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Localisation of neuronal nitric oxide synthase-immunoreactivity in rat and rabbit retinas

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Abstract The distribution of neuronal nitric oxide synthase (NOS) immunoreactivity was examined in rat and rabbit retinas and was compared with the distribution of nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase reactivity and vasoactive intestinal peptide (VIP) immunoreactivity. An antibody raised against a C-terminal fragment of a cloned rat cerebellar NOS was used to localise NOS immunoreactivity. NOS immunoreactive cells were not detected in rat retinas at postnatal day 1 or 4, but were seen from postnatal day 7 onwards. NOS immunolabelling was seen in a small population of cells in the proximal inner nuclear layer. Most of the labelled cells had the position of amacrine cells and were seen to send processes into the inner plexiform layer. A few labelled cells were at times also seen in the ganglion cell layer, which are likely to correspond to displaced amacrine cells. The same NOS-labelling pattern was seen in rat and rabbit retinas.

NADPH-diaphorase staining was observed in both species, in photoreceptor inner segments, in cells with the position of horizontal cells, in a subset of amacrine and displaced amacrine cells, in large cell bodies in the ganglion cell layer, in both plexiform layers, and in endothelium. Colocalisation of NOS immunoreactivity and NADPH-diaphorase staining was only observed among amacrine cells. However, not all NADPH-diaphorase-reactive amacrine cells were found to be NOS immunoreactive. VIP immunoreactivity was also localised in rat retinas in a subpopulation of amacrine cells, but no colocalisation of NOS and VIP immunoreactivity was observed.

Our observations indicate that only amacrine cells contain the NOS form recognisable by the antibody used, and suggest that different isoforms of neuronal NOS may be present in retinal cells. Further, the onset of NOS expression in rat amacrine cells appears to occur independently of neuronal activity.

Key words Nitric oxide synthase · Retina · Immunohistochemistry · Rat · Rabbit

Paper in honour of Professor Rolf Elofsson on the occasion of his retirement from the chair of Zoology at the University of Lund

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Introduction

Nitric oxide (NO) is formed from L-arginine by nitric oxide synthase (NOS). This reaction requires, among other cofactors, the electron donor NADPH (nicotinamide adenine dinucleotide phosphate). Different isoforms of NOS have been characterised, of which constitutive forms are found in nerve cells and vascular endothelium and inducible form(s), for example, in activated macrophages and leukocytes (Förstermann et al. 1991; Moncada 1992; Schuman and Madison 1994). The generated NO is found to then act through stimulation of guanylate cyclase, thereby elevating cGMP levels (Knowles et al. 1989; Moncada et al. 1991).

Messenger RNA for a soluble guanylate cyclase has been found in the rat retina in all cellular layers but in particularly high levels in a subpopulation of ganglion cells (Ahmad and Barnstable 1993). The guanylate cyclase in these cells was recently shown to be sensitive to the NO-donor agent sodium nitroprusside (Ahmad et al. 1994). In addition, this compound was found to increase inward currents in ON-bipolar cells in a dogfish retinal slice preparation (Shiells and Falk 1992), to affect dark voltage and light responses of isolated frog retinal rods (Schmidt et al. 1992), and to selectively block NMDA-induced currents in cultured rat retinal neurones (Ujihara et al. 1993). These observations clearly indicate the pres-

ence of NO-sensitive systems among retinal cells. Furthermore, NOS activity has been detected biochemically in crude preparations of bovine rod outer segments (Venturini et al. 1991), and in homogenates of whole rabbit retina (Osborne et al. 1993).

NOS has been identified as an enzyme capable of producing a NADPH-diaphorase (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; Vincent and Kimura 1992). This assumption has been corroborated by the fact that NADPH-d colocalises with immunohistochemically labelled NOS in various neuronal cell systems (Dawson et al. 1991).

The occurrence of NADPH-d in subpopulations of amacrine cells has been described for all species studies, whereas for other retinal cell types a large variation has been noted among species (Sandell 1985; Sagar 1986; Wässle et al. 1987; Vaney and Young 1988; Straznicky and Gábriel 1991; Mitrofanis et al. 1992; Osborne et al. 1993). However, the different isoforms of NOS as well as other enzymes can exhibit NADPH-d activity. NADPH-d reactivity is thus not associated only with the presence of neuronal NOS.

The presence of NOS has also been demonstrated in retinal cells using immunohistochemistry (Dawson et al. 1991; Yamamoto et al. 1993a, b). However, in these studies, an antibody raised against the whole neuronal NOS molecule was used, which might thus not distinguish between various forms of the enzyme.

The aim of the present study was therefore to examine the distribution of retinal NOS using an antibody specifically directed to neuronal NOS. For this purpose, we employed an antibody raised against a C-terminal fragment of the rat cerebellar NOS cloned by Bredt et al. (1991). The nucleotide sequence in this region appears to be exclusive for the neuronal NOS isoform (Alm et al. 1993). The distribution of NOS was examined in rat and rabbit retinas and compared to that of NADPH-d reactivity.

Further, in order to determine the ontogenic onset in retinal NOS expression, we examined the presence and distribution of NOS immunoreactivity in developing rat retinas. In addition, since NOS and vasoactive intestinal polypeptide (VIP) are frequently found to colocalise in peripheral ganglia (Yamamoto et al. 1993b; P. Alm, B. Larsson, K.-E. Andersson, personal communications), we compared the distribution of the two in the rat retina.

Material and methods

Animals

Light-adapted male Sprague-Dawley rats were used. Retinas obtained from young [postnatal days (PN) 1, 4, 7, 12, 14], and adult (PN 40–60) animals were examined. The young rats were sacrificed by decapitation, and most of the adult animals were anaesthetised with carbon dioxide prior to decapitation. To examine whether perfusion fixation could favour preservation of endogenous NOS pools, a few adult rats were anaesthetised with an intramuscular injection of ketamine (0.45 ml; Ketalar, Parke Davis, 50 mg/ml) and xylazine (0.10–0.20 ml; Rompun, Bayer, 20 mg/kg), and perfused via the ascending aorta with saline, followed by a freshly prepared solution of 4% formaldehyde in phosphate-buffered saline (PBS; 0.1 mM; pH 7.2). Retinas obtained from light-adapted pigmented rabbits (4–6 months old) of either sex were also examined. The animals were sacrificed either by an intravenous injection of air, or by an overdose of pentobarbital (72 mg/kg, i.p.). All animals were treated in accordance with guidelines of the local animal experimentation and ethics committee.

