Nitric oxide synthase immunoreactivity in the developing and adult human retina

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Nitric oxide synthase (NOS) catalyzes the formation of nitric oxide (NO) from L-arginine. In this study, the cellular localization of neuronal NOS (nNOS) activity in the human retina since fetal development was examined by immunohistochemistry. No detectable staining in the fetal retina was present at 14 weeks of gestation (wg), the earliest age group examined. A centro-peripheral gradient of development of nNOS immunoreactivity was evident at 16–17 wg, with the midperipheral retina showing nNOS immunoreactivity in most of the cell types and the inner plexiform layer while the peripheral part demonstrated moderate immunoreactivity only in the ganglion cell layer and photoreceptor precursors. A transient increase in nNOS immunoreactivity in the ganglion cells and Müller cell endfeet between 18–19 and 24–25 wg was observed at the time when programmed cell death in the ganglion cell layer, loss of optic nerve fibres as well as increase in glutamate immunoreactivity and parvalbumin (a calcium binding protein) immunoreactivity in the ganglion cells was reported. These observations indicate that programmed cell death of ganglion cells in the retina may be linked to glutamate toxicity and NO activity, as also suggested by others in the retina and cerebral cortex.

The presence of nNOS immunoreactivity in the photoreceptors from 16–17 weeks of fetal life to adulthood indicates other functions, besides their involvement in photoreceptor function of transduction and information processing.

1. Introduction

The intracellular messenger nitric oxide (NO) has been shown to modulate a number of cellular functions in both neuronal and non-neuronal cells as well as to act as a mediator of cell damage in ischaemic brain injury and a variety of neurological diseases (see reviews in Moncada 1992; Snyder 1992; Paakkari and Lindsberg 1995). Recent physiological studies have indicated the involvement of NO in impulse transduction in the outer retina and in the modulation of visual signal during retinal information processing. In the retinas of lower vertebrates, NO donors or inhibitors of neuronal nitric oxide synthase (nNOS) have been shown to modify light-induced membrane hyperpolarization in photoreceptors (Schmidt *et al* 1992; Kurreny *et al* 1994) and electrically-coupled horizontal cells (De Vries and Schwartz 1989; Miyachi *et al* 1991) as well as to activate cGMP-gated conductances in on-bipolar cells (Scheills and Falk 1992). Recently, NO donors have been shown to modulate cGMP-gated conductances in ganglion cells isolated from rat retina (Ahmad *et al* 1994) as well.

NO is formed by the enzyme nitric oxide synthase (NOS) which catalytically transforms L-arginine into NO and citrulline (Palmer *et al* 1988). Immunohistochemical localization of NOS and histochemical demonstration of NADPH diaphorase (a co-factor of NOS) activity, have been used extensively as indicators of NO producing cells on the assumption that NO being a short-lived gaseous molecule, its biosynthetic enzyme and co-factor must be located either in the target cells or in cells adjacent to the target cells (Vincent 1994).

In the adult retina, NOS has been shown by labelling with antibody to its neuronal isoform – nNOS, to be

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Abbreviations used: NO, Nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; wg, weeks of gestation.

localized in a small subset of amacrine cells and cells within the ganglion cell layer (Dawson *et al* 1991; Yamamoto *et al* 1993), rod photoreceptors (Venturini *et al* 1991), photoreceptor ellipsoids, the distal region of bipolar and glial cells apposing photoreceptor inner segments as well as in the inner and outer plexiform layers (Liepe *et al* 1994). A recent study by electron microscope immunocytochemistry has shown nNOS to be present in some bipolar and horizontal cell processes at the photoreceptor ribbon synapses in turtle and rat retina (Haverkamp and Eldred 1998). The development of the nNOS activity in retina has been studied in cat, rat and rabbit (Vaccaro *et al* 1991; Haberecht *et al* 1994; Perez *et al* 1995) with diverse observations. The present study has examined the developmental appearance and changes in the expression of NOS enzyme by immunohistochemical localization of constitutive nNOS in the retinas of human fetuses ranging in gestational ages from 14 to 25 weeks, a five-month-old infant and an adult subject (35 year-old).

