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Original Article

Dopaminergic amacrine cells in rabbit retina: a confocal microscopic study

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Abstract: Objective: To demonstrate the relationship between dopamicergic amacrine (DA) cells and AII amacrine cells and to characterize DA cells of the rabbit retina. Methods: Retinal whole mounts were double- or triple-labeled using antibodies against tyrosine hydroxylase (TH), calretinin (CR), and vesicular glutamate transporter 1 (VGLUT1) which selectively label DA cells, AII amacrine cells, and all bipolar axon terminals, respectively. Neurochemical property of DA cell was examined by double- or triple-labeling using antibodies against γ-aminobutyric acid (GABA), vesicular GABA transporter (VGAT), and glutamic acid decarboxylase (GAD), all three of which are reliable markers for GABAergic neuron. Confocal images were taken and quantitatively analyzed. Result: In stratum 1, most of DA dendritic varicosities which are presumed sites of neurotransmitter release made contact with somata or lobular appendages of AII amacrine cells. In addition, some of varicosities in strata 3 and 5 contacted arboreal dendrites of AII amacrine cells. Numerous lobular appendages of AII amacrines localized at stratum 2 of the IPL (inner plexiform layer) showed little contact with DA varicosities. Double- or triple-labeling experiment showed that GABAergic markers such as GABA, VGAT, and GAD were not expressed in DA cells of the rabbit retina. Conclusion: DA dendrites stratified at strata 1, 3 and 5 of the IPL; DA cells of the rabbit retina do not use GABA as a neurotransmitter, unlike other mammals.

Keywords: Dopaminergic amacrine cells, ALL amacrine cells, retina, immunofluorescence, confocal microscopy

Introduction

The retina tightly contacts with eyeball posterior wall. It is a part of central nervous system and the origin of all the visual signals. Retina receives the ray from cornea and changes light signal into electrical signal, then the electrical signal is transferred to higher levels of electrical signal and causes vision [1]. The mammalian retina is composed of three layers of nerve cell bodies (ganglion cell layer, inner nuclear layer, and outer nuclear layer) and two layers of synapses (inner plexiform layer and outer plexiform layer) [2-4]. The retina is anatomically divided into 10 layers include pigment epithelium layer, cones and rod cells layer, outer limiting membrane, external granular layer, external plexiform layer, internal granular layer, inner plexiform layer, ganglion cell layer, nerve fiber layer and inner limiting membrane [5]. When light transmit to retina, it is absorbed and transformed into chemical signal by photoreceptors (rod cells and cone cells). Then the chemical signal is transferred to bipolar cell in inner nuclear layer, whose axons and gangliocellular dendrites construct synapses. The signal is further transferred to ganglion cells in ganglion-cell layer, whose axons gathered into a bundle to form optic nerve. Finally the signal is transferred to visual cortex in lateral geniculate body in thalamus by optic nerve. In this process, the amacrine cells mediate lateral connection among bipolar cells in IPL.

The accommodation of chemical signals is essential for the process of retinal neurons adapting light stimulus, among them the dopaminergic pathway plays an important role [6]. Dopamine (DA) is a neuromodulator. In retina, DA is synthesized and released by dopaminergic amacrine cells and interplexiform cells [7]. DA plays a lot of roles in the process of regulating circadian rhythm in retina cells, supporting nutrition for retina tissues, cell survival and eye development [6, 8].

