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SHORT COMMUNICATION

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Immunocytological localization of dopamine in the guinea pig retina

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Abstract We examined dopaminergic neurons in the guinea pig retina; antisera against tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH), phenylethanolamine *N*-methyltransferase (PNMT) and an antiserum against γ-aminobutyric acid (GABA) were used. In the present study, two types of amacrine cells were labeled with an anti-TH antiserum. However, no DBH and PNMT immunoreactivities were seen. The type 1 cell had a larger-sized soma located in the inner nuclear layer with processes ramifying mainly in stratum 1 of the inner plexiform layer (IPL). The type 2 cell had a smaller-sized soma and processes branching in stratum 3 of the IPL. The mean densities were 56.4±11.5/mm^2 for the type 1 cell and 166.6±30.3/mm^2 for the type 2 cell. Double immunocytochemistry using an antiserum against GABA revealed that while none of the type 1 cells showed GABA immunoreactivity, all of the type 2 cells displayed GABA immunoreactivity. Our results suggest that, in the guinea pig retina, the type 1 amacrine cells are pure dopaminergic and the type 2 cells are dopaminergic elements that use GABA as their second transmitter.

Key words Tyrosine hydroxylase · GABA · Co-localization · Immunocytochemistry · Retina · Guinea pig

Introduction

Dopamine seems to be the predominant catecholamine transmitter in the mammalian retina (Kamp 1985). At present, the best marker for dopaminergic neurons in the retina is an antiserum directed against tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis, because norepinephrine and epinephrine occur only in trace amounts in most retinae (Versaux-Botteri and Nguyen-Legros 1986). Two subpopulations of TH-immunoreactive amacrine cells, which actually contain dopamine (Nguyen-Legros et al. 1994), were first demonstrated in the rat retina. The type 1 cell is an amacrine cell with an unusually large cell body and processes ramifying predominantly in stratum 1 of the inner plexiform layer (IPL), and occurs at low density (0.1% of all amacrine cells) (Mitrofanis and Provis 1990; Casini and Brecha 1992). Interplexiform cells are found among 40% of the type 1 cells (Nguyen-Legros et al. 1994). The type 2 cell is a small amacrine cell projecting its processes to stratum 3 of the IPL (Crooks and Kolb 1992; Nguyen-Legros et al. 1994).

The role of γ-aminobutyric acid (GABA) as an inhibitory neurotransmitter in the mammalian retina is well established (Yazulla 1986; Massey and Redburn 1987). The GABAergic markers label 30–50% of all amacrine cells (Mosinger et al. 1986; Wässle et al. 1987), which comprise several morphological classes (Pourcho and Goebel 1983). The co-localization of TH and GABA within the type 1 amacrine cell has been reported in various mammalian retinae (Wässle and Chun 1988; Crooks and Kolb 1992). However, whether the type 2 cell contains GABA has been studied only in the rat with a vascular retina (Young 1994). Thus, we examined dopaminergic cell types and their extent of co-localization with GABA by immunocytochemistry using anti-TH, dopamine β-hydroxylase (DBH), phenylethanolamine *N*-methyltransferase (PNMT) and GABA antisera in the guinea pig, a diurnal mammal with color opponent vision, which has an avascular cone-rich retina.

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Materials and methods

Five adult guinea pigs were used. Principles of laboratory animal care and specific national laws were followed. The guinea pigs were deeply anesthetized by intraperitoneal injection of 4% chloral hydrate (1 ml/100 g body weight). The eyeballs were enucleat-

ed 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 TH, DBH and PNMT immunoreactivities, the retinae were fixed by immersion in 4% paraformaldehyde – 0.05% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4, 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 (PBS), pH 7.4.

