https://doi.org/10.5607/en.2017.26.6.329
Exp Neurobiol. 2017 Dec;26(6):329-338.
pISSN 1226-2560 • eISSN 2093-8144

Original Article

Experimental Neurobiology

Reexamination of Dopaminergic Amacrine Cells in the Rabbit Retina: Confocal Analysis with Double- and Triple-labeling Immunohistochemistry

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Dopaminergic amacrine cells (DACs) are among the most well-characterized neurons in the mammalian retina, and their connections to AII amacrine cells have been described in detail. However, the stratification of DAC dendrites differs based on their location in the inner plexiform layer (IPL), raising the question of whether all AII lobules are modulated by dopamine release from DACs. The present study aimed to clarify the relationship between DACs and AII amacrine cells, and to further elucidate the role of dopamine at synapses with AII amacrine cell. In the rabbit retina, DAC dendrites were observed in strata 1, 3, and 5 of the IPL. In stratum 1, most DAC dendritic varicosities—the presumed sites of neurotransmitter release—made contact with the somata and lobular appendages of AII amacrine cells. However, most lobular appendages of AII amacrine cells localized within stratum 2 of the IPL exhibited little contact with DAC varicosities. In addition, double- or triple-labeling experiments revealed that DACs did not express the GABAergic neuronal markers anti-GABA, vesicular GABA transporter, or glutamic acid decarboxylase. These findings suggest that the lobular appendages of AII amacrine cells are involved in at least two different circuits. We speculate that the circuit associated with stratum 1 of the IPL is modulated by DACs, while that associated with stratum 2 is modulated by unknown amacrine cells expressing a different neuroactive substance. Our findings further indicate that DACs in the rabbit retina do not use GABA as a neurotransmitter, in contrast to those in other mammals.

Key words: Dopamine, AII amacrine cell, Scotopic pathway, GABA, Retinal circuit

INTRODUCTION

Dopamine is the predominant catecholamine transmitter in the mammalian retina [1]. At present, the most effective marker of dopaminergic neurons in the retina is an antibody against tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis, because norepinephrine and epinephrine typically occur only in trace amounts in the retina [2]. Dopaminergic neurons of the retina form a dense dendritic plexus at stratum 1 of the inner plexiform layer (IPL). In rabbit and some other species, dopaminergic neurons are typical amacrine cells whose dendrites do not extend beyond the IPL [3]. In other species, they are interplexiform

Received October 27, 2017, Revised November 17, 2017,
Accepted November 20, 2017

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cells, which send additional processes to the outer plexiform layer (OPL) [4]. Dopaminergic amacrine cells (DACs) are a particularly well-described subtype of retinal neurons, and are among the wide-field amacrine cells that receive input from the cone bipolar cells [5-7]. DACs in the retina establish synapses on AII amacrine cells [6-10], which are involved in the rod pathway that carries dim-light signals to ganglion cells. The balance between rod and cone inputs to the ganglion cells is under the control of AII amacrine cells, which are inhibited by DACs [11, 12]. Light increases dopamine release, and this light-induced release of dopamine is believed to play a role in the inhibitory mechanisms underlying light adaptation [7, 12, 13]. Thus, dopamine acts as a neurotransmitter and modulator in the retina.

AII amacrine cells are crucial interneurons of the scotopic pathway, as these cells transfer rod signals to the photopic pathway and represent the main synaptic target of DACs. AII amacrine cells have distinct bistratified dendrites: Lobular appendages arising from the main dendrite are distributed over sublamina *a* (strata 1 and 2) of the IPL, and the finer "arboreal dendrites" penetrate downward and stratify throughout sublamina *b* (strata 3 to 5) [14]. The lobular appendages of AII amacrine cells form chemical synapses with the axon terminals of OFF-cone bipolar cells in sublamina *a* of the IPL, whereas their primary dendrites and vitreal branches form extensive gap junctions with the axon terminals of ON-cone bipolar cells [15-19]. Research has indicated that dopamine is involved at synapses between lobular appendages and OFF-cone bipolar cells in sublamina *a* [6-10]. However, DAC dendrites very narrowly stratify in the most distal region of stratum 1 in the IPL, while AII lobules are localized throughout strata 1 and 2, raising the question of whether all AII lobules are modulated by dopamine release from DACs.

Colocalization of dopamine with other neurotransmitters appears to be common in the brain. For example, γ-aminobutyric acid (GABA) is localized in some periglomerular cells of the olfactory bulb [20] and in a subpopulation of dopamine-containing neurons of the substantia nigra [21], whereas glutamate is localized in the remaining nigral neurons [22] and ventral tegmental neurons [23]. In the retina of various mammalian species, DACs contain GABA [20, 24, 25]. A previous study in the rodent retina has demonstrated that synapses between DAC and AII amacrine cells are GABAergic [26]. Nevertheless, additional evidence indicates that only very few of DACs contain very low levels of GABA [27], suggesting that DAC synapses are not GABAergic in all mammalian retinas. Moreover, recent reports have demonstrated that DACs also express glutamate [28, 29].

