

Research report

# Neuronal nitric oxide synthase is expressed in the axotomized ganglion cells of the rat retina

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## Abstract

This study investigated the expression and cellular localization of neuronal nitric oxide synthase in the rat retina following optic nerve transection (ONT). In the normal rat retina, nNOS immunoreactivity was localized to amacrine cells and displaced amacrine cells. A few bipolar cells were also labeled. In the axotomized retina, ganglion cells showed nNOS immunoreactivity from 3 days after ONT, and these cells increased in number, peaking 5 days after ONT. Quantitative evaluation using immunoblotting confirmed that nNOS expression showed a peak value (255% of control levels) 5 days after ONT and decreased to 137% of controls by 28 days. These findings suggest that axotomized ganglion cells degenerate via NO-mediated excitotoxicity.

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## 1. Introduction

In the central nervous system (CNS), including the retina, axotomy results in neuronal death. In adult rats, more than 90% of retinal ganglion cells die within 2 weeks after transection of the optic nerve [1,28]. The mechanisms responsible for the loss of axotomized neurons are unclear, but loss of trophic factors from targets, excitotoxicity, and active killing by glial cells have all been suggested [26].

Nitric oxide (NO), a free radical gas with a half-life of a few seconds, has been shown to play various physiological and pathophysiological roles in the nervous system [6,7]. NO is generated by the oxidation of arginine, a reaction catalyzed by the enzyme nitric oxide synthase (NOS) [22]. This is present in three isoforms: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). Localization studies using immunocytochemistry have shown that neuronal NOS is mainly localized to amacrine cells and

displaced amacrine cells in the normal mammalian retina [3,5,16]. However, it is generally accepted that the generation of excessive NO has inconsistent effects on neuronal damage depending on the cellular source and its time of production [2,9]. Thus, NO generated from nNOS or iNOS is neurotoxic, whereas that generated from eNOS is protective [14]. Recently, Koeberle and Ball [15] reported that nitric oxide synthase inhibition delays axonal degeneration and promotes the survival of axotomized retinal ganglion cells. Thus, NO might be involved in ganglion cell death after axotomy.

The purpose of this study was to examine the expression and cellular localization of nNOS in the retina following optic nerve transection to identify the possible role of NO in the death of axotomized ganglion cells.

## 2. Materials and methods

Thirty-five adult, male, albino Sprague–Dawley rats, weighing 200–250 g, were used for this study; five

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animals were used as normal controls and 30 (five per group) for the six experimental groups. The animals were treated according to the regulations of the Catholic Ethics Committee of the Catholic University of Korea, Seoul, which conform to all National Institute of Health (NIH) guidelines.

For retrograde labeling of ganglion cells, rats were deeply anesthetized with an intraperitoneal injection of 4% chloral hydrate (1 ml/100 g body weight). Bilateral stereotaxic injections of 2.4  $\mu$ l (per injection) of Fluoro-Gold (Fluorochrome, Englewood, CO, USA) were made into the superior colliculus; two injections were made into the rostral superior colliculus and one was made into the caudal superior colliculus [24]. Fluoro-Gold is taken up by the axon terminals of retinal ganglion cells and bilaterally transported retrogradely to the cell bodies in the retina. The Fluoro-Gold in the ganglion cells persists for at least 4 weeks without significant fading or leakage [8,26]. Four days after the injection, the rats were deeply anesthetized with an intraperitoneal injection of 4% chloral hydrate, and optic nerve transection (ONT) was performed 5 mm from the posterior pole of the eye without damaging the retinal blood supply. Retinal tissues were collected at 1, 3, 5, 7, 14 and 28 days after ONT and kept for further usage.