2. Materials and methods

2.1 *Tissue samples and fixation*

Retinas were obtained from human fetuses of 14 weeks of gestation (wg, 2), 16–17 wg (3), 18–19 wg (1), 20–21 wg (3) and 24–25 wg (3) as well as from a five-month-old infant and a 35-year-old male subject. The fetuses were procured from cases of hysterotomy and legal spontaneous abortions, whereas the postnatal and adult samples were obtained from autopsies. The required clearance of the Institute's ethical committee and consent of the concerned parents were taken for collection of the fetuses and their use (eyeballs) in research. The fetal ages were determined on the basis of the crown rump length (Hamilton *et al* 1972), the biparietal diameter and foot length. The retinas were immersion fixed in 4% paraformaldehyde or 2·5% glutaraldehyde for 3–4 days at 4°C. After washing in 0·1 M phosphate buffer saline (PBS), the tissues were cryoprotected in 15–30% sucrose overnight, embedded in tissue freezing medium (Leica) and cryosectioned at – 20°C at 20–25 μm thickness.

2.2 *Immunohistochemistry*

The glutaraldehyde-fixed tissue sections were reduced with sodium borohydride (0·1%) for 10 min. The free-floating sections were incubated in 0·3% hydrogen peroxide (H_2O_2) in 90% methanol for 30 min to inactivate the endogenous peroxidase activity. After washing in 0·01 M PBS, sections were blocked for 4 h in 10% goat normal serum and then incubated in the primary antiserum for nNOS (1 : 500 dilution, rabbit polyclonal, Chemicon, Temecula, USA). The secondary antibody used was biotinylated goat anti-rabbit IgG (1 : 200 dilution, Vector Laboratories, Burlingame, CA, USA) for 6 h at 4°C. The antigen-antibody complex was localized employing an avidin-biotin-peroxidase system (ABC kit, Vector Laboratories, USA) according to the manufacturers instructions. Peroxidase staining was developed using 3,3′-diaminobenzidine tetrahydrochloride (0·06%) as chromogen, in 0·1 M acetate-imidazole buffer (pH 7·4) together with H_2O_2 (0·06%) and nickel sulphate (0·5%). To demonstrate the specificity of the antibody, negative control sections were incubated and processed similarly but with the omission of the primary antiserum. The retinas from two adult rhesus monkeys (*Macaca mulatta*), being available fresh from surgery, were also included in this study to better understand the cellular distribution of nNOS immunoreactivity in the primate retina as well serve as positive controls.

3. Results

The human retina follows a centro-peripheral gradient of development from close to the optic disc and shows differentiation of its various cell types in a vitreal to scleral direction. Hence the midperipheral and peripheral retinas from different gestational age periods, the five month-old infant and the adult subject (5–7 mm away from the fovea) were compared. Paraformaldehyde fixed-sections at 14 wg, the earliest age group examined in the present study, revealed negligible staining in the fetal retina (figure 1). At 16–17 wg, the midperipheral retina showed a number of nNOS-immunopositive cells in the ganglion cell layer, presumably ganglion cells and

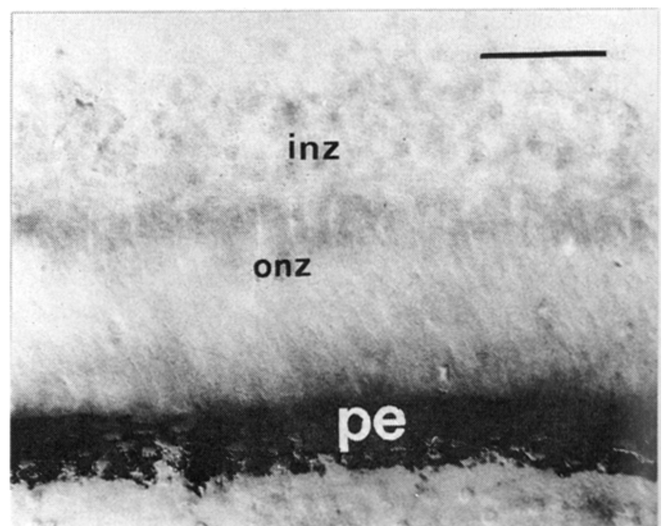


Figure 1. Fetal human retina at 14 weeks of gestation, showing negligible staining for NOS. inz, onz, inner and outer neuroblastic zones; pe, pigment epithelial layer. (Bar, 50 μm.)