Immunostaining was performed by using the avidin-biotin-peroxidase complex (ABC) method. Briefly, the whole-mounted retinae were incubated in the following order: 10% normal goat serum (NGS) in PBS containing 0.5% Triton X-100 for 1 h at room temperature in order to block nonspecific binding activity, rabbit polyclonal antibodies against TH, DBH and PNMT (Eugene Tech., Allendale, NJ) diluted 1:1000 in PBS containing 3% NGS and 0.5% Triton X-100 for 72 h at 4°C, biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA; diluted at 1:50) in PBS for 24 h at 4°C, ABC solution (Vector) in PBS for 24 h at 4°C, and 0.05% 3,3'-diaminobenzidine (DAB) in 0.05 M TRIS-HCl buffer (TB, pH 7.4) containing 0.01% H_2O_2 for 10 min. After 1–2 min, as determined by the degree of staining, the reaction was stopped with several washes of TB and PB. The retinae were mounted onto gelatin-coated slides with the ganglion cell layer (GCL) facing up, and coverslipped with glycerol. In order to test whether TH and GABA are co-localized within the same neurons, retinal pieces were taken from the central region near the optic disk and the sections were dehydrated in a graded series of alcohol, infiltrated with propylene oxide, and embedded in Epon 812. Serial semithin sections (1 µm thick) were made, and collected alternatively on slides coated with 0.5% gelatin. The sections were etched with alcoholic sodium hydroxide for 15 min, and washed in PBS for 5 min. The sections were then treated with 0.5% H_2O_2 in absolute methanol for 30 min to destroy endogenous peroxidase activity. The following immunostaining was performed at room temperature. The sections were incubated for 1 h in PBS containing 10% NGS and 1% Triton X-100. They were subsequently incubated in a rabbit polyclonal antibody directed against GABA (a gift from the Max-Planck Institute for Brain Research, Frankfurt/Main, Germany; dilution rate 1:2000) or TH (Eugene Tech.) in PBS containing 3% NGS for 3 h. Following this incubation, the sections were rinsed in four changes of PBS for 5 min, incubated for 1 h in biotinylated goat anti-rabbit IgG (Vector) with 0.5% Triton X-100, and then rinsed in PBS. They were incubated in ABC solution (Vector) in PBS for 1 h, rinsed in two changes of PBS for 5 min and three changes of TB for 5 min, and further incubated in 0.05% 3,3'-DAB in TB containing 0.01% H_2O_2 for 10 min. To test for antibody specificity, normal rabbit serum (preimmune serum) was applied to the retinal tissues. No immunostaining was observed in control tissue.

Results and discussion

In the present study, TH immunoreactivity was displayed by certain populations of amacrine cells, whereas no DBH or PNMT immunoreactivity was seen in the retinal preparations. Thus, we examined the TH-immunoreactive neurons, and evaluated the co-localization of TH and GABA immunoreactivities within the same neurons. TH-labeled amacrine cell somata were sparsely distributed throughout the retina (Fig. 1). The labeled somata were round or oval. Two types of labeled cells, type 1 and type 2, were distinguishable according to their morphological features. The type 1 cell had a larger-sized soma and showed strong TH immunoreactivity, whereas the type 2 cell had a smaller-sized soma and showed weak immunoreactivity (Fig. 1). The processes emerging from the type 1 cell ramified mainly in stratum 1 of the IPL and formed a dense network, whereas the processes originating from the type 2 cell descended through the IPL and ramified in stratum 3 of the IPL (Fig. 1). However, processes ramifying in the outer plexiform layer were never seen, indicating that TH-labeled cells are amacrine cells in the guinea pig retina. Soma sizes were measured from 100 cells of both TH-labeled type 1 and 2 amacrine cells located in the central regions of the retina. Each cell body was measured by an Image Analyzer. The mean diameter (mean±SD) of the type 1 cell was 12.0±1.7 µm, whereas that of the type 2 cell was 6.4± 1.2 µm. The density (mean±SD) of the TH-immunoreactive amacrine cells was also calculated in the central region of five whole-mounted retinae. The density of the type 1 cell was 56.4±11.5/mm^2, whereas the type 2 cell had a higher density, ranging from 131/mm^2 to 197/mm^2 (mean density, 166.6±30.3/mm^2).