For Western blot analysis, retinal tissues were quickly dissected on an ice-cold plate, frozen in liquid nitrogen, and stored at  $-70^{\circ}\text{C}$ . Each retina, taken at different time points, was homogenized in 10 volumes of sample buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.02% sodium azide, 1 mM PMSF, and 5  $\mu$ g/ml leupeptin). An equal amount of protein for each sample was heated at  $100^{\circ}\text{C}$  for 10 min with an equivalent volume of double strength 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 using a blocking solution containing 5% skim milk powder, 0.05% Tween-20, and phosphate-buffered saline (PBS, pH 7.4). The membrane was then incubated for 15 h at  $4^{\circ}\text{C}$  with primary antibodies against nNOS (1:7000, Sigma, St. Louis, MO, USA) 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 1:100 dilution of peroxidase-conjugated donkey anti-rabbit IgG antibody (Jackson Immuno-Research Laboratories, West Grove, PA, USA). The blot was washed for 10 min three times and then processed for analysis using an Enhanced Chemiluminescence (ECL) detection kit (Amersham, Arlington Heights, IL, USA). The nNOS protein content was calculated by measuring the peak densitometry area with an Eagle Eye™II Still Video System (Stratagene, La Jolla, CA, USA). The optical densities (mean  $\pm$  S.D.) were obtained from five determinations for each band. Statistical evaluation was

based on Student's *t*-test, with  $P < 0.05$  indicating significance.

For immunohistochemistry for nNOS, immunostaining was performed using both the indirect fluorescence and the indirect peroxidase methods. The retinas were immersion-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 2 h at  $4^{\circ}\text{C}$ . After several washes in PB, they were transferred to 30% sucrose in phosphate buffer for 24–48 h. They were then frozen in liquid nitrogen, thawed, and rinsed in 0.01 M PBS (pH 7.4). Vertical vibratome sections (40  $\mu$ m thick) were incubated in 1.4%  $\text{H}_2\text{O}_2$  in methanol for 10 min to block nonspecific binding sites. The sections were then incubated in the following solutions sequentially: 10% normal goat serum (Vector) in 0.01 M PBS (pH 7.4) for 1 h at room temperature; rabbit polyclonal antibody against nNOS (Sigma) diluted 1:7000 in PBS for 10–12 h at  $4^{\circ}\text{C}$ ; peroxidase-conjugated donkey anti-rabbit IgG (Jackson) diluted 1:100 in PBS for 2 h at room temperature; and 0.05% 3,3'-diaminobenzidine/0.01%  $\text{H}_2\text{O}_2$  in 0.05 M Tris-HCl buffer (TB, pH 7.4) for 1 min. For whole-mount immunostaining, the same immunocytochemical procedures described above were used, but for a longer incubation time. For indirect fluorescence immunostaining, whole-mount preparations were rinsed in 0.1 M PBS for 30 min, incubated in nNOS (1:7000) antibody with 0.5% Triton X-100 in 0.1 M PB for 3 days at  $4^{\circ}\text{C}$ . They were then rinsed for 30 min with 0.1 M PB, and incubated in Cy3 conjugated goat anti-rabbit IgG (1:100; Jackson Immuno Labs, West Grove, PA, USA) for 12 h at  $4^{\circ}\text{C}$ . For semi-quantitative analysis of the nNOS immunoreactive ganglion cells, the density (per  $\text{mm}^2$ ) of the labeled ganglion cells was counted in the central region near the optic disc of the whole-mount retinas. The density of the nNOS-labeled cells was also counted in the ganglion cell layer. Using an image analyzer (BMI-PLUS; Bummi Universe, Ansan, Korea), the soma size was measured from nNOS-labeled ganglion cells. Data are given as mean  $\pm$  S.E.M. Statistical significance was assessed using one-way ANOVA and comparisons between groups were performed using a Student-Newman-Keuls test.

### 3. Results

To quantitatively evaluate the postlesional changes in nNOS levels, we performed an immunoblot analysis (Fig. 1). As shown in Fig. 1A, antibodies to nNOS recognized a single band with an apparent molecular mass of 155 kD in the extracts of both control and axotomized rat retinas, but its intensity was much stronger in the axotomized rat retina than in the control retina in accordance with the immunocytochemical observations. Densitometric analysis revealed that nNOS protein levels were upregulated to a peak value of about 255% of controls by day 5. After that, the levels gradually decreased to about 137% of normal retinal levels 28 days after ONT (Fig. 1B).