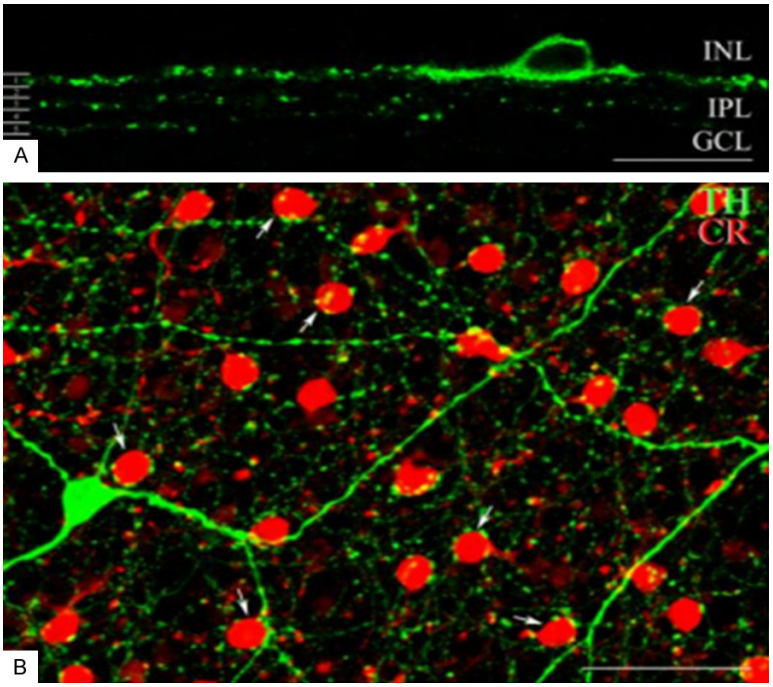

Figure 1. DA cells immunolabeled with TH antibodies and AII amacrine cells immunolabeled with CR antibodies in the rabbit retina. A. An image taken from a vertical section processed for TH immunoreactivities; B. A double-labeled image taken at the border of INL and IPL from a wholemount processed for TH (green) and CR (red) antibodies (Scale bars = 25 μm in A; 50 μm in B).

Dopaminergic amacrine (DA) cells are one of the best characterized neurons in the mammalian retina and their connections and modulations onto AII amacrine cell, an essential interneuron of the rod pathway, have already been reported [9, 10]. Dendrites of DA cells interplexiform cells concentrate upon IPL and they accept the input from bipolar cells and other kind of amacrine cells. At the same time, they regulate entoretina cells by direct synaptic linkage but control ectoretina cells (horizontal cells, photoreceptor and pigment epithelial cells) by diffusion of dopamine following concentration gradient [11, 12]. Synthesis and release of dopamine are influenced by illumination [13-15]. The retina release more dopamine in photopic adaptation state than in dark adaptation state [16, 17]. However, the stratification levels of dendrites of both amacrine cell types are slightly mismatched (the main dendrites of DA cells stratify at the outermost part of stratum 1 of the inner plexiform layer (IPL), while the lobular appendages of AII amacrines are distributed from stratum 1 and stratum 2) and little information on DA cells in the rabbit retina is available. Thus, we aimed to demonstrate the relationship between DA cells and AII amacrine cells and to characterize DA cells of the rabbit retina, by using double- and triple-labeling immunofluorescence and confocal microscopy.

Materials and methods

Materials

Tyrosine hydroxylase (TH), calretinin (CR), and vesicular glutamate transporter 1 (VGLUT1) antibodies were purchased from Santa Cruz Biotechnology; Goat anti rabbit γ-aminobutyric acid (GABA), vesicular GABA transporter (VGAT) and glutamic acid decarboxylase (GAD) antibodies were purchased from Santa Cruz Biotechnology.

Preparation of rabbit retina whole mounts and tissue slices

Tissue section methods followed established protocols. Rabbits were sacrificed by air injection in the marginal ear vein and their eyeballs were enucleated immediately. Retina tissues were fixed, dehydrated and paraffin-embedded by common practice. The whole mounts were sliced into 0.5 slices um along papilla optica and perpendicular to meridian.

Double- and triple-labeling with several antibodies for immunofluorescence and confocal microscopy

Double- and triple-labeling immunofluorescence and confocal microscopy were utilized in this study. Vertical sections from whole mounts of rabbit retina processed for tyrosine hydroxylase TH immunoreactivities were utilized to locate DA somata and their dendrites and that processed for calretinin (CR) immunoreactivities were utilized to locate AII somata and lobules; the whole mounts processed for TH, CR, and vesicular GABA (γ-aminobutyric acid) transporter VGAT immunoreactivities were utilized to study the expression of VGAT and that processed for TH, CR, GABA and glutamic acid decarboxylase (GAD) immunoreactivities were utilized to study the expression of two GABAergic