displaced amacrine cells. The vitreal endfeet of the Müller cells were clearly immunostained. Diffusely distributed reaction product was also seen in the processes of the Müller cells towards the outer limiting membrane as well as in their cell bodies lying near the incipient outer plexiform layer. The inner plexiform layer, and some of the amacrine cells located close to it were immunopositive. In addition, the photoreceptor precursor cells present in the outer neuroblastic zone near the ventricular surface showed faint immunostaining (figure 2A). In the peripheral part of retina, moderate immunoreactivity was observed in the majority of the neurons lying in the ganglion cell layer and photoreceptor precursor cells (figure 2B). At 18–19 wg, in the peripheral retina, while the intensity of immunoreactivity was enhanced in the ganglion cell layer and photoreceptor precursor cells, faint nNOS immunopositivity was detected in the cells of the innermost row of the outer neuroblastic zone – the prospective amacrine cells (figure 3A). The five-month-old infant retina showed nNOS immunoreactivity in the ganglion cell layer and in some scattered cells (presumably amacrine) of the inner nuclear layer. In the photoreceptor layer, the immunoreactivity was present in

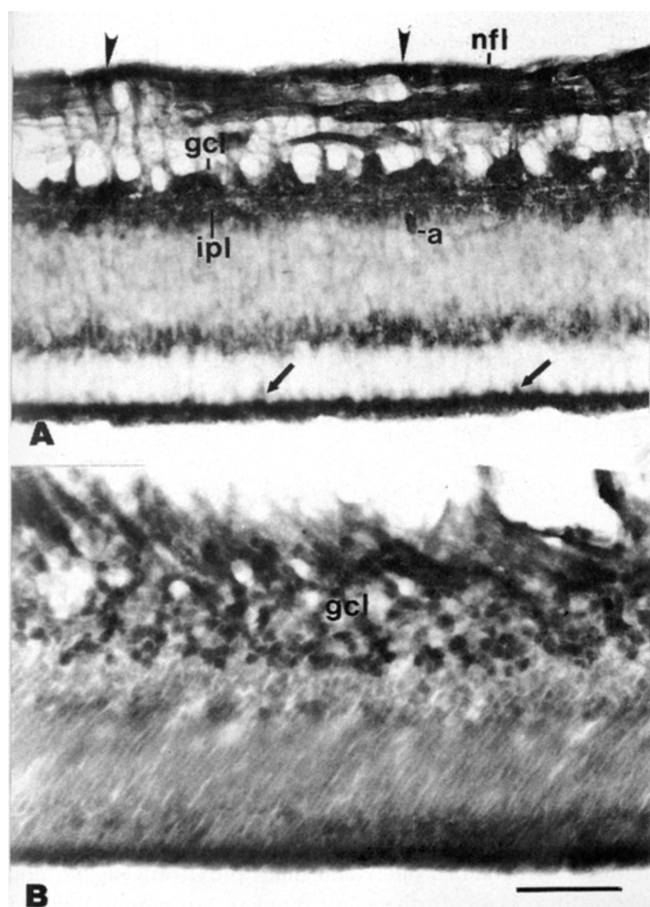


Figure 2. NOS immunoreactivity in fetal human retina at 16–17 weeks of gestation. Paraformaldehyde fixed. (**A**) From the midperipheral part of retina, showing intense staining in nerve fibre layer (nfl), ganglion cell layer (gcl) and inner plexiform layer (ipl). The arrowheads indicate the endfeet of Müller cells. Some amacrine cells (a) are clearly stained. Note that the photoreceptors (arrows) are also immunoreactive. (**B**) From the peripheral part, showing moderate immunoreactivity in the thick ganglion cell layer (gcl). (Bar, 50 μm.)