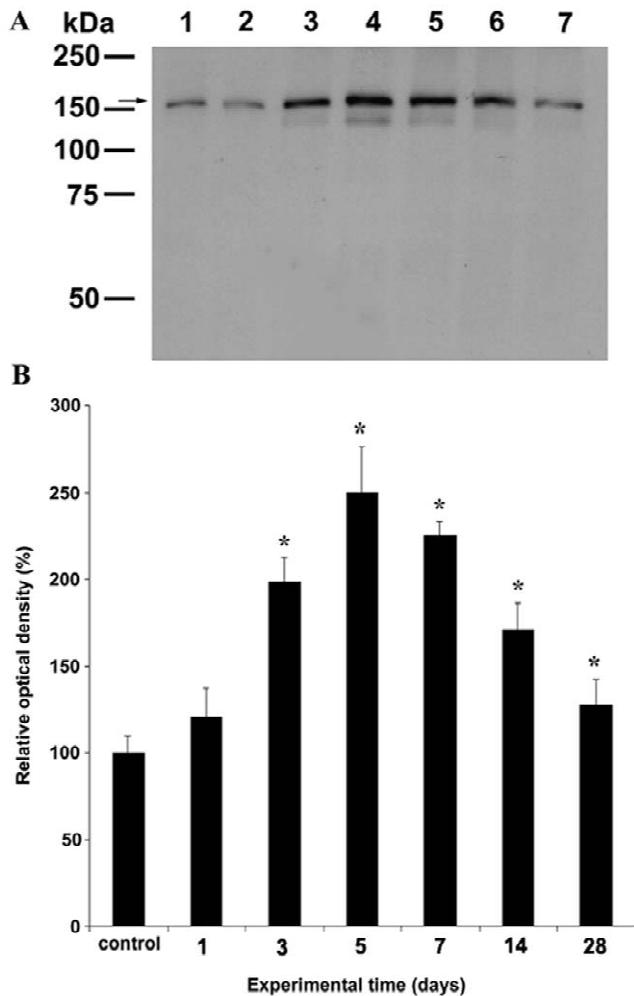


Fig. 1. Immunoblot analysis of nNOS protein levels in the normal and axotomized rat retina. (A) Immunoblot stained for nNOS demonstrating a single band at 155 kDa for all time points tested: normal rat retina (lane 1), 1 (lane 2), 3 (lane 3), 5 (lane 4), 7 (lane 5), 14 (lane 6), and 28 (lane 7) days after optic nerve transection. (B) Densitometric analysis of immunoblots as shown in (A). Data represent mean  $\pm$  S.D. \* $P < 0.05$  (Student's *t*-test).

In the retina of normal rats, NOS immunoreactivity was observed in two types of amacrine cells in the INL and in a class of displaced amacrine cells in the GCL. Some bipolar cells were also labeled (Fig. 2A). These results are in good agreement with previous reports on NOS immunoreactivity in the rat retina [5,16,21,25,29,30]. On the other hand, in the present study, no nNOS immunoreactivity was seen in large somas and axons in the GCL. These observations do not agree with the report of Neufeld et al. [20], who demonstrated the presence of nNOS immunoreactivity in the ganglion cells of the normal rat retina. One possible explanation for this difference is that we employed an antibody against a C-terminal fragment of nNOS of rat brain origin, rather than against an N-terminal fragment of nNOS of human origin. There was no significant change in the morphology of labeled cells of axotomized rat retinas (Fig. 2B), in good agreement with a previous report [23].

