An Unusual Distribution of Neuronal Nitric Oxide Synthase (nNOS) Neurons in the Porcine Retina Next to the Ora Serrata

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Summary

Neuronal nitric oxide synthase (nNOS)-immunoreactive (IR) cells are present in the retina of almost all species. Here we report about an unusual localization of nNOS-IR cells in the porcine retina. While no nNOS-IR amacrine cells were seen in the central porcine retina, a circular arrangement of nNOS-IR cells was observed next to the ora serrata region, particularly in the lower retinal quadrant. The neurons showed co-localization with calretinin. Most of their processes ran parallel to the ora serrata but some were disposed at right angles to the line of the ora serrata, directed either to the epithelial layer of the pars plana or towards the retina. The function of these uniquely located neurons in the porcine retina remains to be determined.

Keywords: Pig; Retina; Nitric oxide; Ora serrata

Introduction

The presence of neuronal nitric oxide synthase (nNOS)-immunoreactive (IR)/nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) positive cells (mainly amacrine cells) is well known in the retina of numerous species [1]. Surprisingly, a unique situation in the distribution of nNOS cells is present in the porcine retina reported here, which was overlooked by a general description of NOS in the porcine eye [2]. Besides this unique finding, the porcine retina is holangiotic and resembles closely that of man but without the formation of a fovea centralis [3-5]. The pig is therefore frequently used as an animal model for ocular research.

Material and Methods

A total of 38 left and four right porcine (*Sus scrofa ssp. domesticus*) eyes from the local abattoir were used for this study. In addition, three eyes from wild pigs (*Sus scrofa*) were kindly provided by a local hunter. The eyes were cut equatorially and fixed in 4% paraformaldehyde for three to four hours. After rinsing in phosphate buffered saline (PBS, pH 7.4) the retinae were either dissected into quadrants and prepared as whole mounts or frozen for serial sagittal sections.

For NADPH diaphorase enzyme histochemistry, 4% paraformaldehyde (PFA)-fixed retinal whole mounts were incubated free-floating in the diaphorase staining solution, containing 2mg Nitroblue tetrazolium, 10mg NADPH, and 30 μl Triton-X-100 in 10 ml PBS buffer for one hour at 37°C. The vitreous was removed carefully from the retina to avoid staining irregularities. Serial 15 μm thick sagittal frozen sections through the central and peripheral retina were also performed, mounted on glass slides, and incubated with the diaphorase staining solution. The reaction was stopped with PBS buffer, the tissue mounted in Kaiser's glycerine jelly and investigated with a microscope. In five eyes, the diameters of 50 stained somata were measured using a Quantimed 500 computer (Leica, Bensheim, D). In addition, the processes of 50 neurons were characterized and the length of their dendritic trees evaluated.

For immunohistochemical procedures, PFA-fixed retinas with and without NADPH diaphorase-staining were cut in sagittal and tangential planes. The 15 μm thick frozen sections were mounted on glass slides, air dried, and incubated in dried milk solution for 20 minutes to reduce nonspecific background staining. Incubation with the primary antibodies (all hosted in rabbits) was performed in a moist chamber for a period of at least 12 hours (overnight) at 4°C. The primary antibodies used were directed to neuronal nitric oxide synthase (nNOS; diluted 1:250; kindly provided by B. Mayer, Graz, Austria), calretinin (diluted 1:500; Chemicon, Hofheim, Germany), gamma-amino butyric acid (GABA, diluted 1:1000; Sigma Chemical Comp., St. Louis, MO), vasoactive intestinal peptide (VIP, diluted 1:400; Euro Diagnostica AB, Malmö, Sweden), tyrosin hydroxylase (TH, diluted 1:400; Chemicon), neuropeptide Y (NPY, 1:400; Biotrend), substance P (SP, diluted 1:500; Biotrend, Köln, Germany), and calcitonin gene related peptide (CGRP, diluted 1:500; Euro Diagnostica AB). After rinsing with TBS the sections were incubated with a goat-anti-rabbit Cy3-conjugated second antibody (diluted 1:1000; Dianova, Hamburg, Germany) for fluorescence microscopy. The sections were mounted with Kaiser's glycerine jelly. Negative controls were performed lacking the primary antibody.