In order to identify whether TH and GABA immunoreactivities are expressed within the same amacrine cells, serial 1-µm-thick vertical semithin sections were cut and then processed for either TH or GABA immunoreactivity. Figure 2 shows an example of three consecutive semithin sections. The sections in Fig. 2A and C show TH immunoreactivity, whereas the section in Fig. 2B exhibits GABA immunoreactivity. Comparison of the three consecutive sections shown in Fig. 2 reveals that the type 2 cell (Fig. 2A,C) displays GABA immunoreactivity (Fig. 2B), whereas the type 1 cell shows no GABA immunoreactivity. All of the type 2 cells (121 cells) investigated in this study were GABA immunoreactive, but the type 1 cells (32 cells) showed no GABA immunoreactivity. Therefore, we conclude that the type 2 cells express GABA immunoreactivity and constitute a subpopulation of GABAergic amacrine cells in the guinea pig retina.

In the present study, TH-immunoreactive neurons were clearly seen to belong to two types, i.e., type 1 and 2 amacrine cells of the guinea pig retina. The type 1 amacrine cell corresponds to well-defined dopaminergic amacrine cells with a large cell body and processes ramifying in stratum 1 of the IPL and forming ring-like structures, as reported for several mammalian retinae (Voigt and Wässle 1987; Mitrofanis and Provis 1990; Casini and Brecha 1992). However, unlike in other mammals, TH-labeled interplexiform cells have not been found in this study. Our results suggest that dopaminergic interplexiform cells are species dependent, together with previous reports that the interplexiform cell system is well developed in rats (Nguyen-Legros 1991), while it is poorly developed in man and macaque monkey (Savy et al. 1991). The type 2 cell is similar to a cell type described in the primates (Crooks and Kolb 1992) and rats (Mitrofanis et al. 1988; Crooks and Kolb 1992; Young 1994). In this study, we could not find cells showing DBH or PNMT immunoreactivity. These results contrast with the reports by Versaux-Botteri et al. (1986) and Nguyen-Legros (1987), who have shown that the type 2 cells exhibit PNMT immunoreactivity. Thus, our results