However, a striking change in nNOS immunoreactivity was observed in the GCL (Figs. 2B, 3 and 4). Fig. 3A shows a normal retina, in which some displaced amacrine cells are labeled. The density of these cells was  $194.8 \pm 31.5$  cells/mm<sup>2</sup> in the central retina. On day 1 after ONT, no changes of labeled cells were observed in the GCL. From 3 days following axotomy, however, NOS-positive cells were dramatically increased, and maximal numbers of labeled cells ( $1927.8 \pm 116.9$  per mm<sup>2</sup>) were observed at 5 days. Thereafter, the density was gradually decreased to  $347.5 \pm 51.5$  cells/mm<sup>2</sup> 28 days after axotomy. During experimental periods, axon bundles showed NOS immunoreactivity, in addition to labeled cells in the GCL (Fig. 3B–F). Seven days post-axotomy, most nNOS-labeled cells showed pyknotic profiles and labeled axons exhibited a beaded appearance (Fig. 3D), suggesting that labeled ganglion cells are degenerating. From 14 days after ONT, labeled ganglion cells showing features of degeneration were rarely seen (Fig. 3E). In addition, large ganglion cells showing nNOS immunoreactivity did not show any degenerative changes (Fig. 3F)

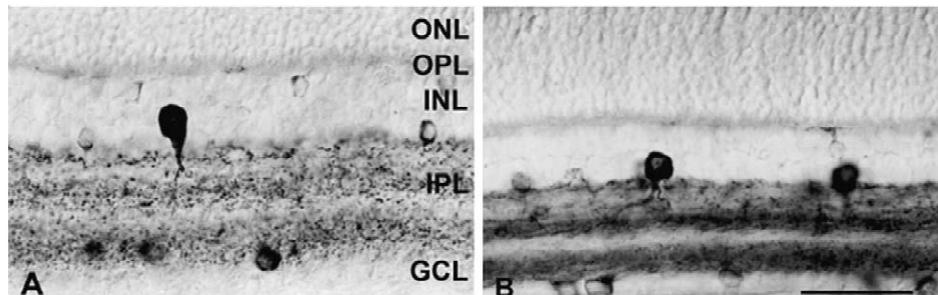


Fig. 2. Light micrographs of vertical vibratome sections taken from the normal retina (A) and the retina 3 days (B) after optic nerve transection. The sections were processed for nNOS immunoreactivity. (A) nNOS immunoreactivity is localized to two types of amacrine cells, a displaced amacrine cell and two bipolar cells. (B) Numerous nNOS immunoreactive cells and labeled processes are visible in the ganglion cell layer (GCL) and in the inner plexiform layer (IPL). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer. Bar: 50  $\mu$ m.