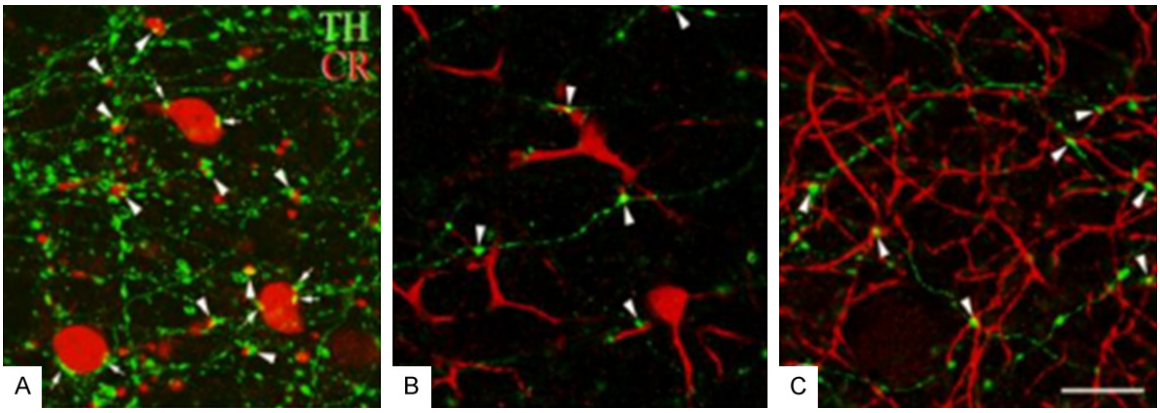

Figure 2. DA dendritic plexus stratified in different strata of the IPL. All the 3 images were taken at different focal planes in the same field of a wholemount processed for TH (green) and CR (red) antibodies. A. Stratum 1. B. Stratum 3. C. Stratum 5. Scale bar = 20 μm.

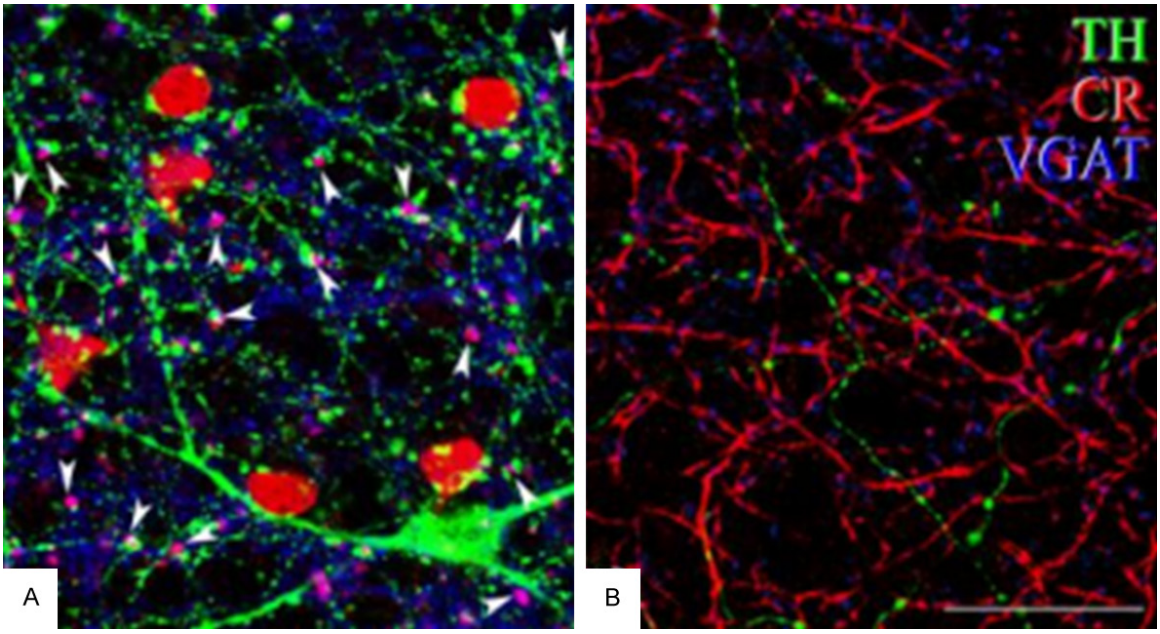

Figure 3. VGAT absent in DA varicosities of the rabbit retina. A. Stratum 1. B. Stratum 5. Scale bar = 50 μm.

markers, GABA and GAD in both DA and AII amacrine cells; the whole mounts processed for TH, CR, and vesicular glutamate transporter 1 (VGLUT1) immunoreactivities were utilized to study how AII lobules were contacted by DA varicosities.

Results

Distribution and contact between DA cells and AII amacrine cells in rabbit retina

In **Figure 1A**, we can see that a TH-labeled DA soma is localized in the INL adjacent to the IPL and its dendrites emerging from the soma are stratified in stratum 1 of the IPL. TH-immunolabled processes are also found in strata 3 and 5 of the IPL. In **Figure 1B**, A DA soma and numerous AII somata are seen. The matrix of DA dendrites with small varicosities covers the retina. Arrows indicate typical examples showing AII somata surrounded by DA varicosities.