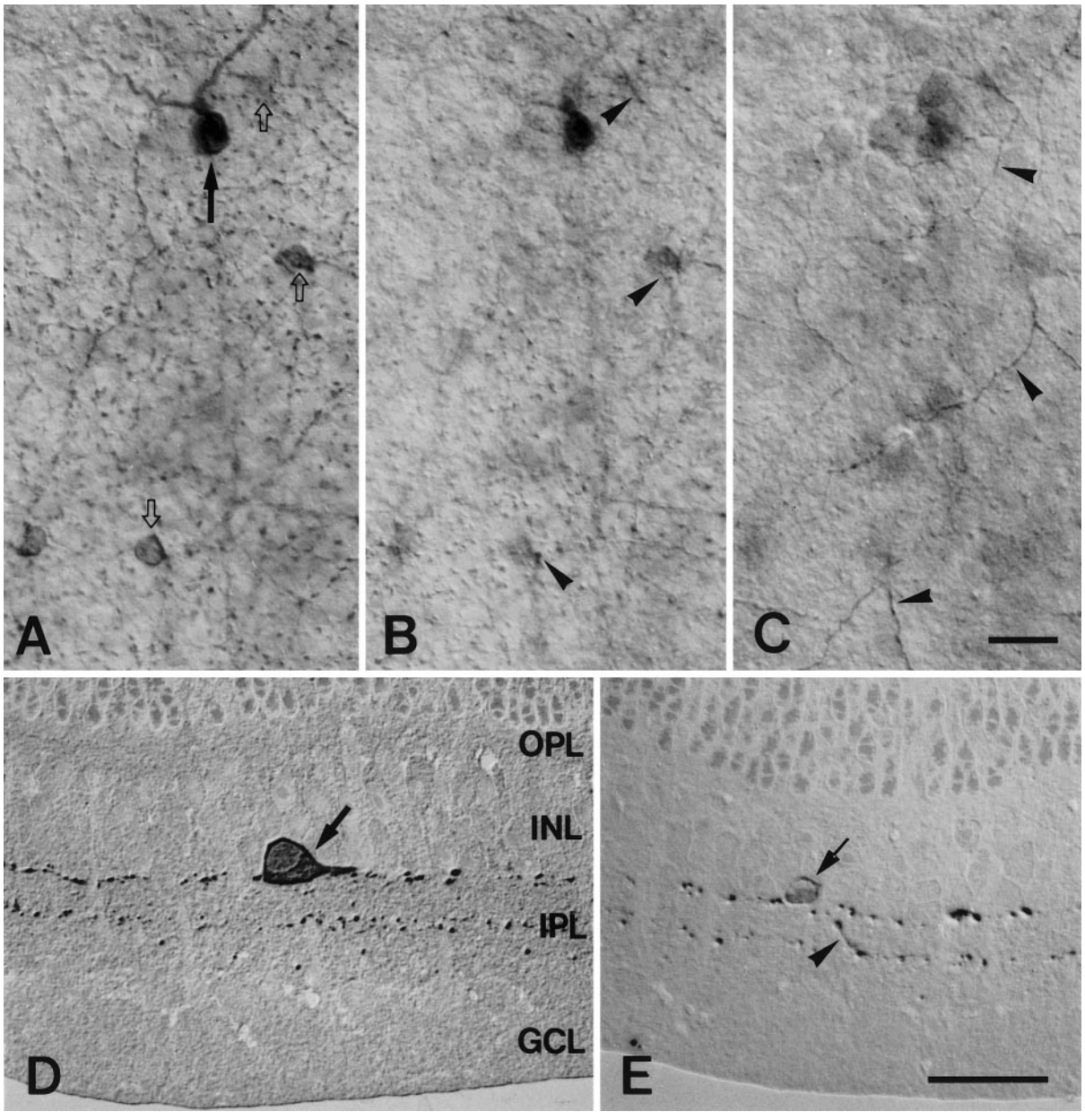

Fig. 1 Light micrographs taken from a whole-mounted preparation **(A–C)** and 1-µm-thick semithin sections **(D,E)** processed for TH immunoreactivity. **A–C** represent the same field. In **A**, the focus is on the inner nuclear layer (*INL*), and type 1 (*arrow*) and type 2 (*open arrows*) amacrine cell bodies are seen. In **B**, the focus is on the inner plexiform layer (*IPL*) near the INL, and fine processes emerging from the type 2 cell bodies (*arrowheads*) are seen. In **C**, the focus is on the middle of the IPL, and fine processes originating from the type 2 cells are clearly visible. In **D**, a type 1 cell (*arrow*) with a larger-sized soma and strong immunoreactivity is seen. In **E**, note a smaller-sized and weakly stained soma (*arrow*) of a type 2 cell. *Arrowhead* indicates a process emerging from the soma of the type 2 cell (*OPL* outer plexiform layer, *GCL* ganglion cell layer). *Scale bar* 25 µm

strongly suggest that TH-labeled amacrine cells of the guinea pig contain dopamine rather than the other catecholamines.

In this study, none of the type 1 cells showed GABA immunoreactivity, while all type 2 cells displayed GABA immunoreactivity, in contrast to the reports that most of the type 1 cells express GABA immunoreactivity in the cat and rat retinae (Wässle and Chun 1988; Wulle and Wagner 1990; Young 1994). Thus, the guinea pig retina possesses diverse dopaminergic systems; i.e., the type 1 cells apparently represent pure dopaminergic amacrine cells, whereas the type 2 cells are dopaminergic amacrine cells that use GABA as their second neurotransmitter. Accordingly, the type 2 cells might have an entirely different role in the IPL compared with the type 1 cells. In conclusion, dopamine is a major catecholamine in the guinea pig retina. However, the actual func-

