container was gently shaken as the reaction proceeded. After 1–2 min, as determined by the degree of staining, the reaction was stopped with several washes of TB and PB.

In order to test the specificity of the antibody, extracts of rat pituitary (Transduction, Lexington, KY) were used as a

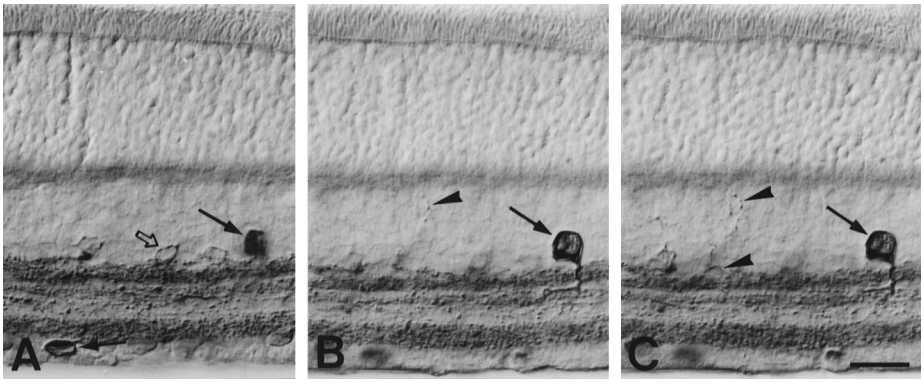

Fig. 3. NOS immunoreactivity of the mouse retina. (A–C) Are taken from the same field at different focal planes. (A) Two types (arrow, open arrow) of labeled amacrine cells are seen in the INL. A displaced amacrine cell in the GCL is indicated by an arrow. (B,C) In addition to type 1 amacrine cell (arrow), fine processes emerging from an interplexiform cell (arrowhead) are seen. Scale bar, 25 μm.

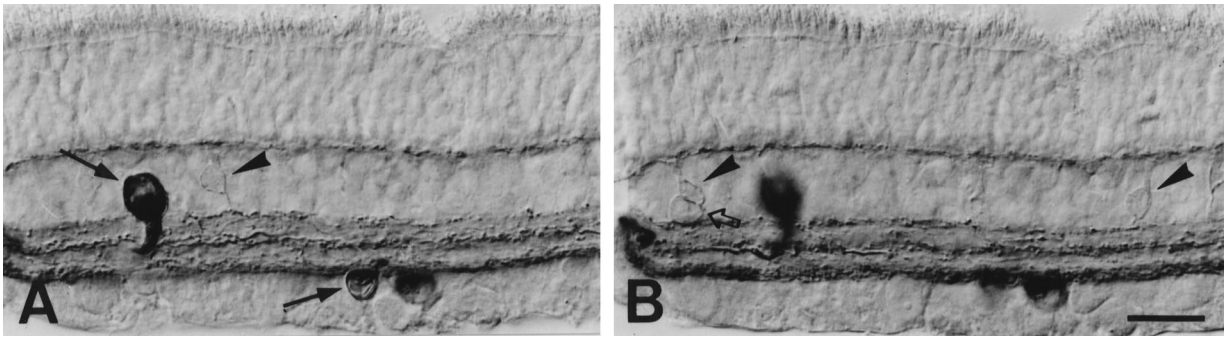

Fig. 4. NOS immunoreactivity of the guinea pig retina. (A,B) Are taken from the same field at different focal planes. Type 1 (arrow) and type 2 (open arrow) of amacrine cells are seen in the IPL. Arrowheads point to labeled bipolar cells. An arrow in the GCL points a displaced amacrine cell. Scale bar, 25 μm.

marker. Intense NOS immunoreactive bands corresponding to rat pituitary NOS were observed in the extracts of all species examined in this study (Fig. 1). The molecular weights of the major bands were about 155 kDa, but its intensity was much stronger in the retinae of the rat, mouse and guinea pig than in those of the rabbit and cat.