We can see that TH-labeled DA dendrites form a dense matrix in **Figure 2A** (Stratum 1). Numerous DA dendritic varicosities contacting CR-labeled AII somata (arrows) and lobules (arrowheads) are clearly seen. In **Figure 2B**

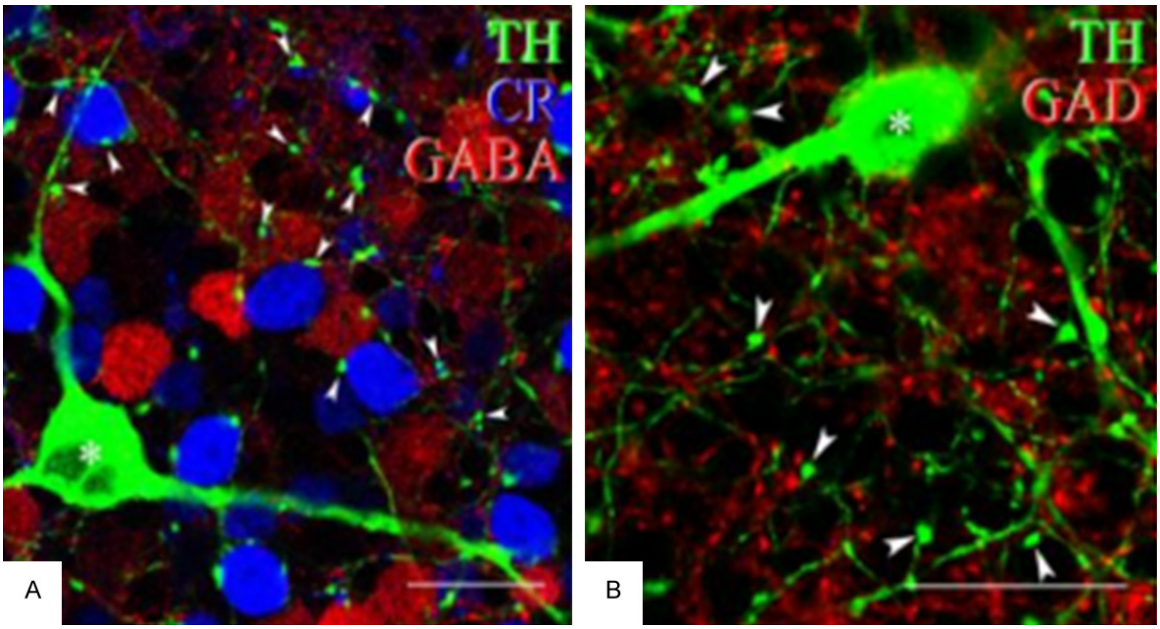

Figure 4. Distribution of two GABAergic markers in rabbit retina. Scale bars = 20 μm.

(Stratum 2), A few DA dendrites with varicosities (arrowheads) are visible. Most of them touch AII dendrites. In **Figure 2C** (Stratum 3), DA dendrites form a sparse matrix. Large varicosities (arrowheads) make contact with AII fine dendrites.

VGAT absent in DA varicosities

Triple-labeled confocal images in **Figure 3** were taken at different focal planes in the same field of a wholemount processed for TH (green), CR (red), and VGAT (blue) immunoreactivities. In **Figure 3A** (Stratum 1), a TH-labeled DA soma and processes with varicosities, and six CR-labeled AII somata and their lobules are seen. VGAT does not colocalize with DA varicosities, while VGAT immunoreactivity is seen in AII lobules (arrowheads), which are seen as pink, but AII somata do not. In **Figure 3B** (Stratum 5), A few DA dendrites with varicosities and the meshwork of AII arboreal dendrites are clearly seen. Both structures do not show VGAT immunoreactivity, indicating that they do not contain VGAT.

GABA and GAD absent in DA cell of the rabbit retina

Figure 4A is a triple-labeled confocal image taken at the border of INL and IPL from a wholemount processed for TH (green), CR (blue), and GABA (red) immunoreactivities. A TH-labeled DA soma and dendrites with varicosities which surround CR-labeled AII somata are clearly seen. The DA cell soma (asterisk) and varicosities (arrowheads) do not demonstrate GABA immunoreactivity. **Figure 4B** is a double-labeled confocal image that taken at the stratum 1 of the IPL from a wholemount processed for TH (red) and GAD (green). In this image, a DA soma (asterisk) and dendrites with varicosities (arrowheads) do not show GAD immunoreactivity.

