Synaptic Pattern of KA1 and KA2 upon the Direction-Selective Ganglion Cells in Developing and Adult Mouse Retina

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The detection of image motion is important to vision. Direction-selective retinal ganglion cells (DS-RGCs) respond strongly to stimuli moving in one direction of motion and are strongly inhibited by stimuli moving in the opposite direction. In this article, we investigated the distributions of kainate glutamate receptor subtypes KA1 and KA2 on the dendritic arbors of DS-RGCs in developing (5, 10) days postnatal (PN) and adult mouse retina to search for anisotropies. The distribution of kainate receptor subtypes on the DS-RGCs was determined using antibody immunocytochemistry. To identify their characteristic morphology, DS-RGCs were injected with Lucifer yellow. The triple-labeled images of dendrites, kinesin II, and receptors were visualized by confocal microscopy and were reconstructed from high-resolution confocal images. We found no evidence of asymmetry in any of the kainate receptor subunits examined on the dendritic arbors of both the On and Off layers of DS-RGCs in all periods of developing and adult stage that would predict direction selectivity.

Key words: direction selectivity, kainate glutamate receptor, retinal ganglion cell, cell injection, immunocytochemistry

I. Introduction

Detection of motion is an essential element in visual processing. Direction-selective retinal ganglion cells (DS-RGCs) fire robustly to stimuli moving along a preferred direction and are strongly inhibited by stimuli moving in null direction [13, 48]. This selectivity represents a classic paradigm of computation by neural microcircuits, but its cellular mechanism remains obscure. DS-RGCs were first identified about 50 years ago from rabbit retina [4]. Ever since, many researches have been carried out to gain understanding of the retinal direction selectivity. However, there is a controversy over whether the directional decision is made presynaptically or postsynaptically [16, 46–48, 50] and whether the starburst amacrine cells (SACs) are required or not for directional discrimination [10, 23, 39, 58].

The On-Off DS-RGCs have been studied using many sophisticated methods [3, 31, 52]. The On-Off DS-RGCs exhibit a characteristic morphology: the dendritic tree is bistratified at 20% and 70% depth of the inner plexiform layer (IPL) in sublamin a (Off) and sublamin b (On), respectively [3, 31, 52]. The dendritic arbors of DS-RGCs curve back toward the cell bodies, forming a similar honeycomb-like appearance [55]. The apical portion of the dendrite has a unique pattern of branching, creating a regular lattice in which some ramifications form closed loops [3, 49, 55, 56].

The directional preference is created by converging the inputs from amacrine and bipolar cells onto the DS-RGCs [5, 32]. Glutamate is the most abundant excitatory neurotransmitter in the vertebrate nervous system, a key neurotransmitter to mediate the transfer of visual information from the bipolar to the ganglion cell [40]. Kainate receptors are non-NMDA ionotropic receptors which respond to the neurotransmitter glutamate. There are five types of kainate receptor subunits (GluR5-7 and KA1-2) [12, 24]. Kainate glutamate receptors have been the subject of many studies.
to determine their important role in the function of ganglion cell responses [38, 43].

Recently, On-Off DS-RGCs were identified in the mouse retina [52]. Mouse is an important animal in research and the data in normal mice will be importantly useful for studies using transgenic and knockout mice. However, no study on the synaptic pattern of DS-RGCs in developing and adult mouse retina has been reported. In our lab, we are currently analyzing the synaptic pattern of AMPA, kainate, and NMDA glutamate receptors to determine any obvious specialization in the distribution of receptor subunits on the DS-RGCs in mouse in order to search for anisotropies which might contribute to a directional preference of the ganglion cells.

In this study, we aimed to identify the spatial distribution of the kainate receptor subtypes KA1 and KA2 on the On-Off dendritic arbors of DS-RGCs in developing and adult mouse retina and whether there are differences in synaptic patterns between developing and adult stages on the dendritic arbors of DS-RGCs. We focused on the data for KA1 and KA2 in the present study to reduce the large quantity of data if we analyze all five kainate receptor subunits simultaneously both in developing and adult animals. In our previous study in adult rabbit, there was no obvious specialized differences in the distribution among the five kainate receptor subunits examined in the dendritic arbors of both the On and Off layers of DS-RGCs in all periods of developing and adult stage.