Analysis of the fluorescence sections (at least 10 sections of the inferior quadrant of four eyes) and quantification of the NADPH-d whole mounts were performed at 400x magnification.

Results

Both, the domesticated and the wild pigs showed the same staining behavior and cell distribution concerning nNOS/NADPH-d positive neurons.

NADPH staining

In the central retina of the pig, intense staining for NADPH-d was found in the outer segments of the photo receptor cells and in the endothelial cells of all retinal vessels. No neuronal staining was

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present, neither in whole mounts nor in sections as reported already previously [2]. Close to the ora serrata region, numerous NADPH-d positive cells formed a circular arrangement along the whole ora serrata region (Figures 1a and c). The distribution of these cells was uneven throughout the circumference with most of the stained cells located in the lower quadrant (Table 1 and figure 1d). Single cells were found to extend as far as 5 mm from the ora serrata into the peripheral retina, predominantly in the nasal quadrant.

The shape and diameter of all cells was regular ranging between 10 and 14 µm. Most of the cells formed 2-3 processes with a diameter of 1-2 µm at the origin of the soma and ran parallel to the ora serrata. The processes branched within a distance of 30 µm. Some processes were running perpendicular towards the epithelial layer of the pars plana region (Figures 1a and c).

Immunohistochemical staining

The staining of porcine retina with antibodies against nNOS revealed an identical pattern as the NADPH-d enzyme histochemistry. Numerous nNOS-IR cells formed a circular arrangement along the whole ora serrata region (Figure 1b) showing the same distribution in the different quadrants (Figure 1d). Beside the photoreceptor outer segments no additional neuronal staining with antibodies against nNOS was seen in the central part of the retina.

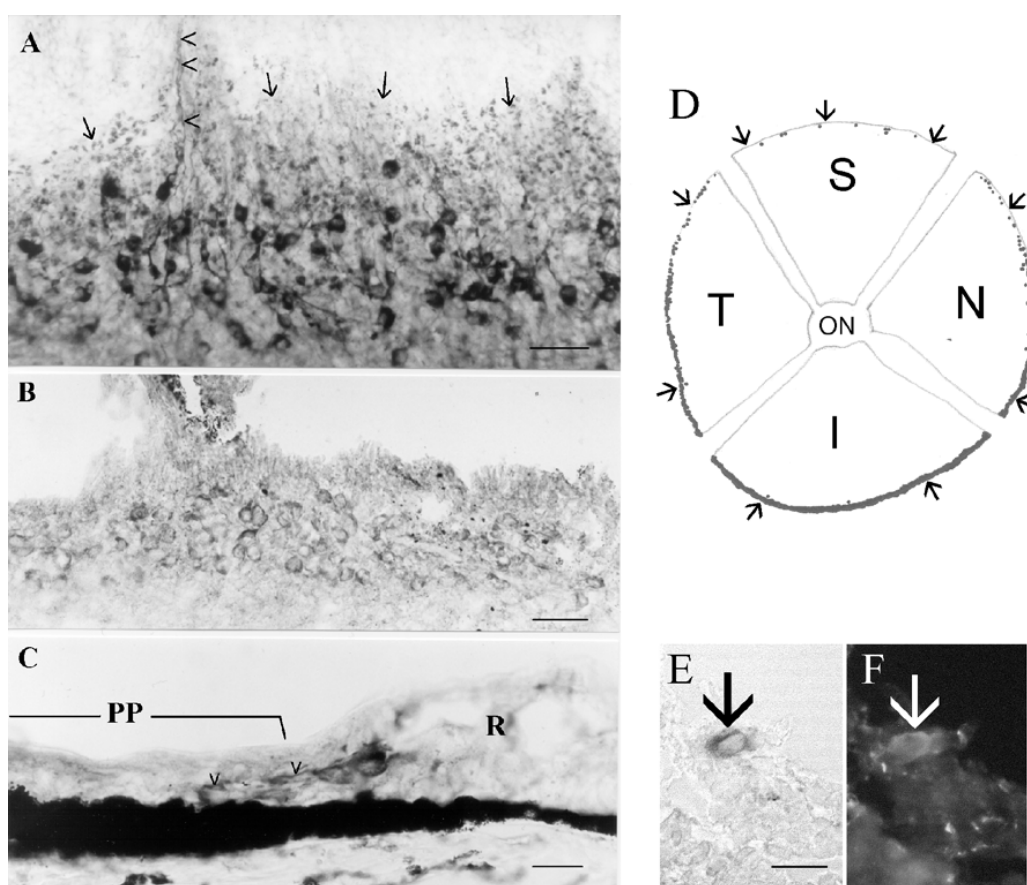Various neurons in the peripheral retina stained positive with