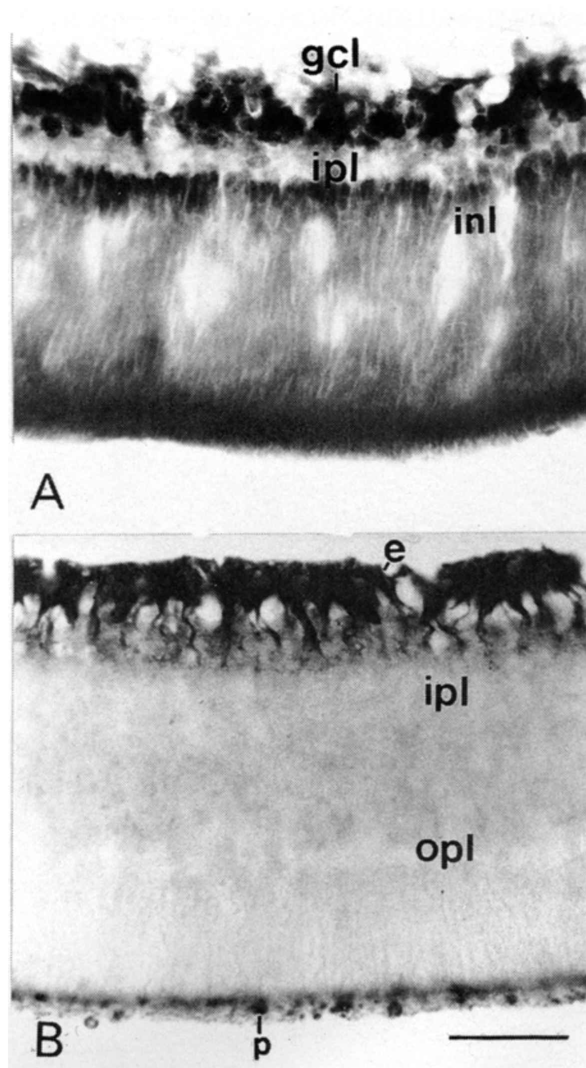


Figure 3. NOS immunoreactivity in 18–19 week-old fetal human retina. (**A**) From the peripheral part of retina, fixed in paraformaldehyde, showing an increase in immunoreactivity in the ganglion cell layer (gcl). Note that the vitrealmost row of the inner nuclear layer (inl) shows moderate staining, while no immunoreactivity is yet developed in the inner plexiform layer (ipl). (**B**) From 20–21 week-old fetal retina, fixed in glutaraldehyde. The endfeet of Müller cells (e) are intensely labelled. ipl, opl, inner and outer plexiform layers, p, photoreceptor precursors. (Bar, 50 μm.)

cell bodies and outer segments (figure 4A). In the adult human retina, the immunoreactivity was present strongly in the nerve fiber layer, and moderately in some amacrine cells and cone ellipsoids. Occasional cells in the ganglion cell layer were stained. In addition, both the inner and outer plexiform layers also showed faint nNOS immunoreactivity (figure 4B). In the monkey retinas fixed in paraformaldehyde, most cells of the ganglion cell layer, cone ellipsoids, inner fibres and the outer segments were strongly labelled (figure 5A, B), perhaps due to the better preservation of the antigen in the fresh retinas.

It is interesting to note that the paraformaldehyde preparations showed nNOS immunoreactivity in both the neuronal and glial elements; on the contrary, the glutaraldehyde-fixed retinal preparations from fetuses of 20–21 (figure 3B) and 24–25 wg (not shown) as well as from the adult human (not shown) and monkeys (figure 5C) showed prominent immunoreactivity mainly in the Müller glial processes and their end feet. Although some photoreceptor precursors were also stained in the early gestational age periods (see e.g., figure 3B), in the adult retinas, nNOS immunoreactivity was absent in the mature photoreceptors (figure 5C). The cell types labelled with nNOS at different ages and using different fixatives are comprehensively tabulated in table 1.