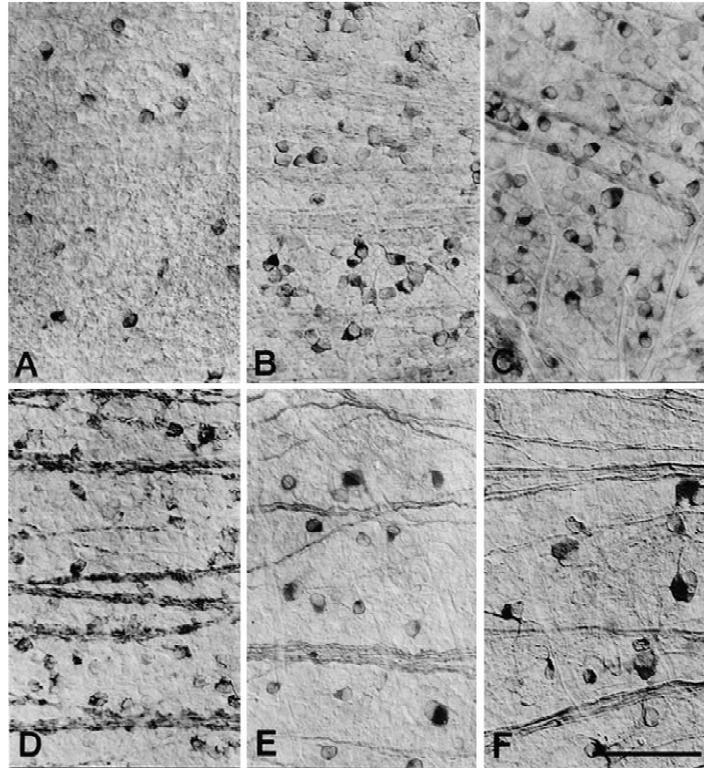


Fig. 3. Light micrographs of whole-mount preparations taken from the normal retina (A) and retinas 3 (B), 5 (C), 7 (D), 14 (E) and 28 (F) days after optic nerve transection, which were processed for nNOS immunoreactivity. The focus is at the ganglion cell layer (GCL). (A) nNOS immunoreactivity is localized to some displaced amacrine cells. (B) Numerous cells are labeled. Axons emerging from small cells are seen to form axon bundles. (C) The number of labeled cells is increased, and axon bundles formed by labeled processes are clearly visible. (D) Labeled cells show a degenerative appearance, and beaded axons are visible. (E) The number of labeled cells is decreased. Axon bundles are seen to be intact. (F) Large ganglion cells showing nNOS immunoreactivity are visible. Degenerative changes are not visible in the labeled cells. Bar: 50  $\mu\text{m}$ .

The density of the Fluoro-Gold-labeled ganglion cells was calculated in unit areas ( $1 \text{ mm}^2$ ) in the central region near the optic disc. In the normal retina, the mean density of labeled ganglion cells was  $2542 \pm 182 \text{ cells/mm}^2$ . These values correspond to the previous report of Danias et al. [4]. The density of labeled cells was altered from 3 days after ONT. At 3 and 5 days after the ONT, the density of the labeled cells was about  $2257 \pm 146$  and  $1925 \pm 162 \text{ cells/mm}^2$ , respectively. At 14 and 28 days after ONT, the density of the labeled cells fell to 8.5% ( $215 \pm 21 \text{ cells/mm}^2$ ) and 6.4% ( $162 \pm 27 \text{ cells/mm}^2$ ) of control values, respectively. These results are in agreement with a previous report by Berkelaar et al. [1], who demonstrated that most ganglion cells die 14 days after ONT. In the normal retina and the retina 1 day after ONT, Fluoro-Gold-labeled ganglion cells did not show nNOS immunoreactivity (Fig. 4A–F). However, from 3 days following ONT, Fluoro-Gold-labeled ganglion cells showed nNOS immunoreactivity. Sixty-six percent ( $1487 \pm 186 \text{ cells/mm}^2$ ) of Fluoro-Gold-labeled ganglion cells exhibited nNOS immunoreactivity (Fig. 4G–L) 3 days after ONT (Fig. 4G–I) and about 80% 2 and 4 weeks ( $173 \pm 28$  and  $127 \pm 13 \text{ cells/mm}^2$ ) after ONT (Fig. 4J–L). Thus, most labeled cells in the GCL were most likely ganglion cells.

In the present study, the size of nNOS-labeled ganglion cells was analyzed to identify whether there are differences in their response to the axotomy insult according to cell size (Fig. 5). Soma sizes were measured from nNOS-labeled ganglion cells located in a unit area ( $1 \text{ mm}^2$ ) of the central region by an Image Analyzer. Three days after ONT, ganglion cells with diameters ranging from 7 to 13  $\mu\text{m}$  (mean diameter  $10.5 \pm 2.2 \mu\text{m}$ ) showed nNOS immunoreactivity. Seven days after ONT, the mean diameter of the labeled cells was  $11.0 \pm 2.2 \mu\text{m}$  (ranging from 8 to 14  $\mu\text{m}$ ). Fourteen and 28 days post-axotomy, the labeled ganglion cells had diameters  $>9 \mu\text{m}$ . These results demonstrate that relatively small ganglion cells express nNOS immunoreactivity at an early stage (up to 7 days after ONT), whereas large ganglion cells express nNOS immunoreactivity at a later stage, and this suggests that large ganglion cells may be resistant to axotomy insults.