Tissue preparation

Eyes from perfused animals were enucleated and post-fixed in 4% formaldehyde solution for 2 h at 4° C. Eyes from non-perfused animals were quickly enucleated and immersed in 4% formaldehyde solution at 4° C for 1–6 h. Some rat and rabbit eyes were immersion-fixed in a 2% formaldehyde solution for 30 min. Following an incision in the cornea made under the fixation medium, the lens and the vitreous body of rat retinas were carefully removed. Rabbit eyes were hemisected under fixation solution through a coronal cut behind the limbus, and the anterior segment, lens and vitreous body were discarded. The remaining eyecups were transferred to fresh fixation medium and kept at 4° C for the times indicated above. All tissue was thoroughly rinsed and cryoprotected by transferring stepwise to solutions of 5, 10, 15, and 20% sucrose in PBS, for at least 24 h. Rabbit eye cups were divided across the optic nerve head, perpendicularly to the myelinated streak. Sections (10–15 µm thick) were cut on a cryostat. Rabbit retinal pieces were cut along the sagittal axis, and the sections obtained included superior and inferior retina and the myelinated streak. The sections were collected on albumin/chrome alum- or poly-L-lysine-coated slides, air dried, and stored at –20° C until used.

NOS immunohistochemistry

The NOS antisera used have been previously described (Alm et al. 1993). A sequence from the C-terminal (FIEESKKDADEVFSS) of a cloned rat cerebellar NOS was synthesised and coupled to bovine serum albumin (BSA) before immunisation of rabbits and guinea pigs. No differences were noted between the two antisera.

Sections were brought to room temperature and air dried before pre-incubation for 90 min with 0.1 mM PBS containing 0.25% or 1% BSA and 0.25% Triton X-100. The sections were then incubated with rabbit or guinea pig anti-NOS serum (1:320–1:2560, diluted in PBS/BSA/Triton X-100; Milab, Malmö, Sweden) for 12–18 h at 4° C. After rinsing, the sections were incubated with secondary antibody: TRSC (Texas red sulfonyl chloride)-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, Pa., USA), or FITC (fluorescein isothiocyanate)-conjugated goat anti-guinea pig IgG (Sigma, St. Louis, Mo., USA), diluted 1:80, for 90 min at room temperature. The sections were then rinsed with 0.1 mM PBS and mounted with buffered glycerol containing phenylendiamine (Merck, Darmstadt, Germany) to prevent fluorescence fading.

No NOS immunoreactivity could be seen in sections incubated with antisera pre-absorbed with the synthesised antigen-BSA complex (100 µg/ml of diluted antiserum for 15–24 h at 4° C), or pre-adsorbed with a purified homogenate from rat cerebellum (Knowles et al. 1989).

NADPH-d histochemistry

In most experiments, tissue which had first been processed for NOS immunoreactivity was examined for the localisation of NADPH-d. Thus, sections exhibiting NOS-immunoreactive cells were photogaphed, after which the slides were soaked in 0.1 mM PBS for at least 4 h in order to remove the coverslip. This was followed by incubation for 1–4 h at 37° C in 100 mM Tris HCl buffer (pH 8.0) containing 0.2% Triton X-100, 0.5 mM nitroblue tetrazolium, and 1 mM β-NADPH, or 1 mM β-NADP, together with 15 mM malic acid and 1 mM $MnCl_2$. In other experiments, the retina was carefully isolated from the choroid following the cryoprotection step and was reacted for diaphorase activity in the same me-

dia as above (4 h, 37° C). The isolated retina was flat mounted with Kaiser's glycerol gelatine and coverslipped. When omitting β-NADPH or β-NADP from the incubation medium, no staining was observed in any retinal layer. All the chemicals were obtained from Sigma.

VIP immunohistochemistry

For the simultaneous demonstration of NOS and VIP immunoreactivity in rat retinas, sections were normally first processed for NOS immunohistochemistry and subsequently for VIP immunohistochemistry. In some cases, this order was reversed, which resulted in weaker VIP immunostaining, but without significantly affecting the number or the distribution of labelled cells. Neither did incubation of sections with a mixture of both antisera alter the results.

Thus, in most cases, sections were processed for NOS immunohistochemistry, after which they were rinsed and incubated for 90 min with 0.1 mM PBS/1% BSA/0.25% Triton X-100. This was followed by incubation with normal goat serum (10%, Vector Laboratories, Burlingame, Calif., USA) for 30 min. Antiserum to VIP raised in guinea pig (1:1000–1:2000 in PBS/BSA/Triton X-100; Milab, Malmö, Sweden) was then applied to the sections for 12–18 h at 4° C. After rinsing, incubation with FITC-conjugated goat anti guinea pig IgG (1:80, Sigma) for 90 min at room temperature, and further rinsing with PBS, the sections were mounted with buffered glycerol containing phenylendiamine.

The immunolabelled structures are referred to as NOS- and VIP-IR (immunoreactive), as cross-reactions with other antigens sharing similar amino acid sequences cannot be completely excluded.

Results

NOS immunoreactivity

Rat

No visible differences (in terms of labelling intensity, or total number of labelled cells) were noted between rats