Two kinds of AII lobules in sublamina a of the IPL

Two CR-labeled AII amacrine somata in the INL adjacent to the IPL and their dendrites through the IPL are seen in **Figure 5A**. Lobular appendages (arrows) arising from the main dendrite are distributed through strata 1 and 2 of the IPL, while the finer arboreal dendrites penetrate down into strata 3 to 5 close to the GCL. In **Figure 5B**, where the focus is at stratum 1 of the IPL, CR-labeled AII lobules (arrowheads) are contacted by TH-labeled DA varicosities and VGLUT1-labeled cone bipolar terminals. In **Figure 5C**, where the focus is at stratum 2 of the IPL, AII lobules (arrowheads) are still touched by cone bipolar terminals, whereas are not made contacts by DA varicosities. **Figure**

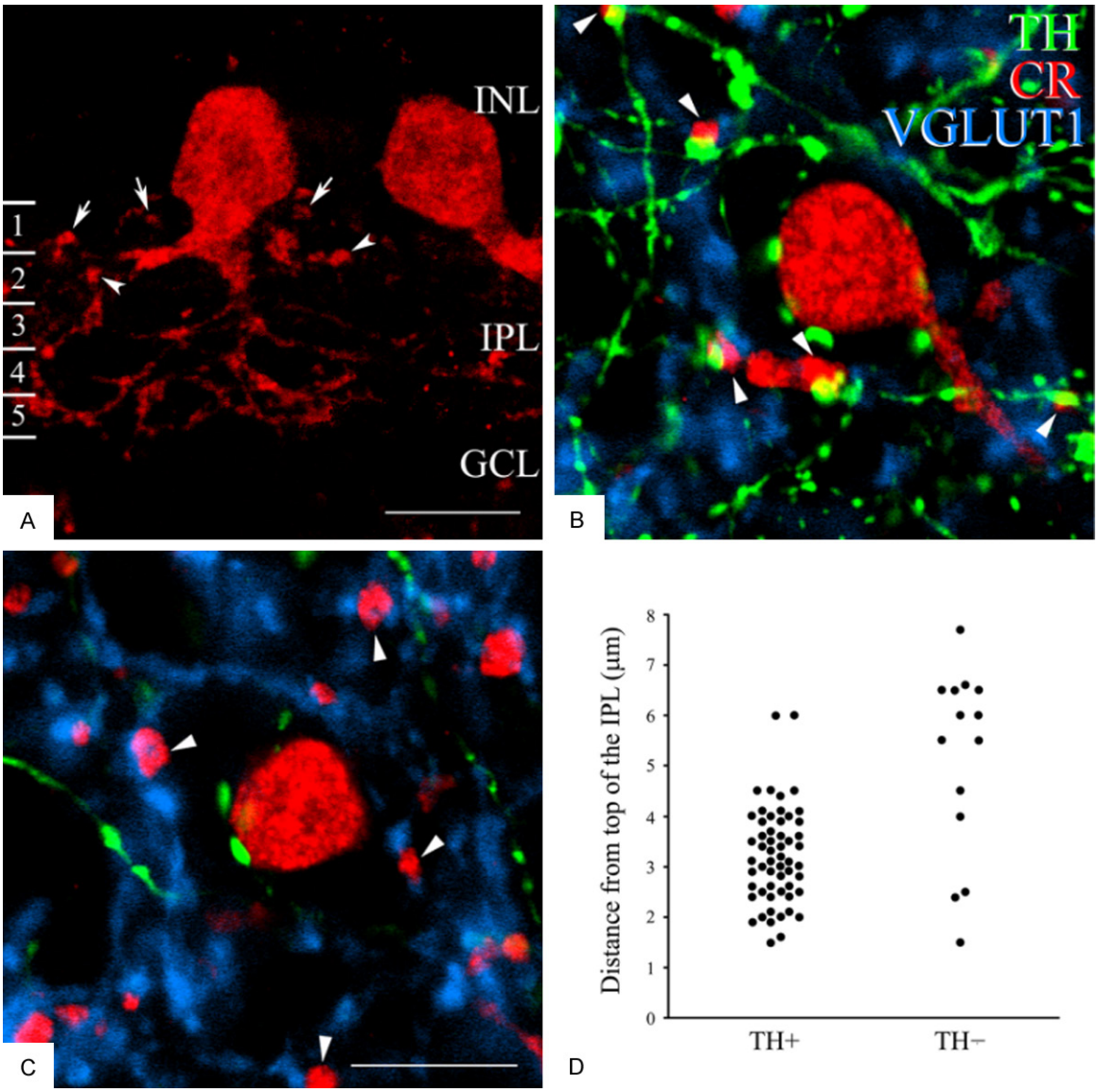

Figure 5. Two kinds of AII lobules in sublamina of the IPL. A. An image taken from a vertical section processed for CR immunoreactivities. B, C. Triple-labeled confocal images taken at different focal planes in the same field of a wholemount processed for TH (green), CR (red), and VGLUT1 (blue) immunoreactivities. D. Distribution of 70 AII lobules which are contacted by DA contacted varicosities (TH+) and not ones (TH-), according to the depth of the IPL. Scale bars = 10 μm.

5D shows that almost AII lobules in stratum 1 of the IPL are contacted by DA varicosities while AII lobules at deeper portion are not contacted by DA varicosities.

Discussion

Production of dopamine in retina cells is very conservative; most of them are located at inner nuclear layer. Tyrosine hydroxylase (TH) is a rate-limiting enzyme for the multi-procedure reaction of dopamine biosynthesis. Dopamine is synthesized in the distal end of synapse with two steps of enzymatic reaction: At beginning, tyrosine is transited into dopa with the help of TH; the dopa is further transited into dopamine with the help of aromatic amino aciddecarboxylase. In this study, the TH was utilized as the maker for DA cells. Calcium binding protein calretinin can be used to label the population of AII amacrine cells selectively. AII amacrine cells are small, distinctly bistratified cells, with so-called lobular appendages in sublamina a of the IPL and bushy dendritic fields in sublamina