#### 4. Discussion

The present study demonstrates that optic nerve transection induces widespread expression of nNOS immunoreactivity in ganglion cells which normally do not express

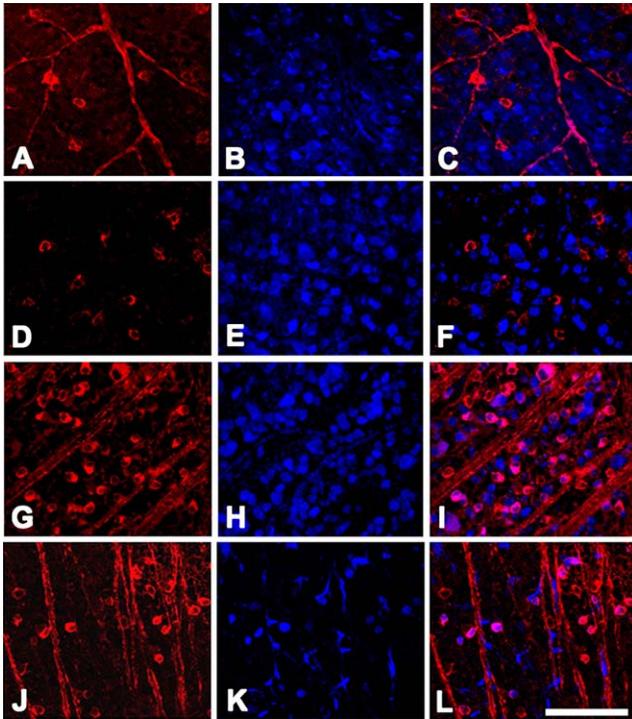


Fig. 4. Confocal micrographs taken from whole-mount preparations of normal retina (A, B, C) and retinas 1 day (D, E, F), 5 days (G, H, I) and 2 weeks (J, K, L) after optic nerve transection (ONT). Neuronal nitric oxide synthase (nNOS) immunoreactivity was visualized using a Cy3-conjugated secondary antibody (red) in (A), (D), (G) and (J). Retrogradely Fluoro-Gold labeled ganglion cells were visualized using a UV filter (blue) in (B), (E), (H) and (K). No ganglion cell bodies showing nNOS immunoreactivity are seen in the normal retina (C) and the retina 1 day (F) after ONT, whereas several cell bodies showing nNOS immunoreactivity are visible 5 days (I) and 2 weeks (L) after ONT. Bar: 50  $\mu\text{m}$ .

this enzyme activity in the rat retina, indicating that NOS expression may be related to degenerative events in axotomized ganglion cells in the rat retina. Previously, induction of NOS immunoreactivity in neurons has been reported in cell bodies in brain stem nuclei several weeks after axotomy [19,31,32]. However, Huxlin and Bennett [13] have reported that section of the optic nerve does not alter the levels of nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d), identical to nNOS [5],

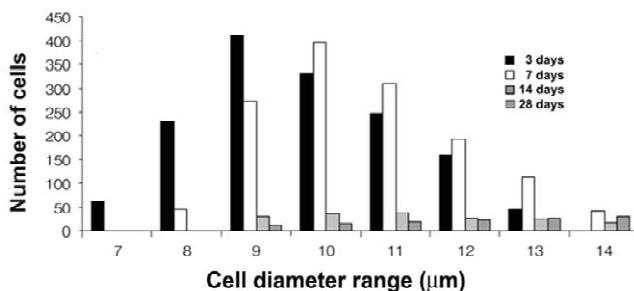


Fig. 5. Size distribution of nNOS immunoreactive ganglion cells located in a unit area (1  $\text{mm}^2$ ) of the central region of retinas 3, 7, 14 and 28 days after optic nerve transection.