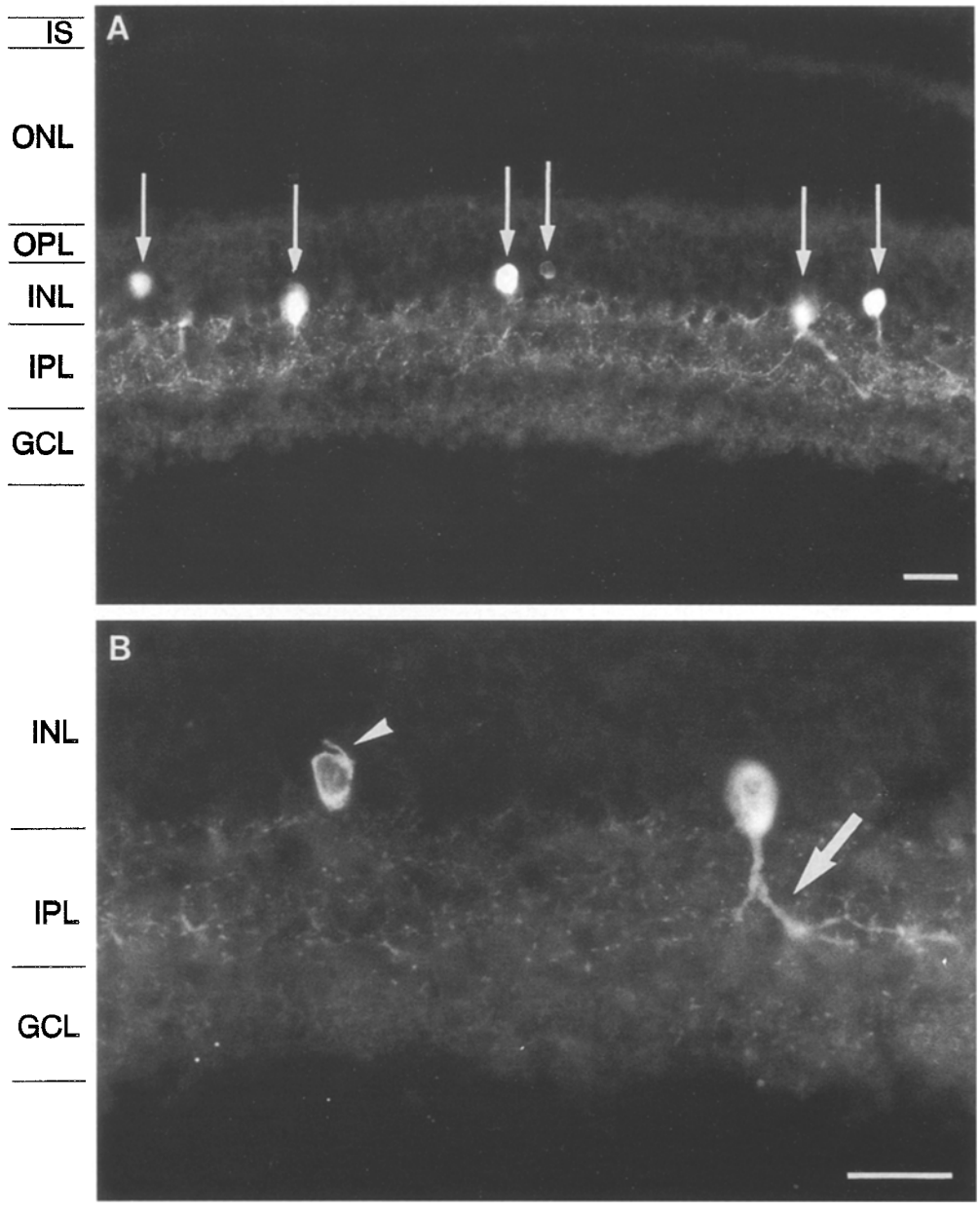

Fig. 1A, B Fluorescence micrographs illustrating the distribution of NOS immunoreactivity in the adult rat retina. **A** Labelled cell bodies are situated in the inner half of the inner nuclear layer, mainly next to the inner plexiform layer (*small arrows*). **B** Higher power micrograph showing labelling in two cell bodies located in the innermost cell row of the inner nuclear layer. From one cell, a dense process is seen to project to the innermost sublamina of the inner plexiform layer (*large arrow*). From the other cell, a short process is seen to project outwards (*arrowhead*). Labelled processes appear throughout the inner plexiform layer without a distinct sign of sublayering. *IS* Photoreceptor inner segments, *ONL* outer nuclear layer, *OPL* outer plexiform layer, *INL* inner nuclear layer, *IPL* inner plexiform layer, *GCL* ganglion cell layer. *Bar* 25 μm