In the present study, we aimed to further elucidate the relationship between DACs and AII amacrine cell, as well as the role of dopamine at synapses between the two cell types. In addition, we aimed to determine whether DACs in the rabbit retina use GABA as a neurotransmitter, similar to those in other mammals. Our findings demonstrated that DACs exert a substantial influence on AII amacrine cells, although DACs may not form synapses at all AII lobules. We further observed that DACs in the rabbit retina do not contain any reliable GABAergic markers, such as GABA, vesicular GABA transporter (VGAT), or the catalytic enzyme glutamic acid decarboxylase (GAD). Thus, our findings indicate that DACs in the rabbit retina are not GABAergic, in contrast to those of other mammals.

MATERIALS AND METHODS

Animals

Eight adult albino rabbits of both sexes (weight: 3~5 kg) were used for the experiments of the present study. Retinas were prepared in accordance with previously described methods [30]. Briefly, animals were deeply anesthetized via an intraperitoneal (i.p.) injection of urethane (loading dose: 1.5 g/kg). Following enucleation, animals were sacrificed *via* anesthetic overdose, and the eyes were hemisected. The cornea, lens, and vitreous humor were removed, and the posterior portion of the eye was cut into several pieces and immersed in bicarbonate-based Ames' solution, which was gassed with 95% O_2 and 5% CO_2. Use of animals was approved by the IACUC (Institutional Animal Care and Use Committee) in College of Medicine, The Catholic University of Korea (No. 2013-0088-06).

Immunohistochemistry

The retina was removed from the sclera, placed onto nitrocellulose filter paper, and fixed in 4% paraformaldehyde for 2 h. After fixation, the retina was washed with phosphate-buffered saline (PBS) containing 0.5% Triton and 0.1% sodium azide.

Immunohistochemistry was conducted in accordance with previously described methods [30]. Briefly, whole-mount retina or free-floating vibratome vertical preparations were blocked with 3% donkey serum in PBS containing 0.5%Triton X-100/0.1% sodium azide for 2 h to reduce nonspecific labeling. The tissues were then incubated in mixture of primary antibodies—usually mouse monoclonal antibodies to TH (1:3,000; provided by Dr. John Haycock, Louisiana State University, Baton Rouge, LA) and goat polyclonal antibodies to calretinin (CR) (1:4,000; Chemicon, El Segunda, CA)—in the presence of 1% donkey serum/PBS/ with 0.5%Triton X-100/0.1% sodium azide for 5~10 days. Samples that lacked primary antibodies were used as blank controls. Following extensive washing with PBS containing 0.5%Triton X-100/0.1%

https://doi.org/10.5607/en.2017.26.6.329

sodium azide, the tissue was incubated overnight with a 1:200 dilution of secondary antibodies conjugated to the appropriate fluorochromes, which were Cy-3 (Jackson Labs, West Grove, PA), and Alexa 488 (Molecular Probes, Eugene, OR). After several washes in PBS, the fluorescent specimens were mounted with Vectashield mounting medium (Vector, Burlingame, CA). Digital images (1,024×1,024 pixels) were acquired using a Zeiss LSM 510 confocal microscope and processed in Photoshop (Adobe Systems, San Jose, CA).

For triple-labeling experiments, the retina was incubated in antibodies against TH (1: 3,000), CR (1:4,000), and one of the following, as appropriate: vesicular glutamate transporter 1 (VGLUT1) (guinea pig polyclonal, 1:1,000; Chemicon), VGAT (guinea pig polyclonal, 1:1,000, Chemicon), GABA (rabbit polyclonal, 1:1,000; provided by Dr. David Pow, University of Queensland, Brisbane, Austrailia), or GAD65/67 (rabbit polyclonal, 1:1,000; Chemicon). Appropriate secondary antibodies conjugated with Cy-3, Cy-5, or Alexa 488, were used.

Image analysis

Image-Pro Plus and custom software were used to analyze the association between DAC varicosities and AII lobules [31]. Boxes (50×50 pixels) were clipped from the image by centering a sampling box around the CR-immunoreactive AII lobules in stratum 1 of the IPL. Alignment and averaging of these boxes produced a plot of color intensity against the location of pixels in the *x-y* plane. This method determines the average distribution of each labeled channel around a repeated neuronal structure (e.g., AII lobules in the present study). Controls were established by rotating one channel by 180° or transposing one channel from left to right.

RESULTS

DAC in the rabbit retina

An antibody against TH clearly labeled the somata and dendrites of DACs, as previously reported [2, 32]. In the rabbit retina, DACs exhibited large somata and dendrites, which were located adjacent to the inner nuclear layer (INL) in stratum 1 of the IPL (Fig. 1A). In addition, a small number of TH-labeled dendrites were observed in strata 3 and 5 of the IPL. While these rather sparse processes may have represented dendrites arising from DACs, they may also have represented those of type II catecholaminergic amacrine cells [3]. In whole-mount preparations, each DAC soma gave rise to 3 to 5 primary dendrites, as well as fine dendrites that formed ring-like structures around AII amacrine somata labeled with an antibody against CR (Fig. 1B). These findings are in accordance with the previously reported properties of typical DACs [2, 8, 9].