Figure 1: A. Retinal whole mount (NADPH-d) showing numerous stained cells next to the oraserrata (marked by arrows). At one place, stained processes run into the pars plana region (arrowheads). **B**. Tangential section stained with antibodies against nNOS at the same position. **C**. NADPH-d. Sagittal section showing stained processes (arrowheads) of the positive neurons towards the pars plana (PP) region. R=retina. **D**. Schematic drawing of a whole mount showing the different distribution of NADPH-d/nNOS positive cells (black dots) in the porcine retina. T=temporal, S=superior, N=nasal, I=inferior quadrant. ON=optic nerve region. Arrows mark the oraserrata. **E, F**. Double-staining of a single cell (arrow) with NADPH-d (E) and Calretinin (F). Scale bar in A and B: 50 µm, in C, E and F: 20 µm.

	temporal	superior	nasal	inferior	total number
DT, retina 1	218	32	202	721	1461
DT, retina 2	148	32	444	1024	1648
DT, retina 3	584	9	397	1081	2071
DT, retina 4	412	12	246	1215	1885
WT, retina 1	101	9	577	755	1442
WT, retina 2	107	31	458	849	1445
WT, retina 3	152	21	568	698	1439

Table 1: Number of NADPH-d positive cells in the oraserrata region, counted in four different quadrants comprising the whole circumference. Retinal whole mounts of the wild (WT) and domesticated type (DT) of *Susscrofa* were used.

antibodies against gamma-amino butyric acid, vasoactive intestinal peptide, tyrosin hydroxylase, neuropeptide Y, substance P, and calcitonin gene related peptide, but the localization was different compared to that of the NADPH-diaphorase and nNOS-IR cells.

Double staining procedures

NADPH-d stained sections were incubated with antibodies against nNOS and calretinin and confirmed a complete co-localization (Figure 1e and f). No co-localization was found with any of the other antibodies tested.

Discussion

The nNOS-IR neurons at the ora serrata region of the porcine retina can be described as unique and not classifiable. Concerning the uniqueness, I was not able to demonstrate these cells in the following species: mouse and rat (different strains), guinea pig, cat, dog, cow, chicken, eagle, monkeys (*Tupaia glis, Aotus trivirgatus, Cercocebus, Macaca fascicularis, Macaca mulatta*), and human (unpublished results). Concerning their classification, nNOS-IR neurons in the retina are mainly attributed to amacrine cells [1]. However, due to the unusual location, morphological criteria fail [6]. Calretinin has been found in amacrine subpopulations of the pig retina [7], but no co-localization of calretinin and nNOS is reported in the retina of any other species, although it can be found in brain neurons [8,9].

Interestingly, a significant superior – inferior difference was observed concerning the number of the nNOS-IR neurons showing only single cells in the superior quadrant and numerous cells in the inferior quadrant. The number of cells showed some individual variation (Table 1) but the distribution was constant. For statistical analysis the number of eyes quantified was too low. Since there was no difference between wild and domesticated animals, the observed cells seem to be representative for pigs in general.

Some speculations on the function of the nNOS-IR neurons in the porcine retina other than light response modulation, usually ascribed to these cells in other species [1], include reception of non-neuronal signals, secretion of factors, or modulation of the vascular tone. Calretinin is known to be present in rapid adapting mechano receptors [10] and in neurosecretory cells [8]. Both functions are not documented for the retina. If the release of nitric oxide of these cells

influences aqueous humor formation [11] remains questionable, since their processes only reached the posterior pars plana region. Still, a cross-talk between the anterior and posterior eye segment seems likely.

Concerning regulatory properties of the vascular tone, a large anterior border venule exists in the porcine retina [3,5] that forms an almost complete ring next to the ora serrata, which is more developed temporally. It is unknown whether this venous vascular ring has any special functions and/or might be influenced by the described nNOS-IR cells.

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