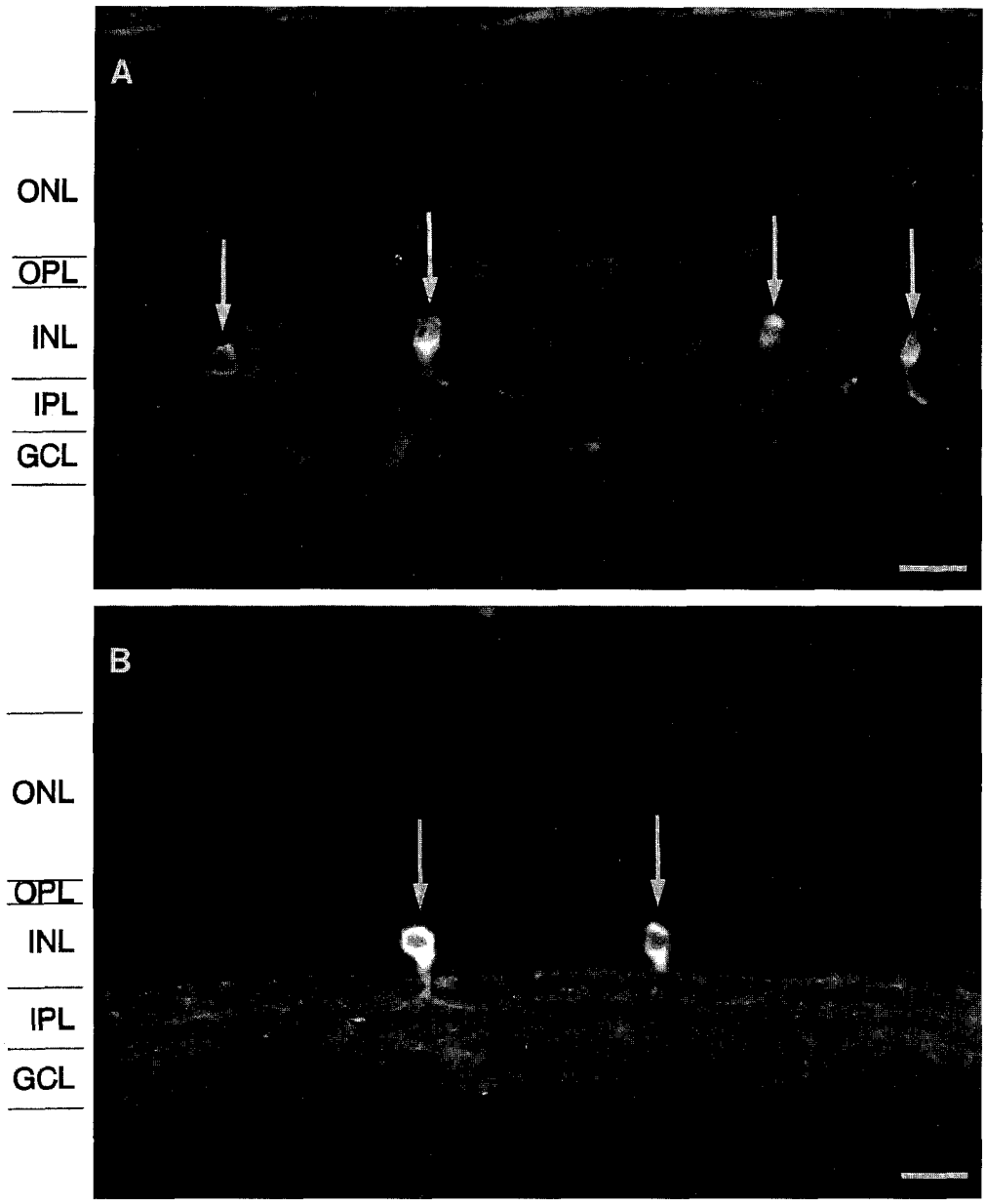

Fig. 2 Distribution of NOS immunoreactivity in the rat retina at PN 12 (**A**) and PN 14 (**B**). Stained cell bodies are located in the inner nuclear layer, next to the inner plexiform layer (*small arrows*), giving off processes that project into the inner plexiform layer. Immunoreactive processes appear mainly in the outermost and innermost laminas of the inner plexiform layer (**B**). Designation of layers as in Fig. 1. Fluorescence micrographs. *Bar* 25 μm

sacrificed by decapitation with or without carbon dioxide exposure. Similarly, perfusion-fixation of the animals was not found to produce any difference in the labelling pattern. As NOS immunoreactivity was found to be sensitive to overfixation (over 8 h fixation significantly reduced labelling intensity), carbon dioxide anaesthesia followed by decapitation and immersion fixation was used for most rats. No differences in the distribution of NOS immunoreactivity were noted between retinas fixed in 4% formaldehyde solution for 1–3 h and those fixed in a 2% solution for 30 min.

In the adult rat retina (n=13), most NOS-IR cells were seen in the two innermost cell rows of the inner nuclear layer, but more commonly in the cell row next to the inner plexiform layer (Fig. 1A). The labelled cells were of variable size and exhibited different intensities of immunoreactivity. Given the position and the shape of the labelled cell bodies, most of them are likely to be amacrine cells. Dense, fibre-like structures were often found to emerge from these labelled cells and to enter the inner plexiform layer, branching off mainly at the outermost and innermost sublaminas (Figs. 1A, B). NOS-IR terminals could also be seen throughout the inner plexiform layer (Figs. 1A, B).

A short process emerging from NOS-IR cell bodies located in the inner nuclear layer could occasionally be seen to project outwards. These cells could therefore correspond to interplexiform and/or bipolar cells (Fig. 1B). However, an ascending process was never seen running across the inner nuclear layer and reaching the outer plexiform layer. Further, no NOS immunoreactivity was detected in the outer plexiform layer.

A small number of NOS-IR cells could be seen in the ganglion cell layer, located mainly next to the inner plexi-