In the present study, we aimed to demonstrate the relationship between DAC varicosities and both types of AII dendrites—lobules and arboreal dendrites—which are associated with chemical and electrical synapses, respectively. Fig. 2A shows the relationship between DAC varicosities and AII lobules in stratum 1 of the IPL. In this stratum, DAC dendrites (green) with varicosities form a dense plexus, and the base of AII somata and lobules (red) can be observed. The merged image indicates that almost all AII lobules and AII somata appear to be localized close to DAC varicosities. Therefore, these findings demonstrate that AII lobules form synapses with DAC varicosities in stratum 1 of the IPL.

The relationships between DAC varicosities and AII arboreal dendrites in strata 3 and 5 of the IPL are shown in Figs. 2B and 2C, respectively. Although a few DAC dendrites with varicosities were observed in stratum 3, several AII primary and secondary arboreal dendrites were identified in this region. DAC varicosities with a diameter >0.5 μm appeared to associate with AII dendrites in stratum 3. Among DAC varicosities in stratum 3, 66% (76/116) made

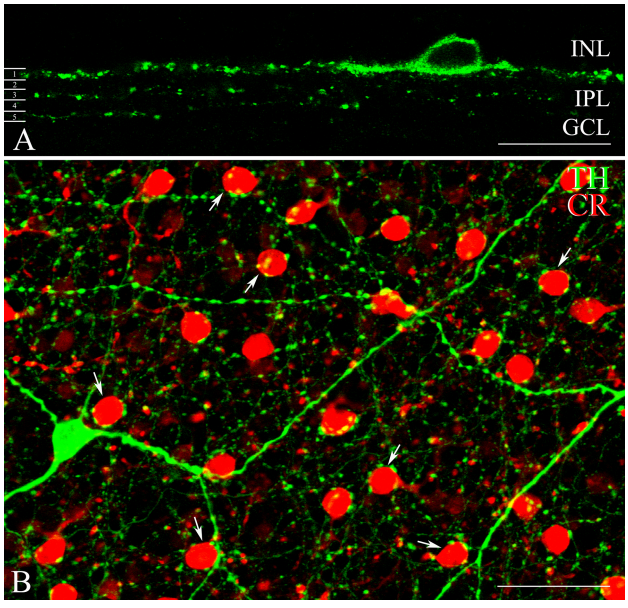

Fig. 1. DACs immunolabeled with TH antibodies in the rabbit retina. (A) An image obtaind from a vertical section processed for TH immunoreactivity. A TH-labeled DAC soma is localized in the region of the INL adjacent to the IPL, and dendrites emerging from the soma are stratified in stratum 1 of the IPL. TH-immunolabeled processes can also be observed in strata 3 and 5 of the IPL. (B) A double-labeled image obtained at the border between the INL and IPL in a whole-mount preparation processed for TH (green) and CR (red) antibodies (an AII marker). A DAC soma and numerous AII somata can be observed. The matrix of DAC dendrites with small varicosities covers the retina. Arrows indicate typical examples of AII somata surrounded by DAC varicosities. Scale bars=25 μm in A; 50 μm in B. DACs: Dopaminergic amacrine cells; TH: tyrosine hydroxylase; INL: inner nuclear layer; IPL: inner plexiform layer; CR: calretinin.