In the rat retina, two populations of amacrine cells showed NOS immunoreactivity (Fig. 2): type 1 cells had large somata which were located in the inner nuclear layer (INL) with long and sparsely branched processes ramifying mainly in stratum 3 of the inner plexiform layer (IPL). Smaller cell bodies of type 2 cells were also located in the INL. Their fine processes branched mostly in stratum 1 of the IPL. A class of displaced amacrine cells in the ganglion cell layer (GCL) was also labeled; their processes stratified mainly in strata 4 and 5 of the IPL. Some NOS-labeled cell bodies were seen in the outer margin of the INL; they sent processes into the outer plexiform layer (OPL) and IPL (Fig. 2B), thus indicating that they correspond to bipolar cells. In the mouse retina, two types of amacrine cells and a population of displaced amacrine cells showed NOS immunoreactivity (Fig. 3). Stratification patterns of labeled processes were similar to those of the rat retina. Fine processes emerging from the type 2 amacrine cells occasionally projected toward the OPL; these type 2 amacrine cells correspond to interplexiform cells. In the guinea pig retina (Fig. 4), as in the rat retina, NOS immunoreactivity was localized to two types of amacrine cells, a class of displaced amacrine cells and some bipolar cells. In the IPL, three distinct lamination (strata 1, 3 and 5) of the labeled processes were seen. Axons and cell bodies of bipolar cells were weakly labeled, whereas dendrites of bipolar cells showed strong NOS immunoreactivity in the OPL. In the rabbit retina, two types of amacrine cells showed NOS immunoreactivity (Fig. 5). These cells corresponded to the type 1 and 2 of the rat, mouse and guinea pig retina, respectively. A class of displaced amacrine cells also showed NOS immunoreactivity. In addition, some photoreceptor cells were clearly labeled. In the cat retina (Fig. 6), unlike other mammals, one type of amacrine cells and two types of displaced amacrine cells showed NOS immunoreativity. Endfeet of Müller cells were also labeled intensely.

Soluble guanylate cyclase, which synthesizes cGMP upon activation by NO was present in ON-bipolar cells and ganglion cells [11,12]. NO released from the NOS amacrine cells stimulated cGMP synthesis in these cells, resulting in activation of cGMP gated channels [13]. The results of the present study suggested that amacrine cells might be the most prominent source for NO in the cells

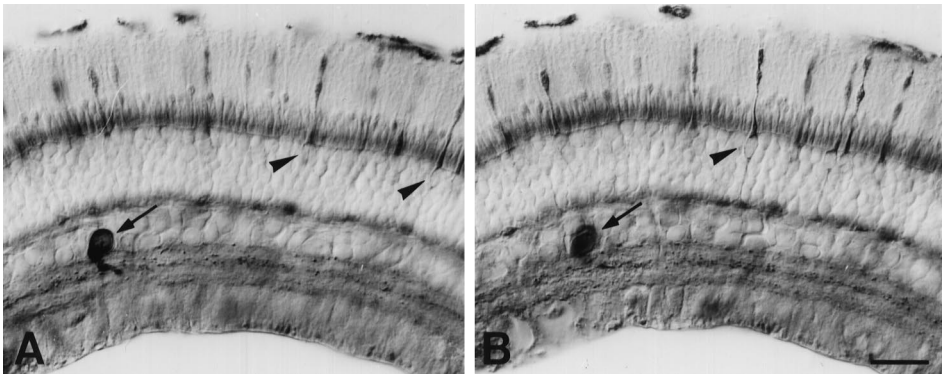

Fig. 5. NOS immunoreactivity of the rabbit retina. (A,B) Are taken from the same field at different focal planes. Type 1 amacrine cell (arrow) is seen. Arrowheads point labeled photoreceptor cells. Scale bar, 25 μm.

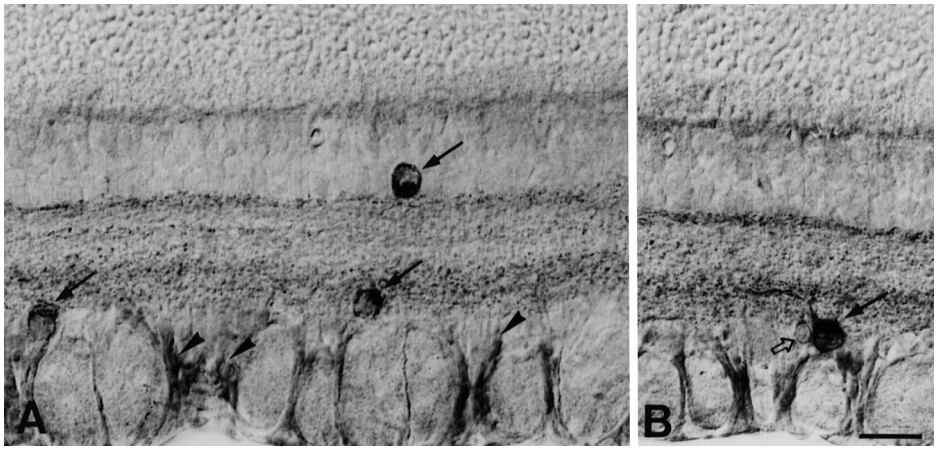

Fig. 6. NOS immunoreactivity of the cat retina. (A) A type 1 amacrine cell (arrow) is seen in the INL. In the GCL, two displaced amacrine cells (arrows) and endfeet of Müller cells (arrowheads) are seen. (B) Two types of displaced amacrine cells (arrow, open arrow) in the GCL. Scale bar, 25 μm.

which are located in the INL and ganglion cell layer of the mammalian retina.