b. Cells of this characteristic shape have been recognized in practically all mammalian retinae studied, including those of marsupials [18, 19]. Calretinin-positive amacrine cells had the morphological attributes of AII amacrine cells [20]. GABA is a major inhibitory transmitter in retina [21, 22]. GABA induces inhibition in vision formation process and plays a neurotrophic role in retina development process [23-25]. GAD and VGAT play a part in GABA synthesis and transport process respectively. GABA is contained and synthesized in not only GABAergic retinal cells but also dopaminergic retinal cells in mammals [26]. However, in this study, double- or triple-labeling experiment showed that GABAergic markers such as GABA, VGAT, and GAD were not expressed in DA cells of the rabbit retina. Thus, DA cells of the rabbit retina do not use GABA as a neurotransmitter, unlike other mammals.

Accurate synapse contacts are constructed among different types of nerve cells in mammal retina. These synapse contacts are the keys for visual information processing and accurate visual sensation and perception. Amacrine cells and bipolar cells construct functional nerve ring pathway with classical dendrite apophysis prominence pattern in inner plexiform layer. This area is conventionally divided into 5 paralleled layers [27]. Retina amacrine cells are fractionated into many isoforms, including dopamicergic amacrine cells. All dopamicergic amacrine cells can be dyed in dopamine synthetase and tyrosine hydroxylase immunohistochemical experiment, which suggesting that the dopamicergic amacrine cells are a widely distributed amacrine cells. Extensive dendrite branches exist in the layer close to amacrine cell soma. The thinnest dendrites and axons spread out several hundreds of micrometer and their distal ends surround the soma and dendrites of AII amacrine cells and other kinds of amacrine cells. Abundant synapses join the main vision pathway. In addition, the dopamicergic amacrine cells accept synapses from other amacrine cells and cop bipolar cells. Dopaminergic neuron is a kind of characteristic amacrine cells in retina. Dopamine release in the cells is regulated by outside light and biological clock. Besides, dopamine is nutrition for retina [28, 29]. In mammalian retina, DA cells characteristically contact with rod cells and widely contact with AII amacrine cells which insert in rod bipolar cells and ganglion cells. In this study, DA dendrites stratified at strata 1, 3 and 5 of the IPL. In stratum 1, most of DA dendritic varicosities which are presumed sites of neurotransmitter release made contact with somata or lobular appendages of AII amacrine cells. In addition, some of varicosities in strata 3 and 5 contacted arboreal dendrites of AII amacrine cells. Numerous lobular appendages of AII amacrines localized at stratum 2 of the IPL showed little contact with DA varicosities.

Disclosure of conflict of interest

None.

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