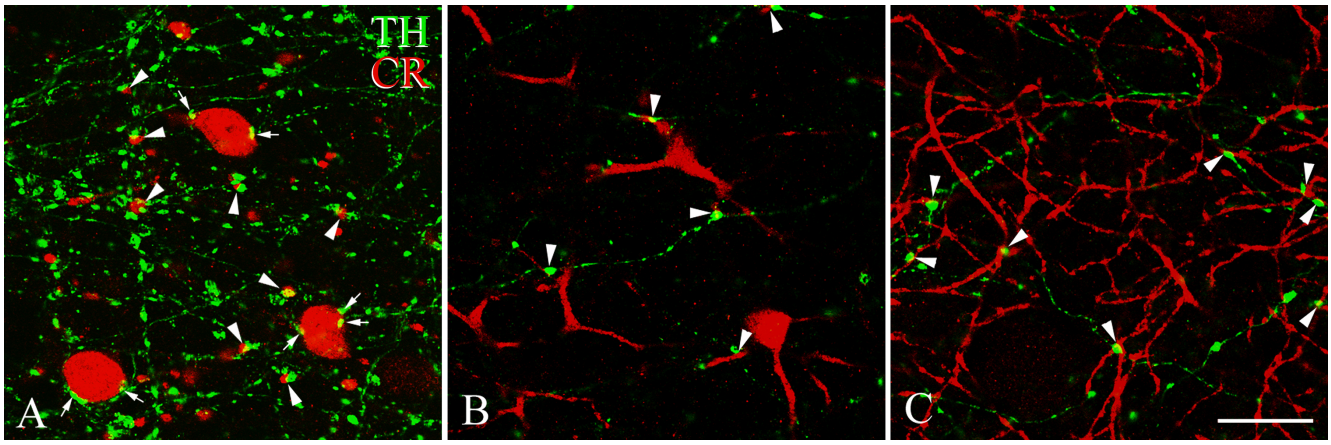

Fig. 2. DAC dendritic plexus stratified in different strata of the IPL. Images were obtained form different focal planes in the same field of a whole-mount preparation processed for TH (green) and CR (red) antibodies. (A) Stratum 1. TH-labeled DAC dendrites form a dense matrix. Numerous DAC dendritic varicosities contact CR-labeled AII somata (arrows) and lobules (arrowheads). (B) Stratum 3. A few DAC dendrites with varicosities (arrowheads) are visible, most of which contact AII dendrites. (C) Stratum 5. DAC dendrites form a sparse matrix. Large varicosities (arrowheads) make contact with fine dendrites of AII cells. Scale bar=20 μm. DACs: Dopaminergic amacrine cells; TH: tyrosine hydroxylase; CR: calretinin.

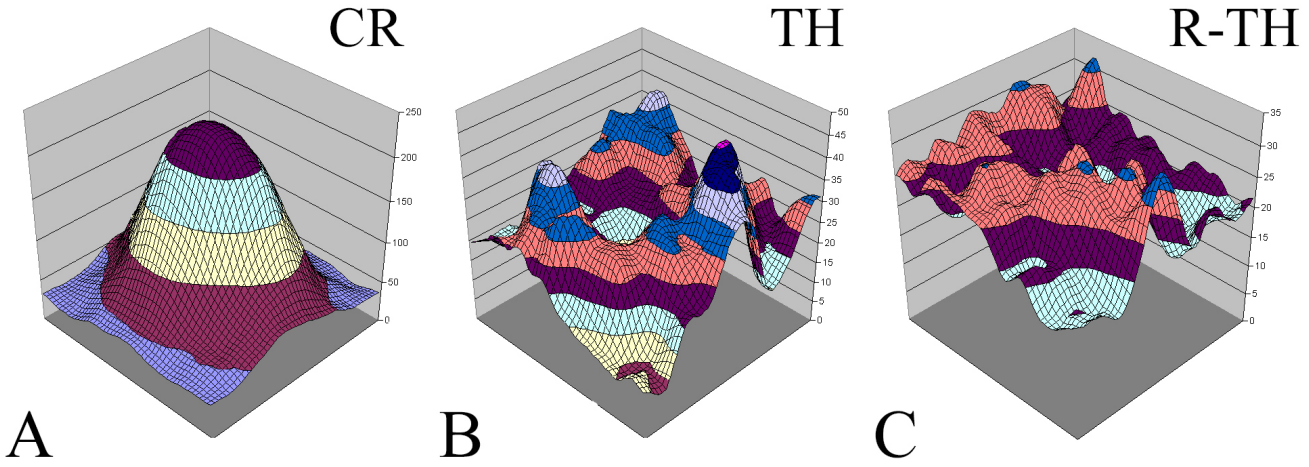

Fig. 3. Analysis of the relationship between DAC varicosities and AII lobules. The analysis was conducted in a retinal whole-mount preparation processed for TH (green) and CR (red) immunoreactivity in stratum 1 of the IPL, where TH-labeled DAC cells form a main dendritic matrix. Surface plots show the average distribution of labeling in each channel. Sampling boxes (50×50 pixels; n=54) were clipped from the image by centering the sampling box around the CR-immunoreactive AII lobules in stratum 1 of the IPL. This analysis verified the contacts depicted in Fig. 2A. (A) The average AII signal is represented as a surface plot of the intensity of red pixels (*z* axis) vs. the location of the pixels (*x* and *y* axes). A sharp peak can be observed at the center of the plot due to the alignment of the sampling boxes with AII lobules. (B) The caldera of green pixels indicates that the probability of finding DAC varicosities adjacent to AII lobules is high. (C) Controls were developed by rotating the DAC image 180° out of phase. When the same analysis was performed around AII lobules, no discernible structure was observed in the plot, indicating an absence of the spatial relationship observed in (B). DACs: Dopaminergic amacrine cells; TH: tyrosine hydroxylase; IPL: inner plexiform layer; CR: calretinin.

contact with AIIs. In stratum 5, several DAC dendrites were visible, along with a dense arboreal meshwork of AII cells, 67% (104/156) made contact with AII dendrites.

We further quantitatively assessed the relationship between DAC varicosities and AII lobules in stratum 1 of the IPL, using custom-made software [31]. A cursor was centered on each AII lobule, and a 50-×-50-pixel box was clipped from the image. During this manual selection process, the red and green channels were turned off to reduce operator bias. These image sections were then aligned and averaged. In effect, this method enables one to analyze the average distribution of labeling in the other two channels around a repeated neuronal structure. In the present study, we evaluated the average distribution of DAC varicosities around AII lobules (Fig. 3). Fig. 3A shows the central peak of AII lobules with very low noise, while Fig. 3B shows the average distribution of DAC varicosities around the AII lobules. In Fig. 3A, a distinct volcanic caldera can

https://doi.org/10.5607/en.2017.26.6.329

be observed around a central cavity coincident with the average AII lobular peak. In this case, the height of the caldera immediately adjacent to the AII lobules exceeds the local noise at a distance from the central cavity. This result demonstrates that, on average, there is a high probability of encountering DAC varicosities immediately adjacent to an AII lobule. Controls for this analysis were developed by rotating the DAC 180° from the AII image. Following rotation, we observed no peaks in association with the AII lobular signal (Fig. 3C), indicating that AII lobules form synapses with DAC varicosities in this stratum.