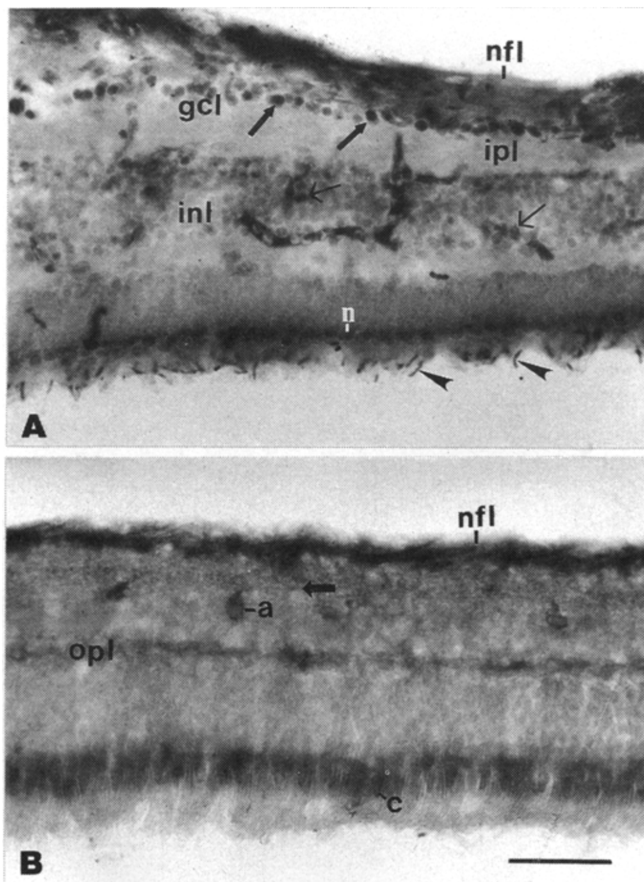


Figure 4. NOS immunoreactivity in human retina. Paraformaldehyde fixed. (**A**) From the five month-old infant retina closer to the optic disc. The immunoreactivity is present in the nerve fibre layer (nfl), in some cells (arrows) of the ganglion cell layer (gcl) and inner nuclear layer (inl). Note that the cone nuclei (n) and outer segments (arrowheads) are stained. (**B**) From the 35 year-old adult human retina, showing strong immunoreactivity in the nerve fibre layer (nfl). The inner plexiform layer (arrow), amacrine cells (a), outer plexiform layer (opl) and the cones (c) are moderately stained.

4. Discussion

4.1 *Fixatives and nNOS*

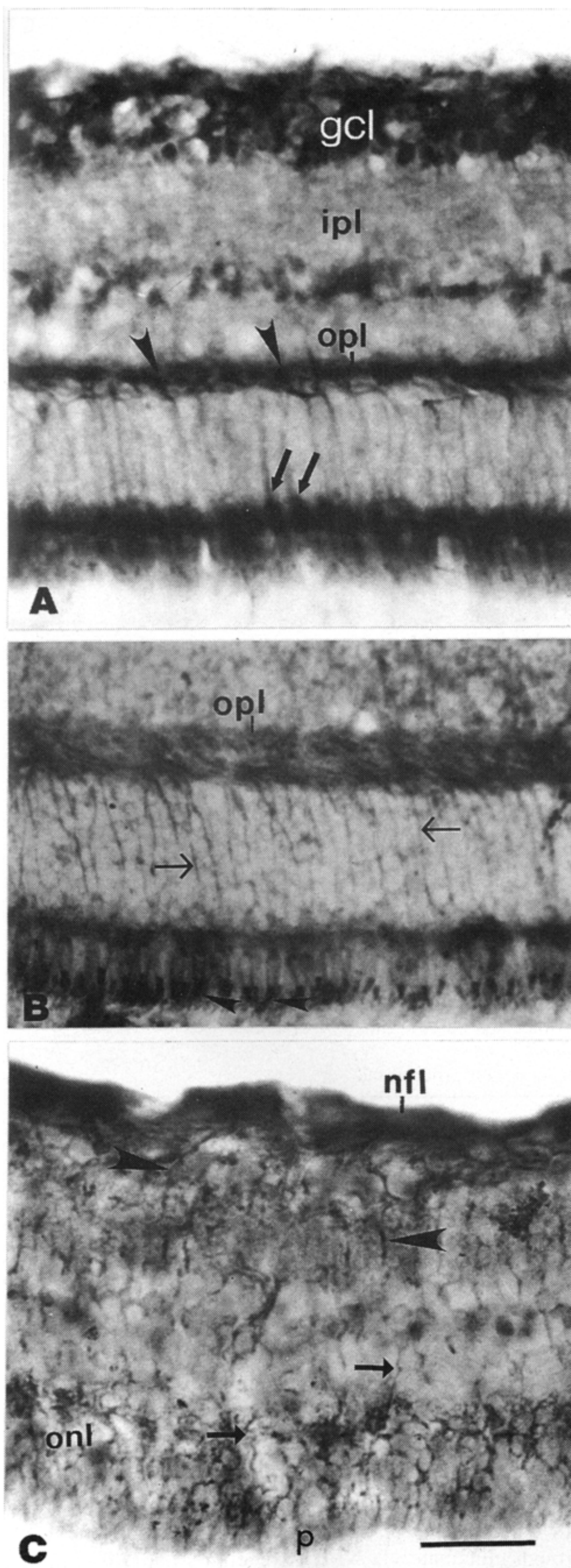
The present study has demonstrated that in retinas fixed in paraformaldehyde, nNOS immunoreactivity was revealed in both neurons and glia, while glutaraldehyde fixation preferentially allowed prominent staining of Müller glial cells though some neuronal elements (i.e., photoreceptor precursors) also were variably nNOS-immunoreactive. It is interesting to note that such an observation was also made by Huxlin (1995) when labelling adult rat retinal samples for NADPH-diaphorase activity with different fixatives and time protocols, suggesting thereby that the nature of fixatives, fixation conditions and exposure time are indeed critical factors in the cellular localization of NOS and its cofactor, NADPH-diaphorase. We have not stained sections for NADPH-diaphorase, but localization of NOS immunoreactivity is considered equivalent to the demonstration of localization of NADPH-diaphorase (Valtschanoff *et al* 1993).