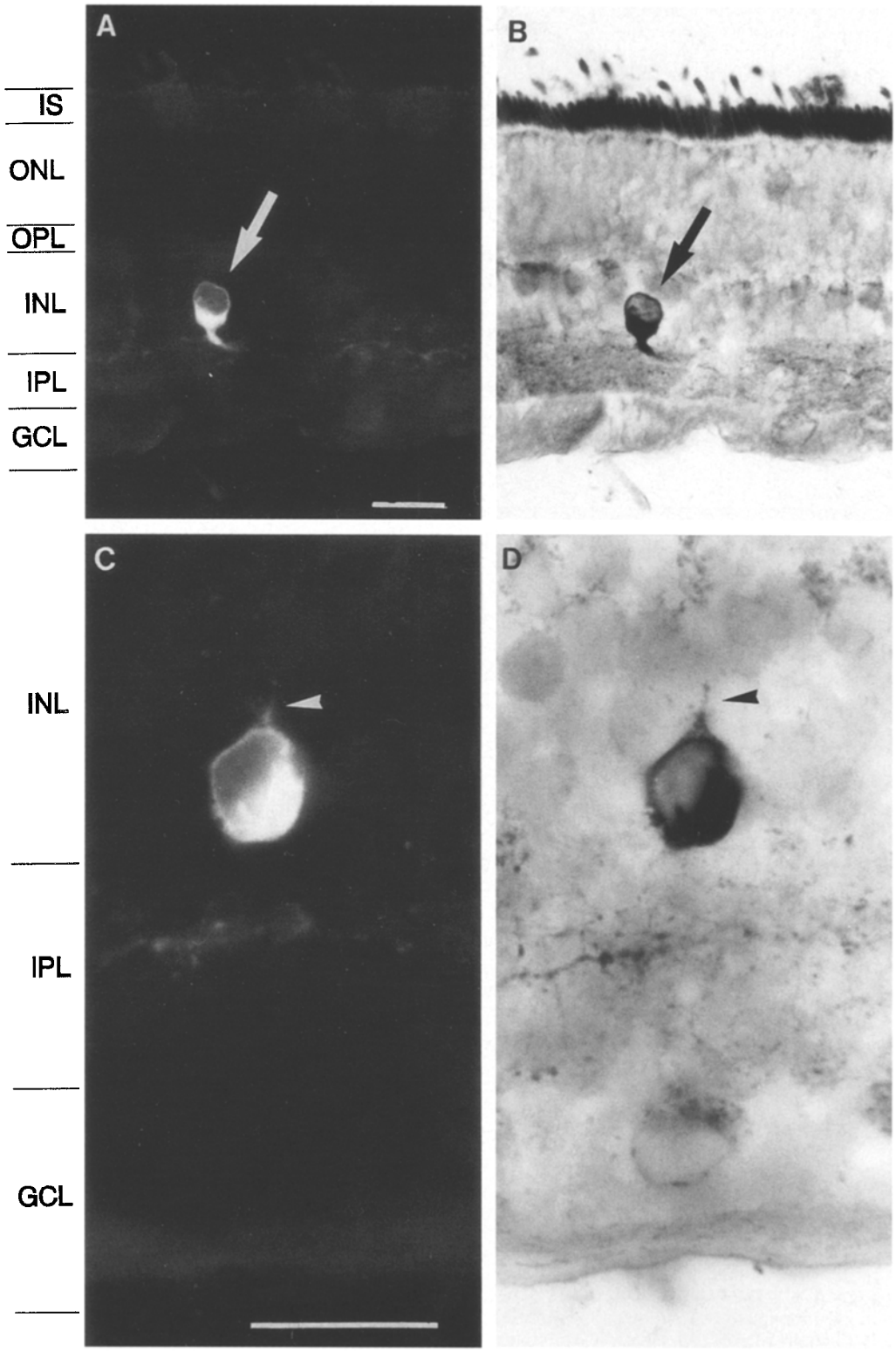

Fig. 3A–D Distribution of NOS immunoreactivity and of NADPH-d in the rabbit retina. **A** NOS-IR cell body appears next to the inner plexiform layer (*arrow*). A short dense process is directed towards the inner plexiform layer. Only very faint immunoreactive processes are seen in the inner plexiform layer. **B** The same field as in **A** illustrating NADPH-d staining. **C** NOS immunoreactivity in a cell body emitting a thin process towards the outer retina (*arrowhead*). **D** NADPH-d reactivity in the same cell body as in **C**. Note staining of the fine ascending process (*arrowhead*). Staining is also seen in a thin network of varicose fibres in the inner plexiform layer and in a cell body in the ganglion cell layer. The retinal tissue shown in these micrographs derives from rabbit retinas fixed for 3 h in 4% paraformaldehyde. Designation of layers as in Fig. 1. **A**, **C** Fluorescence micrographs; **B**, **D** Bright field micrographs. *Bar* 20 μm

form layer (Fig. 3A). Weak labelling of large cell bodies could also be seen in the proximal ganglion cell layer in a few retinas (Fig. 6C), suggesting that some ganglion cells may contain NOS. However, no NOS-IR structures were seen in the nerve fibre layer or in the optic nerve head region, indicating that most of the labelled cells in the ganglion cell layer correspond to displaced amacrine cells. No specific staining corresponding to NOS-IR was observed in photoreceptors or in horizontal cells.

The same labelling pattern was observed at PN 12 (n=3) and PN 14 (n=3) as in adult retinas. Most NOS-immunolabelled cells were found in the innermost cell

row of the inner nuclear layer, and many were seen to send processes into the inner plexiform layer (Figs. 2A, B), suggesting that the majority of them are likely to correspond to amacrine cells. At PN 14, the sublayering in the inner plexiform layer appeared more distinct than that of the adult rat retina (Fig. 2B), probably because collaterals are not yet fully developed. NOS-IR cells were only occasionally seen in the ganglion cell layer at PN 12 and PN 14.

A few NOS-positive cells were also seen at PN 7 (n=3). However, at this developmental stage, cells were generally more weakly labelled (not shown). No NOS immunolabelling of cells in the ganglion cell layer or in the inner plexiform layer was seen at PN 7. At PN 1 (n=3) and PN 4 (n=3), no NOS immunolabelling could be detected in any retinal layer.

Rabbit

There were no observable differences in the distribution of NOS immunoreactivity related to the method used to sacrifice the animals. Further, immersion fixation of retinas in 4% FA solution for 1–6 h or in 2% FA for 30 min was found to produce the same results. In the rabbit retina (n=10), most NOS-IR cells were found in the inner third of the inner nuclear layer (Fig. 3A), and are thus likely to correspond to amacrine cells. These cells were seen both in superior and inferior retina, but were more rare in the far inferior region. Some of these NOS-IR cells were seen to project into the inner plexiform layer mainly to the innermost and outermost sublaminas. Moderately immunoreactive structures were found throughout the inner plexiform layer. Similar to rat retinas, a short process could, at times, be seen projecting outwards from cells located in the inner nuclear layer (Fig. 3C). However, a fully labelled process extending to the outer plexiform layer was never observed, nor was there any NOS immunolabelling of terminals seen in the outer plexiform layer. Some NOS-IR cells were also found in the ganglion cell layer, mostly next to the inner plexiform layer. No specific labelling was detected in the outer retina.

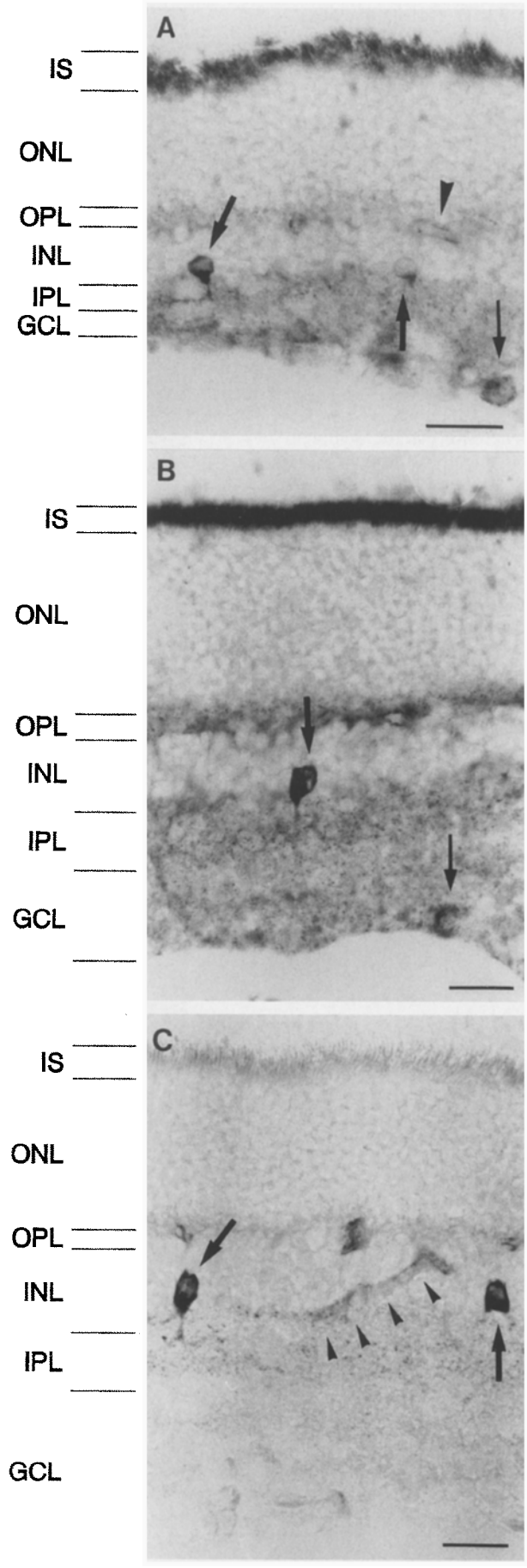

Fig. 4A–C NADPH-d staining in the adult rat retina. **A** Fixation in 2% formaldehyde solution for 30 min; incubated with NADPH. **B** Fixation in 4% formaldehyde solution for 60 min; incubated with NADPH. **C** Fixation in 4% formaldehyde solution for 3 h; incubated with NADP/malate/Mn. **A–C** NADPH-d reactive cell bodies are seen in all retinas in the inner nuclear layer, next to the inner plexiform layer (*large arrows*). In **A** and **B**, stained cell bodies are also seen in the ganglion cell layer (*small arrows*). Labelled structures can be seen distally in the inner nuclear layer and at the level of the outer plexiform layer. The vascular endothelium appears also stained (*arrowheads*). Intense labelling of photoreceptor inner segments and overall staining throughout the retina is seen in **A** and **B**. Designation of layers as in Fig. 1. Bright field micrographs. *Bar* 25 μm