Two types of AII lobules: synapses with or without DAC varicosities

AII amacrine cells are crucial interneuron of the scotopic pathway, and their morphology has been well-characterized in previous studies [14, 19, 33]. Fig. 4A shows an AII amacrine cell with distinct bistratified dendrites in a vertical section of the rabbit retina: Lobular appendages arising from the main dendrite are dis-

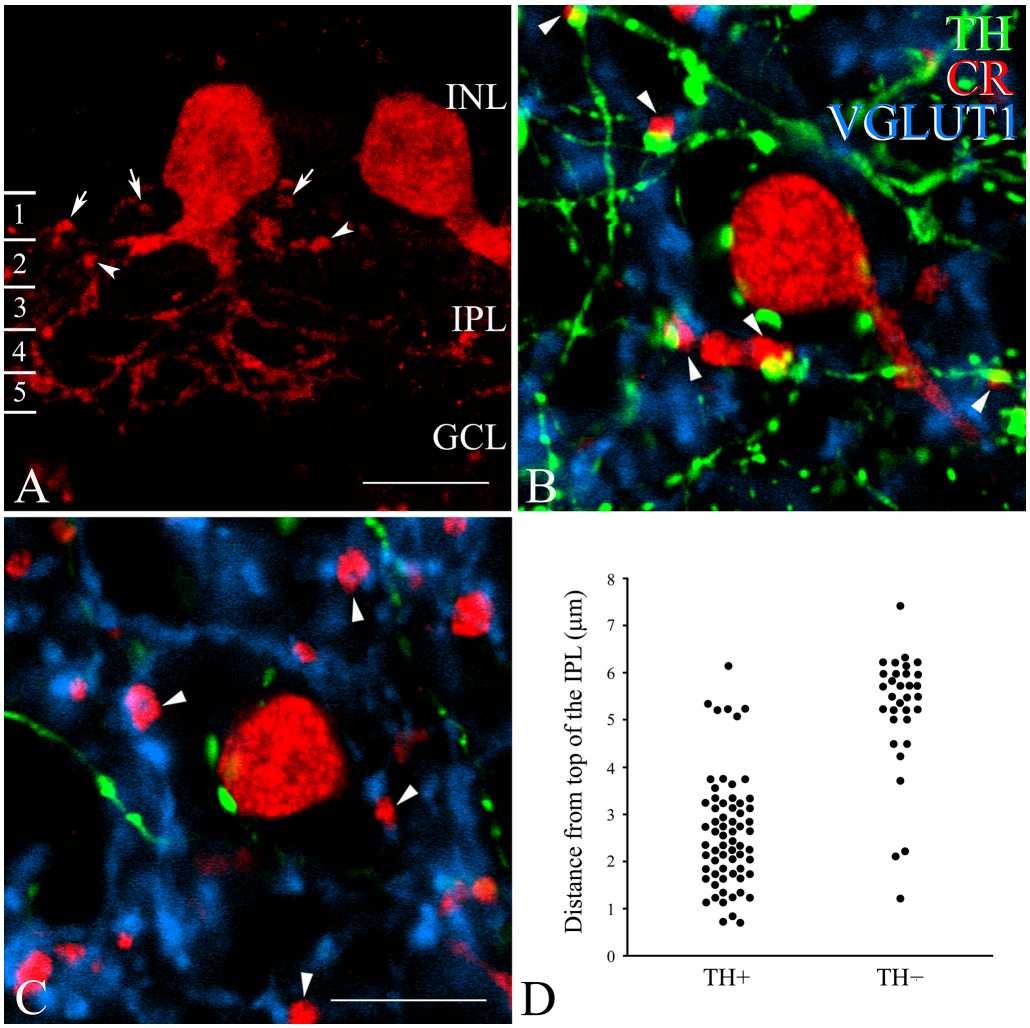

Fig. 4. Two types of AII lobules in sublamina *a* of the IPL. (A) An image obtained from a vertical section processed for CR immunoreactivity. Two CR-labeled AII amacrine somata can be observed in the region of the INL adjacent to the IPL, with dendrites extending through the IPL. Lobular appendages (arrows) arising from the main dendrite are distributed throughout strata 1 and 2 of the IPL, while the finer arboreal dendrites penetrate downward into strata 3 through 5, extending towards the GCL. (B, C) Triple-labeled confocal images obtained at different focal planes in the same field of a whole-mount processed for TH (green), CR (red), and VGLUT1 (blue) immunoreactivity. (B) Depicts stratum 1 of the IPL, in which CR-labeled AII lobules (arrowheads) are contacted by TH-labeled DAC varicosities and the terminals of VGLUT1-labeled cone bipolar cells. (C) Depicts stratum 2 of the IPL, in which AII lobules (arrowheads) remain in contact with the terminals of cone bipolar cells, but not with DAC varicosities. (D) Distribution of AII lobules in (TH+) and out of (TH-) contact with DAC varicosities, according to depth in the IPL. The graph shows that most AII lobules in stratum 1 of the IPL are contacted by DAC varicosities, while AII lobules in deeper regions are not contacted by DAC varicosities. Scale bars=10 µm. DACs: Dopaminergic amacrine cells; TH: tyrosine hydroxylase; INL: inner nuclear layer; IPL: inner plexiform layer; CR: calretinin; GCL: granule cell layer; VGLUT1: vesicular glutamate transporter 1.