4.2 *Adult retina*

The adult retina of different vertebrates consistently shows nNOS immunoreactivity and NADPH-diaphorase in the amacrine cells (Vaccaro *et al* 1991; Liepe *et al* 1994; Perez *et al* 1995). However, the localization of nNOS/NADPH-diaphorase in other cell types as well as in the outer and inner plexiform layers has not been consistently reported in different vertebrates and in the same animal species by different authors. In the present study, the adult human retina revealed staining in Müller glia, ganglion and amacrine cells, cone inner (ellipsoids) and outer segments, outer and inner plexiform layers, and nerve fibre layer. In the adult monkey, all components of the cone photoreceptors were more prominently stained in both the para- and peri-foveal region (figure 5A, B).

4.3 *Developing retina*

Studies on the developing rat retina have shown that although the mature expression of nNOS and its cofactor does not occur until the end of second postnatal week, the



developmental expression of the cellular label of NADPH-diaphorase or nNOS immunoreactivity begins at postnatal age three (Mitrofanis 1989) to seven days (Perez *et al* 1995). In rabbit and cat retinas, NADPH-diaphorase reactive cells, on the other hand, have been detected prenatally (Vaccaro *et al* 1991; Mitrofanis *et al* 1992). The activity of this second messenger system has also been shown to be present early in the chick retina (Ientile *et al* 1996) and in dissociated murine cortical cultures (Southam and Garthwaite 1991). The present study too, showed prenatal appearance of nNOS immunoreactivity in the human retina. At 16–17 wg, the nNOS antigen is exhibited by the ganglion and displaced amacrine cells as well as Müller glial endfeet in the inner retina and by the photoreceptor precursor cells at the ventricular surface.

4.4 *Role for NO in developing and adult retina*

A transient increase in nNOS-immunoreactivity in the ganglion cells and Müller cell endfeet of fetal human retinas between 18–19 and 24–25 wg has been observed in the present study at the time when naturally occurring programmed cell death is reported to occur in the developing human ganglion cell layer (Provis *et al* 1985a). The loss of ganglion cells in fetal human retina is substantiated by the concurrent excessive reduction in the number of optic nerve fibres (axons of ganglion cells) between 16–17 and 19–20 weeks of gestation followed by gradual loss up to 26 weeks of prenatal life (Provis *et al* 1985b; Wadhwa and Bijlani 1987). At 16–17 wg, glutamate immunoreactivity is also observed to increase in the retinal ganglion cell layer (Wadhwa *et al* 1994; Jotwani *et al* 1998) as well as a spurt is noted in parvalbumin (protein which binds glutamate released Ca^{+}) immunoreactivity in fetal human retina (Nag and Wadhwa 1996). Collectively, these observations indicate that programmed cell death of ganglion cells which occurs in the retina may be linked to excessive glutamate and NO activity. Involvement of NO in cell death has been suggested in rat retina (Huxlin and Bennett 1995; Nichol *et al* 1995) and cerebral cortex (Bredt and Snyder 1994).