in ganglion cells. These conflicting results might be due to the fact that the NADPH-d staining pattern is variable according to the fixation conditions such as the time of fixation, methods applied (direct or indirect) and the nature of the fixatives [12]. In addition, Patel et al. [23] investigated the effects of an optic tract section on nNOS expression in ganglion cells of the developing rat retina. Contrary to our results, there was no upregulation of nNOS in the axotomized ganglion cells. One possible explanation for this discrepancy is that they performed optic tract section on postnatal day 7, when synaptogenesis and nNOS immunoreactive neurons are not fully developed. It has been reported that synaptogenesis of the IPL is fully established during the second postnatal week [11] and nNOS immunoreactive neurons reach maximum differentiation on postnatal day 14 or 15, the time of eye opening in the developing rat [17]. Thus, upregulation of nNOS may vary with the time of section of the optic nerve or tract, the maturity of the lesioned neurons, and the nature of the lesion.

The precise mechanism responsible for the upregulation of nNOS in axotomized neurons is still not clear. However, there is growing evidence that excitotoxicity is involved in axotomized neuronal death. Excitation of neurons by the binding of glutamate to the NMDA receptor increases the influx of  $\text{Ca}^{2+}$ , which then binds to calmodulin [10] and increases NOS activity [5]. Thus, upregulation of nNOS may be attributed to excessive influx of  $\text{Ca}^{2+}$  into axotomized ganglion cells via the activation of glutamate receptors located in the axotomized ganglion cells. The excitotoxicity hypothesis suggests that neurons deprived of a functional connection to their targets become more sensitive to toxic agents in their environment [13]. In the cat retina the beta ganglion cells are the first of all ganglion cells to degenerate because they receive high glutamatergic input from bipolar cells [27]. Taken together, it can be inferred that glutamate might act as a neurotoxic agent to axotomized ganglion cells, even if the level of glutamate is normal in the retina after axotomy.

In the present study, most ganglion cells showing nNOS activity degenerated within 2 weeks. These results clearly indicate that excessive NO liberated from the up-regulated nNOS might be one of the mechanisms responsible for the degeneration of axotomized ganglion cells. This explanation is supported by the report of Bolaños et al. [2], who showed that brief exposure of neuronal cultures to glutamate receptor agonists only transiently elevates intracellular calcium, which returns to basal levels within seconds. This short-term calcium elevation presumably initiates irreversible processes that lead to neuronal cell death via peroxynitrate, which is formed by the reaction of NO and  $\text{O}_2^-$ , and initiates unspecific protein and lipid peroxidation, DNA damage, and mitochondrial impairment. Thus, NO may be involved in axotomized ganglion cell death. Actually, conflicting evidence has accumulated regarding the neurotoxicity of neuronally derived NO. Lipton et al.

[18] suggest that the cell redox status may determine whether NO formation, following activation of NMDA receptor activation, is neurotoxic or even neuroprotective. An intracellular oxidizing environment favors the formation of  $\text{NO}^+$ , which may down-regulate the NMDA receptor by S-nitrosylation (neuroprotection). In contrast, a reducing intracellular environment favors the reduction of  $\text{NO}^+$  to NO, which does not react with the thiol groups of the redox modulatory site of the NMDA receptor (neurodegeneration). The mechanism responsible for the resistance of large ganglion cells to axotomy insults may reflect intracellular responses to NO that are different from those of vulnerable ganglion cells. However, more detailed study is clearly needed to elucidate the exact mechanism.

In conclusion, optic nerve transection leads to the loss of most ganglion cells in the ganglion cell layer. Most axotomized ganglion cells express neuronal nitric oxide synthase immunoreactivity, and they degenerate within 14 days after optic nerve transection. We suggest that the excessive NO generated from axotomized ganglion cells might be involved in ganglion cell death in the rat retina after optic nerve transection. However, the vulnerability of ganglion cells to axotomy insults may depend on their cellular response to NO.

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