tributed through strata 1 and 2 of the IPL, while the finer arboreal dendrites penetrate strata 1 and 2, and stratified throughout strata 3 to 5, in accordance with the findings of previous studies [14, 33]. Previous studies have suggested that the synapses between AII lobules and the varicosities of DACs in stratum 1 are dopaminergic [6-10]. However, DAC dendrites very narrowly stratify in the most distal portion of stratum 1 in the IPL, while AII lobules are localized throughout strata 1 and 2. Therefore, we hypothesized that AII lobules exhibit two types of synaptic circuits in sublamina *a* of the IPL: dopamine-related and dopamine-non-related. To test this hypothesis, we examined the distribution of AII lobules in sublamina *a* of the IPL.

Fig. 4B and 4C include stack images (thickness: 1 μm) obtained at different focal planes in the same field of a whole-mount preparation, showing the relationship among AII lobules (red), DAC varicosities (green) and the terminals of OFF-cone bipolar cells (blue) according to the level of sublamina *a* of the IPL. In Fig. 4B, which focuses on the level of stratum 1 adjacent to the INL, an AII soma and several lobules are contacted by DAC varicosities and OFF-cone bipolar terminals labeled with an antibody against VGLUT1. In Fig. 4C, which focuses on stratum 2, different lobules make contact with OFF-cone bipolar terminals, but not with DAC varicosities. These results are consistent with our working hypothesis. We further investigated 97 lobules around 6 AII somata (Fig. 4D). Among these lobules, 21.3% (32/97) were not contacted by DAC varicosities. Most such lobules were located in deeper regions of sublamina *a*, while the lobules contacting DAC varicosities (65/97) were superficially distributed in sublamina a.

Expression of GABAergic markers in DACs

DACs are known to contain GABA and are thus regarded as a GABAergic subpopulation in the mammalian retina [20, 24, 25]. Recently, Contini and Raviola demonstrated that synapses formed between DACs and AII amacrine cells in the rodent retina are GABAergic [26]. However, some evidence suggests that that only very few of DACs contain very low level of GABA [27]. Moreover, recent reports have demonstrated that DACs express glutamate rather than GABA [28, 29]. Thus, we aimed to confirm whether DAC synapses in the rabbit retina are GABAergic.

We first investigated whether GABA is localized in DAC varicosities using an antibody against VGAT, a transporter that delivers GABA to synaptic vesicles at presynaptic terminals [34]. Fig. 5 depicts images obtained from a whole-mount preparation triple-labeled with antibodies against TH (green), CR (red), and VGAT (blue). VGAT immunoreactivity was not observed in DAC varicosities, soma, or dendrites; however, VGAT-labeled cells were observed in all lobules (but not somata) in stratum 1 of the IPL (Fig. 5A, arrowheads). In stratum 5, VGAT did not colocalize with TH and CR, indicating that DAC processes and AII arboreal dendrites in this stratum do not contain GABA (Fig. 5B).

We then aimed to confirm whether DACs of the rabbit retina are GABAergic *via* double-labeling with antibodies against GABA