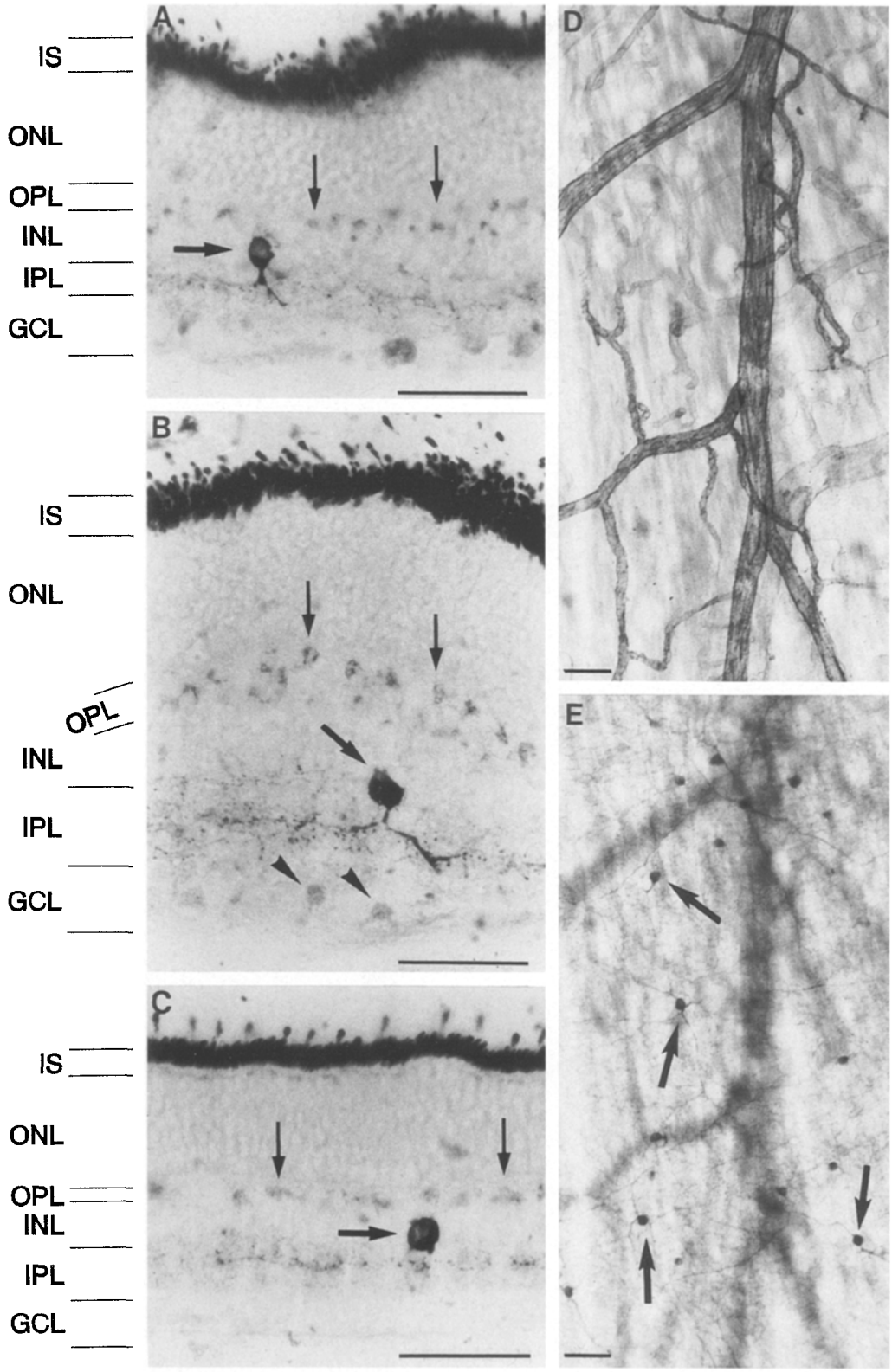

Fig. 5A–E NADPH-d staining in the rabbit retina. **A** Fixation in 2% formaldehyde solution for 30 min; incubated with NADPH. **B** Fixation in 4% formaldehyde solution for 3 h; incubated with NADPH. **C** Fixation in 4% formaldehyde solution for 6 h; incubated with NADP/malate/Mn. **D, E** Fixation in 4% formaldehyde solution for 2 h; incubated with NADP/malate/Mn. **A–C** NADPH-d reactivity is seen in cell bodies in the proximal (*large arrows*), and distal inner nuclear layer (*small arrows*), and in the ganglion cell layer (*arrowheads*). Labelled processes are seen to ramify in the inner plexiform layer (**A, B**). **D** Wholemount of rabbit retina from a region near the optic disc with the focus at the vitreal surface of the retina showing staining of the vascular endothelium. **E** The same field as in **D** with the focus at the proximal inner nuclear layer showing NADPH-d reactive cell bodies (*white arrows*). Intense labelling of photoreceptor inner segments is seen in **A–C**. Bright field micrographs. Designation of layers as in Fig. 1. *Bar* 50 μm

NADPH-d reactivity and colocalisation with NOS immunoreactivity

The overall distribution of staining appeared more intense in retinas fixed in a 2% formaldehyde solution for 30 min (n=4) or in a 4% solution for 1 h (n=4) (Figs. 4A, B, 5A) than in retinas fixed in 4% formaldehyde solution for longer periods of time (2–3 h for rats, n=5, and 2–6 h for rabbits, n=6, Fig. 5B). Incubation of sections with NADPH (n=5), also resulted in a more intense overall staining than incubation with NADP/malate/Mn (n=3).