Figure. 5. Paraformaldehyde (**A, B**) and glutaraldehyde-fixed (**C**) adult monkey retina, approximately 8–9 mm (**A,C**) and 4–5 mm (**B**) away from the fovea. Intense immunoreactivity is present in the photoreceptor pedicles (arrowheads) and nuclei (arrows) in (**A**), whereas the outer segments (small arrowheads) and inner fibres (arrows) are clearly labelled in (**B**). In both regions, the outer plexiform layer (opl) and the ganglion cell layer (gcl, shown in figure **A**) are strongly NOS-immunopositive. In (**C**), the staining is present in Müller cell vitreal (arrowheads) as well as scleral processes (arrows). Note that photoreceptors (p) are not stained. The staining in the nerve fibre layer (nfl) is due to the Müller cell end feet. onl, outer nuclear layer (Bar, 50 µm.)

Table 1. Age, fixation, number of samples used and cell types labelled.

Age	No. of eyeballs	Fixation		Labelled cell types and layers
14 wg	4	Paraformaldehyde	(2)	None
16–17 wg	6	Paraformaldehyde	(2)	*Midperipheral retina*: Nerve fibre layer, ganglion and displaced amacrine cells, some amacrines, inner plexiform layer, some photoreceptor precursors, Müller cells and their end feet *Peripheral retina:* Nerve fibre layer, ganglion and displaced amacrine cells and some photoreceptor precursors
		Gluteraldehyde	(2)	Müller cells and few photoreceptors
18–19 wg	2	Paraformaldehyde	(2)	*Peripheral retina:* Nerve fibre layer, ganglion and displaced amacrine cells, some amacrines and photoreceptor cell bodies
20–21 wg	6	Paraformaldehyde	(2)	Same as at 18–19 wg
		Gluteraldehyde	(2)	Müller cell processes and some photoreceptors
24–25 wg	6	Paraformaldehyde	(2)	Same as at 18–19 wg
		Gluteraldehyde	(1)	Müller cell processes
5 month old infant	2	Paraformaldehyde	(2)	*Midperipheral retina:* Nerve fibre layer, ganglion and displaced amacrine cells, some amacrines as well as photoreceptor cell bodies and outer segments
35 year old adult human	2	Paraformaldehyde	(1)	*Midperipheral retina:* Nerve fibre layer, some ganglion and displaced amacrine cells, some amacrines, faint inner plexiform and moderately stained outer plexiform layer as well as prominent cone photoreceptor outer segments
		Gluteraldehyde	(1)	Müller cells and their processes
Adult monkey	4	Paraformaldehyde	(2)	*Midperipheral retina*: Nerve fibre layer, most cells of ganglion cell layer, some amacrines, prominently stained outer and inner plexiform layers, cone ellipsoids with inner fibres and outer segments
		Gluteraldehyde	(2)	Müller cells and their processes

In a recent study, Haberecht *et al* (1997) have examined the development of retinal susceptibility to glutamate toxicity as well as the protective effects of two N-methyl-D-aspartate (NMDA) antagonists and NOS inhibitor, N^G-methyl-L-arginine (met ARG) using one day *in vitro* retinal explants of adult and neonatal (postnatal day 1) rabbits. Their observations suggest that NMDA/NO excitotoxic pathway may not be active at birth since the NOS inhibitor had little effect at this stage, thereby indicating that NO may play a role other than that of a toxic agent in developing retina. In their earlier study, Haberecht *et al* (1994) have shown that NOS containing cells are present in their mature form only around second postnatal week. Thus it appears that since this study was conducted in explants from postnatal day 1, the developmental neurotoxicity of glutamate/NO which peaks at the end of first postnatal week in rabbit may not have been addressed optimally by the authors. It is interesting to note that during the period of 16 to 26 wg in the human fetus, the next higher visual relay centre, i.e., lateral geniculate nucleus shows segregation of its cells and retinal terminals into the adult like laminar pattern (Damayanti *et al* 1983) which is dependent on correct matching and refinement of connectivity of optic nerve fibres coming from the retinal ganglion cells. There is evidence to show that the formation of activity-dependent synaptic connections and refinement of projections from retina to lateral geniculate nucleus in the ferret (Cramer *et al* 1996) as well as in developing and regenerating olfactory neurons (Roskams *et al* 1994) also occurs through glutamate via NMDA receptors and NO. A dose dependent role for NO similar to that shown for glutamate

(Lipton and Kater 1989) resulting in dendritic growth and synapse development at low concentrations and its neurotoxic effects at high titres needs further evaluation.

Continued presence of NOS-immunoreactivity in the photoreceptors from 16–17 weeks of fetal life to adulthood indicates other functions besides their definitive involvement in the photoreceptor function of transduction and information processing.

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