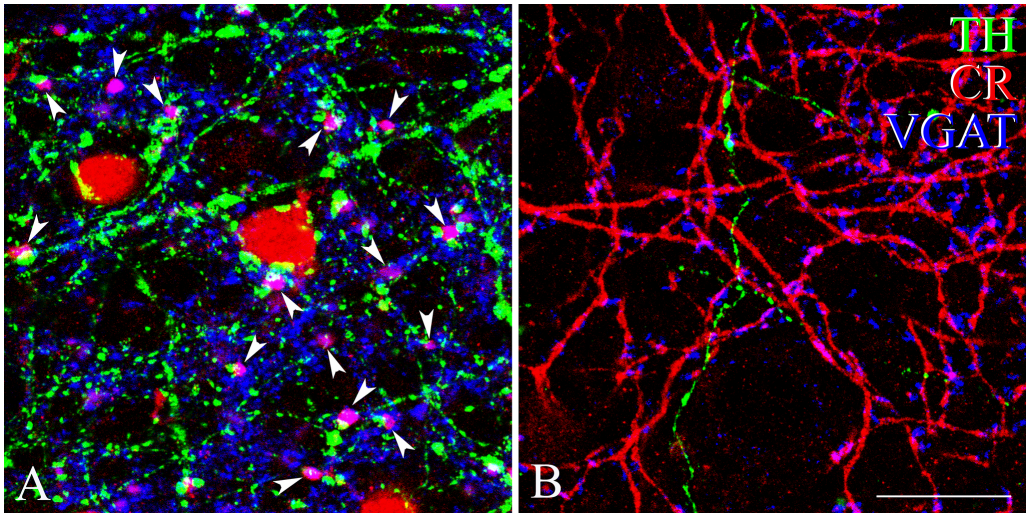

Fig. 5. VGAT is absent in DAC varicosities of the rabbit retina. Triple-labeled confocal images obtained at different focal planes in the same field of a whole-mount preparation processed for TH (green), CR (red), and VGAT (blue) immunoreactivity. (A) Stratum 1. A TH-labeled DAC soma with dendritic varicosities can be observed, along with 6 CR-labeled AII somata and their lobules. VGAT does not colocalize with DAC varicosities, while VGAT immunoreactivity can be observed in AII lobules (pink, arrowheads), but not in AII somata. (B) Stratum 5. A few DAC dendrites with varicosities can be observed, although the meshwork of AII arboreal dendrites is clearly visible. Neither structure exhibits VGAT immunoreactivity, indicating an absence of VGAT. Scale bar=50 μm. DACs: Dopaminergic amacrine cells; TH: tyrosine hydroxylase; CR: calretinin; VGAT: vesicular GABA transporter.

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and GAD. Fig. 6A depicts the border between the INL and IPL, at which a DAC soma with dendritic varicosities can be observed, along with AII somata and GABA-labeled somata/processes. As no overlap was observed among the three profiles, these findings indicate that DACs in the rabbit retina do not contain GABA in their processes, varicosities, or somata. Confocal imaging of sections double-labeled with TH (red) and GAD (green) failed to identify localized GAD immunoreactivity in DAC somata or dendrites (Fig. 6B). These results clearly demonstrate that DACs in the rabbit retina are not GABAergic.

DISCUSSION

Recent studies have revealed that DACs innervate AII amacrine cells in a compartment-specific manner and make contact with various types of amacrine cells, including AII cells [35, 36]. In the present study, we investigated the relationship between DACs and AII amacrine cells in the rabbit retina, as well as the neurochemical property of DACs, using immunohistochemistry and confocal microscopy. In accordance with the findings of previous studies, our results indicate that DACs exert a substantial influence on AII somata and lobules. Interestingly, however, we also identified AII lobules that were not contacted by DAC varicosities in stratum 2 of sublamina *a* of the IPL. Furthermore, our findings indicate that DACs in the rabbit retina are not GABAergic, in contrast to those of other mammals.

In the present study, the stratification and morphological characteristics of DAC somata and dendrites were consistent with those reported in previous reports [2, 32]. The characteristic rings surrounding AII somata at the border between the INL and IPL were clearly observed. Although the synaptic circuits associated with these characteristic rings have been well-described, the precise circuitry associated with synapses between DACs and AII cells remains to be elucidated.

The lobular appendages are the major chemical synaptic output sites of AII amacrine cells [15, 17]. These lobules form reciprocal inhibitory synapses with the terminals of OFF-cone bipolar cells in strata 1 and 2 [17, 18, 37, 38]. These synapses account for 90% of the output from AII amacrine cells, which rarely synapse directly onto OFF-ganglion cells in the rabbit retina [17]. Both AII somata and dendrites are postsynaptic to other amacrine cells in stratum 1. Double labeling studies have revealed that the dendrites of DACs contact lobules and encircle AII somata, forming characteristic rings [6, 8, 10]. These DAC inputs are believed to mediate changes in AII/AII coupling that occur during light adaptation [11, 12].

In the present study, we demonstrated that AII lobules in stratum 1 of sublamina *a* of the IPL were exclusively contacted by DAC varicosities, while most AII lobules in stratum 2 are not. This finding implies that rod signals are processed by AII amacrine cells in sublamina *a via* two different synaptic circuits. In accordance with