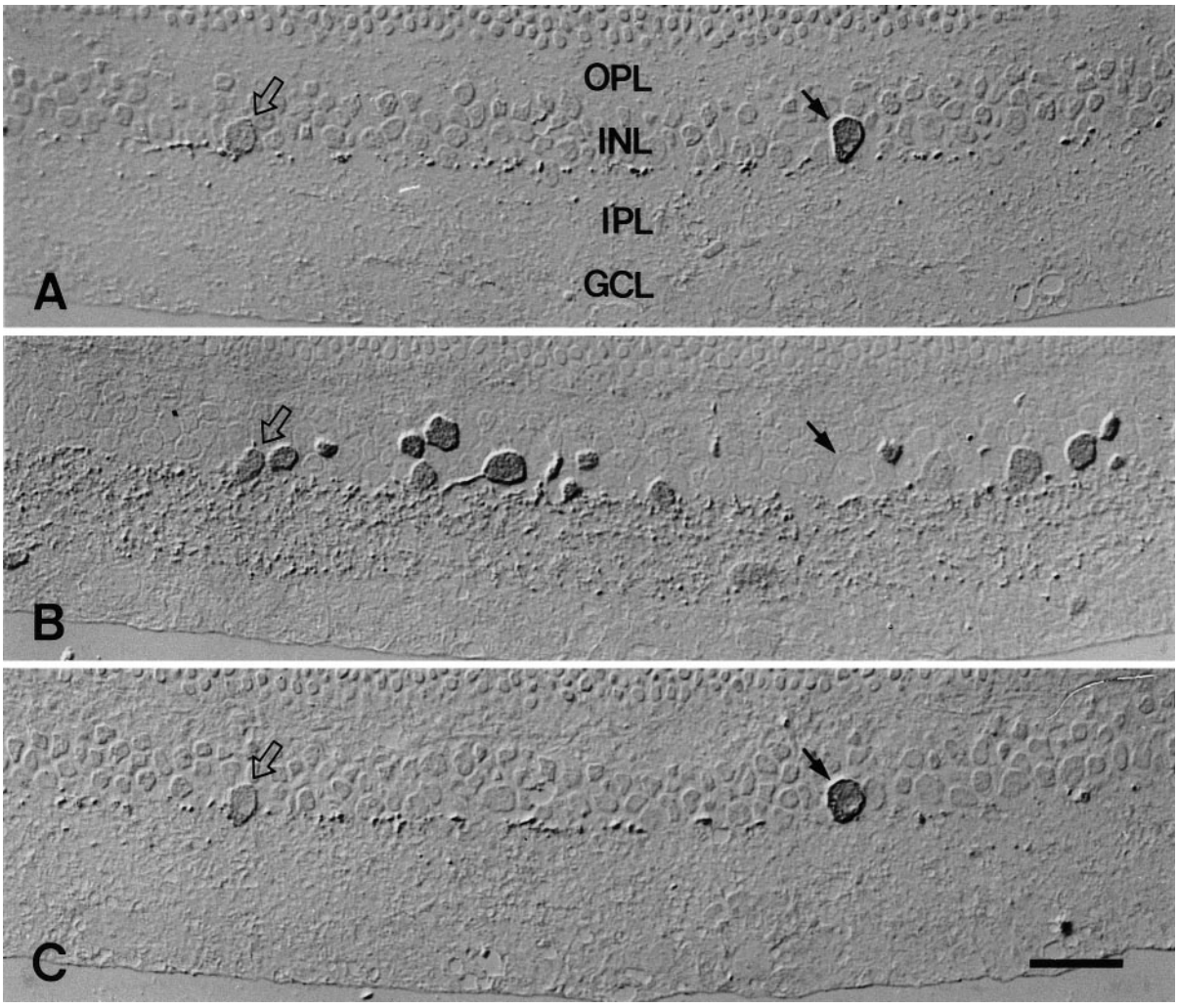

Fig. 2 Three consecutive vertical semithin (1-µm-thick) sections through a guinea pig retina processed for TH **(A,C)** and GABA **(B)** immunoreactivities. In **A** and **C**, a type 1 cell (*arrow*) with a larger cell body and stronger immunoreactivity and a type 2 cell (*open arrow*) with a smaller cell body and weaker immunoreactivity are seen. In **B**, the type 2 cell indicated by the *open arrow* (cf. **A,C**) shows GABA immunoreactivity, whereas the type 1 cell (*arrow*) shows no GABA immunoreactivity. *Scale bar* 25 µm

tion of GABA within the type 2 cell remains to be elucidated.

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