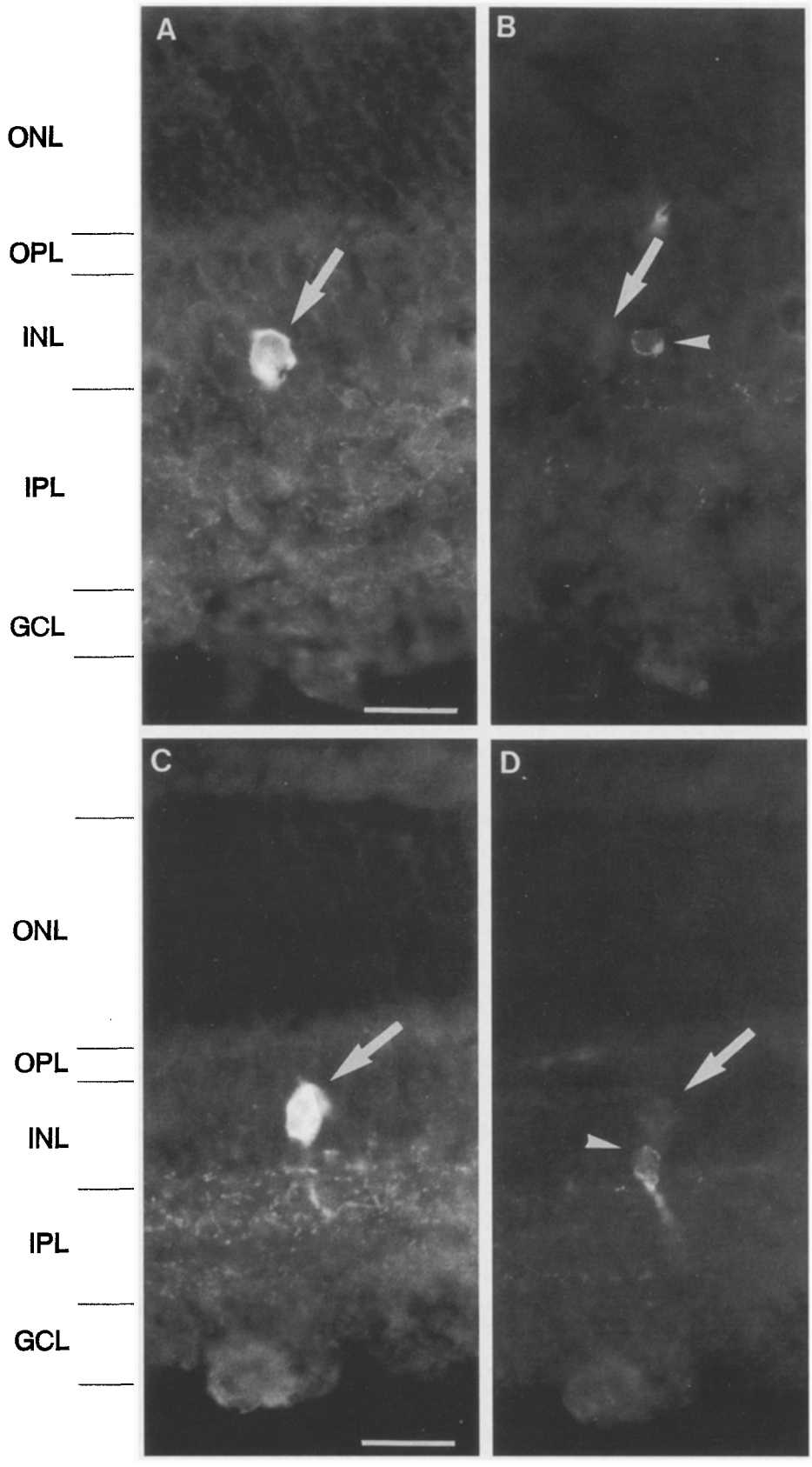

Fig. 6A–D Localisation of NOS immunoreactivity in the adult rat retina employing TRSC immunofluorescence (**A, C**). The same sections processed also for VIP immunohistochemistry employing FITC (**B, D**). Note that VIP-IR cells (*arrowheads*) are adjacent to NOS-IR cell bodies (*arrows*). Fluorescence micrographs. Designation of layers as in Fig. 1. *Bar* 25 μm

In rat retinas, strong NADPH-d reactivity was found in photoreceptor inner segments, particularly in tissue incubated with NADPH, irrespective of the strength of fixative (2% or 4% formaldehyde solution), or the fixation time (30 min to 3 h, Fig. 4A, B). Weak NADPH-d staining was also seen in photoreceptor inner segments of retinas incubated with NADP/malate/Mn, and was found to become fainter with longer fixation times (Fig. 4C). In rabbit retinas, intense staining could be seen over the photoreceptor inner segments, using NADP or NADPH as substrate (Fig. 5A–C). In both species, NADPH-d reactive cell bodies were seen in the proximal inner nuclear layer and in the ganglion cell layer, next to the inner plexiform layer (Figs. 3B, D, 4, 5). Most of these are likely to correspond to amacrine cells and displaced amacrine cells, respectively. In addition, weaker NADPH-d reactivity was seen at times in larger cell bodies located in the proximal ganglion cell layer (Fig. 5A). NADPH-d staining was also seen in the inner plexiform layer and at the level of the outer plexiform (Figs. 3–5). Both in rats and rabbits, the retinal vascular endothelium was found to be NADPH-d reactive (Figs. 4A, C, 5D, E).

All NOS-IR cell bodies found in rat and rabbit retinas were seen to be NADPH-d reactive. However, in both species, a small number of NADPH-d positive cells were found in the ganglion cell and in the amacrine cell layers that did not exhibit NOS immunoreactivity. Further, no NOS immunoreactivity was seen in structures that stained for NADPH-d in the outer retina (photoreceptor inner segments or outer plexiform layer), in the horizontal cell layer, or in endothelium, irrespective of the fixation solution used (2% or 4% formaldehyde), or of the time of fixation (30 min to 6 h) (Figs. 3–5).

Colocalisation of NOS and VIP immunoreactivities

Similar to NOS-IR cells, the VIP-IR cells were found in the rat retina in the inner margin or the inner nuclear layer (Fig. 6B, D), and were seen to project into the inner plexiform layer. However, VIP immunoreactivity was not found to colocalise with NOS immunoreactivity in the rat retina, although, notably, NOS- and VIP-IR cells were at times seen next to each other (Fig. 6A–D).