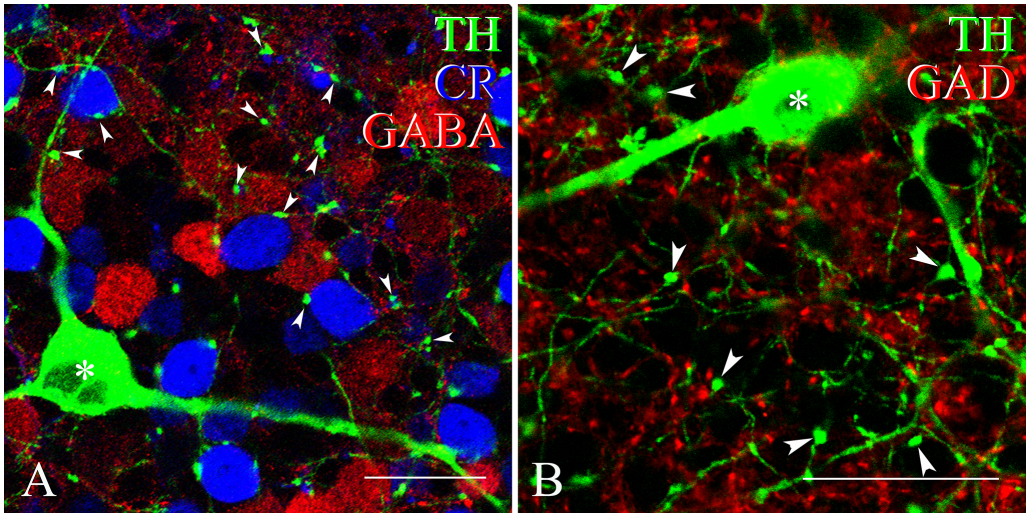

Fig. 6. Two GABAergic markers were absent in DACs of the rabbit retina. (A) A triple-labeled confocal image obtained at the border between the INL and IPL in a whole-mount preparation processed for TH (green), CR (blue), and GABA (red) immunoreactivity. A TH-labeled DAC with dendritic varicosities surrounding CR-labeled AII somata can be observed. The DAC soma (asterisk) and varicosities (arrowheads) do not demonstrate GABA immunoreactivity. (B) A double-labeled confocal image obtained at the stratum 1 of the IPL from a whole-mount preparation processed for TH (red) and GAD (green). Neither the DAC soma (asterisk) or dendritic varicosities (arrowheads) exhibit GAD immunoreactivity. Scale bars=20 μm. DACs: Dopaminergic amacrine cells; TH: tyrosine hydroxylase; INL: inner nuclear layer; IPL: inner plexiform layer; CR: calretinin; GAD: glutamic acid decarboxylase.

these findings, Merighi et al. demonstrated that at least two different types of OFF-cone bipolar cells (DAPI-Ba1 and DAPI-Ba2) are postsynaptic to AII lobules in different regions of sublamina *a* of the IPL in the rabbit retina [38]. Moreover, at least 6 different kinds of OFF-cone bipolar cells have been reported in the rabbit retina [39, 40]. A recent connectomic analysis by Marc et al. demonstrated that all OFF-cone bipolar cells make contact with AII lobules [41]. Taken together, these findings indicate that AII amacrine cells transfer rod signals onto ganglion cell channels *via* various types of OFF-cone bipolar cells in sublamina *a* of the IPL, and that dopamine released by DACs may modulate synaptic connections between AII lobules and OFF-cone bipolar terminals within specific circuits.

GABA is colocalized with dopamine in DACs of the retina in various mammalian species [20, 24, 25]. A clever experiment by Contini and Raviola demonstrated that DAC varicosities express both GABA and VGAT, and that postsynaptic GABAA receptors are localized between the dendrites of DACs and AII somata in the rodent retina, indicating that DAC synapses onto AII amacrine cells are GABAergic [26]. While postsynaptic dopamine receptors are not localized on the surface of AII somata, vesicular monoamine transporter-2 colocalizes with VGAT in the same DAC varicosities, suggesting that dopamine diffuses far from the synapse and exerts paracrine effects at the extrasynaptic sites of metabotropic receptors [13, 25, 42, 43].

However, in the present study, DACs in the rabbit retina did not express GABAergic markers such as GABA or GAD (an enzyme involved in GABA synthesis). VGAT, a transporter that delivers GABA to synaptic vesicles, was not localized in the soma, dendrites, or varicosities of DACs. However, previous studies have indicated that glycinergic AII lobules contain VGAT, indicating that VGAT also delivers glycine to synaptic vesicles [34]. In accordance with the findings of previous studies [27-29], our results support the notion that DACs in the rabbit retina contain dopamine only, and suggest that synapses between DAC varicosities and AII somata/lobules in stratum 1 of the IPL are dopaminergic rather than GABAergic. Taken together, these findings indicate that synaptic input from DACs in the rabbit retina may be mediated only by dopamine, which is known to modulate the permeability of tracers for AII-AII gap junctions [44, 45]. Further study should be needed to provide the physiologic implication of the absence of GABAergic neurotransmission from DACs in the rabbit retina in contrast to those in other mammals.

Lastly, we want to comment on distinct characteristics of DACs in the rabbit retina. As mentioned above, previous studies have revealed that DACs are present in various species and share common morphological and synaptic features [5-10]. However, rabbit DACs are typical amacrine cells whose dendrites do not extend beyond the IPL [3], while those in other species are interplexiform cells that send additional processes to the OPL [2]. In addition, rabbit DACs do not use GABA as a co-neurotransmitter. These specific features suggest that synaptic wiring and the mechanisms underlying visual processing in the retina differ between rabbits and other mammals. This hypothesis is in accordance with the findings of a recent report by Ding et al., who demonstrated that direction-selectivity—a common mechanism in the mammalian retina—occurs *via* species-specific connections [46]. Indeed, the authors reported that the distribution of inhibitory inputs differed between mouse and rabbit retinas. Taken together, these findings indicate that the role of dopamine in retinal function and the synaptic features of DACs should be revisited in future studies.

ACKNOWLEDGEMENTS

We thank Prof. Stephen C. Massey, Department of Ophthalmology and Visual Science, Medical School at Houston, University of Texas, for the technical support and valuable comments. This study was supported by National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (NRF-2017R1A2B2005309) and Seoul St. Mary's Hospital (R&D 2016 program).

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