There is evidence that NO participates in the regulation of cGMP levels in photoreceptor cells, thereby affecting membrane conductance in these cells [14,15]. Even though there is no direct evidence of sGC in horizontal cells, several studies suggest a role for NO-activated sGC in the regulation of electrical coupling via gap junctions in these cells [16]. However, a source of NO to these cells has not been clarified in the mammalian retina. In the present study, NOS immunoreactivity was observed not only in amacrine cells but also some bipolar cells of the rat and guinea pig, some interplexiform cells of the mouse, some photoreceptor cells of the rabbit and Müller cells of the cat. These cells might also serve as a source of NO to photoreceptors or horizontal cells in these animals. Our results further indicated that Müller cells, bipolar cells and photoreceptor cells might modulate photoreceptor function via NO through a retrograde or anterograde signaling pathway in vertebrate retina [15,17–19].

This work was supported by KOSEF grant from Korea Science and Engineering Foundation.

[1] Palmer, R.M.J., Ashton, D.S. and Moncada, S., Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature (Lond.), 333 (1988) 664–666.
[2] Bredt, D.S. and Snyder, S.H., Nitric oxide: a novel neuronal messenger. Neuron, 8 (1992) 8–11.
[3] Dawson, T.M., Bredt, D.S., Fotuhi, M., Hwang, P.M. and Snyder, S.H., Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. Proc. Natl. Acad. Sci. USA, 88 (1991) 7797–7801.
[4] Goldstein, I.M., Oswald, P. and Roth, S., Nitric oxide: a review of its role in retinal function and disease. Vision Res., 36 (1996) 2979–2994.
[5] Perez, M.T.R., Larsson, B., Alm, P., Andersson, K.E. and Ehinger, B., Localization of neuronal nitric oxide synthase-immunoreactivity in rat and rabbit retinas. Exp. Brain Res., 104 (1995) 207–217.
[6] Darius, S., Wolf, G., Huang, P.L. and Fishman, M.C., Localization of NADPH-diaphorase/nitric oxide synthase in the rat retina: an electron microscopic study. Brain Res., 690 (1995) 231–235.
[7] Yamamoto, R., Bredt, D.S., Snyder, S.H. and Stone, R.A., The localization of nitric oxide synthase in the rat eye and related cranial ganglia. Neuroscience, 54 (1993) 189–200.
[8] Oh, S.J., Kim, I.B., Lee, M.Y., Chun, M.H. and Chung, J.W., NOS-like immunoreactive neurons express GABA-like immunoreactivity in rabbit and rat retinae. Exp. Brain Res., 120 (1998) 109–113.
[9] Mitrofanis, J., Development of NADPH-diaphorase cells in the rat's retina. Neurosci. Lett., 102 (1989) 165–172.
[10] Huxlin, K.R., NADPH-diaphorase expression in neurons and glia of the normal adult retina. Brain Res., 692 (1995) 195–206.
[11] Shiells, R.A. and Falk, G., Retinal on-bipolar cells contain a nitric oxide-sensitive guanylate cyclase. NeuroReport, 3 (1992) 845–848.
[12] Ahmad, I., Leinders-Zufall, T., Kocsis, J.D., Shepherd, G.M., Zufall, F. and Barnstable, C.J., Retinal ganglion cells express a cGMP-gated cation conductance activatable by nitric oxide donors. Neuron, 12 (1994) 155–165.
[13] Gotzes, S., de Vente, J. and Müller, F., Nitric oxide modulates cGMP levels in neurons of the inner and outer retina in opposite ways. Vision Neurosci., 15 (1998) 945–955.
[14] Ehret-Hilberer, S., Nullans, G., Aunis, D. and Virmaux, N., Mono ADP-ribosylation of transducin catalyzed by rod outer segment extract. Fed. Eur. Biochem. Soc., 309 (1992) 394–398.
[15] Kurenny, D.E., Moroz, L.L., Turner, R.W., Sharkey, K.A. and Barnes, S., Modulation of ion channels in rod photoreceptors by nitric oxide. Neuron, 13 (1994) 315–324.
[16] McMahon, D.G., Modulation of electrical synaptic transmission in zebrafish retinal horizontal cells. J. Neurosci., 14 (1994) 1722–1734.
[17] Liepe, B.A., Stone, C., Koistinaho, J. and Copenhagen, D.R., Nitric oxide synthase in Müller cells and neurons of salamander and fish retina. J. Neurosci., 14 (1994) 7641–7654.
[18] Elphick, M.R., Localization of nitric oxide synthase using NADPH diaphorase histochemistry. Methods Mol. Biol., 72 (1997) 153–158.
[19] Bicker, G., NO news from insect brains. Trends Neurosci., 21 (1998) 349–355.