Discussion

A relatively small subpopulation of rat and rabbit retinal cells was seen to contain detectable amounts of NOS. Most labelled cells are likely to correspond to amacrine and displaced amacrine cells. In addition, a few interplexiform and/or bipolar cells may also contain NOS. The localisation of NOS immunoreactivity to all these cells agrees with previous reports on the distribution of NOS immunoreactivity in the rat retina (Dawson et al. 1991; Yamamoto et al. 1993a, b), and correlates well with the demonstration of NO-sensitive systems among retinal cells (Shiells and Falk 1992; Ahmad and Barnstable 1993; Ahmad et al. 1994; Bugnon et al. 1994).

On the other hand, in the present study, no NOS immunoreactivity was seen in horizontal cells, in the outer plexiform layer or in endothelium. These observations do not agree with earlier studies on the distribution of NOS immunoreactivity in the retina (Dawson et al. 1991; Yamamoto et al. 1993a, b). One possible explanation to this difference is that we have employed an antibody against a specific portion (a C-terminal fragment) of a neuronal form of rat NOS, rather than against the whole molecule, as was the case in the previous studies (Dawson et al. 1991; Yamamoto et al. 1993a, b). The C-terminal fragment appears to be unique to neuronal NOS (Alm et al. 1993), which could explain why no labelling of endothelial cells was observed in our study. On the other hand, being specific for neuronal NOS, labelling of horizontal cells could be expected. As NOS levels are likely to vary from cell to cell, they may have been too low in the case of the horizontal cells to allow detection by our system. Another possibility is that NOS stores in horizontal cells may have been depleted during manipulation of the tissue, which would imply that these cells are more sensitive than amacrine cells.

Osborne et al. (1993) have recently demonstrated NADPH-d reactivity in the horizontal cells of the rabbit retina. In their study, retinas were only lightly fixed (2% formaldehyde solution for 30 min) which, according to the authors, favoured the development of NADPH-d reaction in the horizontal cells. We have also localised NADPH-d reactivity in the distal inner nuclear layer and in the outer plexiform layer, both in rat and rabbit retinas. This was seen in our study using the conditions described above (2% formaldehyde solution for 30 min), as well as with longer fixation times employing 4% formaldehyde. However, we were still not capable of detecting NOS immunoreactivity in horizontal cells or in the outer plexiform layer, under any of the conditions tested. As seen in the present study and observed by others (Alm et al. 1993), not all NADPH-d reactive cells were seen to express NOS immunoreactivity. It thus appears that NADPH-d staining does not distinguish between different isoforms of NOS, therefore labelling more structures than those seen to express NOS immunoreactivity in our study.

Application of L-arginine (used by endogenous NOS as substrate) has been shown to eliminate the electrical coupling between retinal horizontal cells of certain non-mammalian species (Miyachi et al. 1991), suggesting that horizontal cells and/or neighbouring cells may be capable of synthesising NO. Thus, the lack of immunolabelling in horizontal cells in our study, in which an antibody against a unique portion of the molecule was used, could reflect the fact that these cells may use a different form of the enzyme, which is then not recognised by our antibody.

There is evidence that NO participates in the regulation of cGMP levels also in photoreceptors, thereby affecting membrane conductance in these cells. In isolated frog retinal rods, both the dark voltage and the recovery of the light responses were seen to be affected by sodium

nitroprusside, effects which are likely to result from an alteration of cGMP levels in the cells (Schmidt et al. 1992). Further, ADP-ribosylation of rod outer segment proteins, one of which was identified as the α-subunit of G-proteins, has also been shown to be regulated by endogenous NO (Pozdnyakov et al. 1993). NOS activity has been demonstrated in a crude preparation of bovine rod outer segments (Venturini et al. 1991), and NADPH-d has been localised in photoreceptor inner segments in retinas of various species (Sagar 1986, 1990; Sato 1990; Osborne et al. 1993). A particulate guanylate cyclase from bovine rod outer segments has been solubilised and also found to be NO-sensitive (Horio and Murad 1991). It is thus perhaps surprising that NOS immunoreactivity has normally not been localised in photoreceptors (Dawson et al. 1991; Yamamoto et al. 1993a, b) and the present study. In one recent report, NOS immunoreactivity has, however, been shown to be present in photoreceptor inner segments and to a certain extent also in cone outer segments in the rabbit retina (Koch et al. 1994). It is possible that basal NOS levels in photoreceptors are too low to be detected by most immunohistochemical methods available at the moment, or that the NOS form present in these cells is yet another isoform, which can not be recognised by the antibodies employed.

The present study also shows that cellular labelling corresponding to NOS immunoreactivity could not be detected in the rat retina at an early postnatal age (PN 1 or PN 4). This contrasts with an earlier report on the distribution of NADPH-d in the rat retina, which demonstrated that labelled cells could be seen from PN 3 (Mitrofanis 1989). In rabbit and cat retinas, NADPH-d reactive cells have been detected already pre-natally, and a role for NADPH-d in synaptogenesis has been proposed (Mitrofanis 1989; Vaccaro et al. 1991; Mitrofanis et al. 1992). It is possible that the levels of NOS are too low in the neonatal retina to be detected by our NOS antisera. However, it is also conceivable that NADPH-d reactivity seen in the retina at PN 3 or earlier may occur in cells using a different NOS isoform, which is not recognisable by our antibodies.

Nevertheless, NOS immunoreactivity could be detected in a few cell bodies at PN 7 and was very distinct already at PN 12 and PN 14. In the rat retina, neuronal activity is first detectable at about PN 9, when a small a-wave has been observed (Grün 1982). However, the peak of synapse formation is seen to occur at around PN 12 in the rat retina, and a complete electroretinogram is only recorded around PN 15, by the time of eye opening (Weidman and Kuwabara 1968; Grün 1982). Thus, although the levels of endogenous NOS appeared to increase with time during the first postnatal days, the onset of expression of this enzyme in retinal cells does not seem to correlate with visual input.

NOS has been shown to colocalise with VIP in numerous neuronal cell systems, including the pterygopalatine ganglion, which supplies the eye (Yamamoto et al. 1993b). However, in contrast to these reports, no colocalisation of the two was seen in the rat retina in our study. Interestingly, NOS- and VIP-IR cells were often found next to each other. However, it remains to be examined whether the incidence of these "pairs" is significantly high and, if so, whether it can be correlated with a functional interaction between the two systems.

In summary, the present study has demonstrated the localisation of immunoreactivity associated with neuronal NOS in rat and rabbit retinas. Cells located in the inner retina, most of which are likely to correspond to amacrine cells, appeared labelled. NOS immunoreactivity was not found in the neonatal rat retina, but was detected before the time when retinal activity is first recordable in rat retinas. Moreover, our observations open the possibility that different forms of neuronal NOS could exist in retinal cells.

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