II. Materials and Methods

Retinal isolation and preparation

C57BL6 mice (P5, P10, and Adult) were anesthetized using a mixture of ketamine hydrochloride (30–40 mg/kg) and xylazine (3–6 mg/kg). To suppress blink reflexes, proparacaine hydrochloride (100–200 µl) was applied to the cornea. The eye was hemisected at the equator, and the lens and vitreous humor were lifted away. The retina was placed away from the pigmented epithelium. The retina was mounted on a non-fluorescent filter paper and vitreous humor were lifted away. The retina was teased away from the pigmented epithelium. The retina was placed in Ames’ medium (Sigma-Aldrich, St. Louis, MO, USA) that had been equilibrated with 95% O₂ and 5% CO₂ [1, 56]. The fundamental procedure for isolating and maintaining a living retina in vitro was developed and described in detail by Ames and Nesbett (1981). All investigations involving animals conformed to the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

Living cell injection using Lucifer yellow

DS-RGCs were targeted and injected by conventional injection techniques [23, 30, 34, 56]. To identify the DS-RGCs, the isolated retinas were exposed to DAPI. This procedure labeled the SACs brightly and the ganglion cells weakly. The retina was mounted on a non-fluorescent Millipore (0.45 mm, Black, HABP 47 mm) filter paper and preserved under flowing Ames’ medium. The DS-RGCs were recognized after injection with Lucifer yellow, based on their distinctive morphology, as shown in Figure 1 [25, 49, 51, 55, 56].

Fluorescence immunocytochemistry

After intracellular injections with Lucifer yellow, the whole retina was fixed in 4% cold paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 2 hr. The conventional immunocytochemical techniques were as previously described in detail [29]. Tissues filled with Lucifer yellow were labeled with antibodies against KA1 (SC-8917, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and KA2 (SC-8915, 1:100, Santa Cruz Biotechnology). The secondary antibody used was Cy5-conjugated anti goat IgG (1:100, Jackson ImmunoResearch, West Grove, PA, USA) for GluRs (KA1 and KA2).

Ribon synapses were labeled using a marker for the membrane traffic motor protein kinesin, mouse anti kinesin II (MMS-198P, 1:100, Covance, Berkeley, CA, USA). The secondary antibody used was Cy3-conjugated anti mouse IgG (1:100, Jackson ImmunoResearch, West Grove, PA, USA) for kinesin II. After immunocytochemistry, the tissues were cover-slipped with a vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). As a negative control, some sections were incubated in the same solution without the addition of the primary antibody. These control tissues showed no GluR immunoreactivity.

Data analysis

We used a confocal scanning module (LSM 700, Carl Zeiss Meditec Inc., Jena, Germany) mounted on a fluorescence microscope (Axio Observer Z1, Carl Zeiss) using a C-Apochromat 40×/1.2 W or 63×/1.2 W Corr UV-VIS-IR M27 objective (Carl Zeiss). The Z-series of the confocal images established a 3D image with a ZEN 2009 program (Carl Zeiss) for identifying the whole dendritic arbor of DS-RGCs. We used a triple-labeling technique to identify the synapse on the dendrites. We used laser line and emission filters: Lucifer yellow (488 nm), kinesin II (568 nm), and GluRs (647 nm). We obtained approximately 60–70 fields of confocal images on each On-Off dendritic layer in each cell and joined these to form a 1024×1024 montage image. Methods of judging for counting a punctum as a synapse have been described in our previous studies [30, 34].

For each kainate receptor image, we calculated kainate receptor immunopuncta on On-Off dendrites for each of the eight cardinal directions. Immunopuncta were included in the histogram for a given cardinal direction within 45° (Fig. 7A). Each punctum was necessarily included in the analysis for one cardinal direction. First, the density of kainate GluR immunopuncta was expressed in terms of a linear dendritic extent (puncta/µm dendrite). For the histo-
gram, the average densities of immunoreactive puncta/µm² dendrites were calculated at 10 µm intervals from the center of the soma to the outer dendrites within each cardinal direction. Second, the density of the kainate receptors immunopuncta was also expressed in terms of the average density of the immunoreactive puncta/total length of µm dendrites within each cardinal direction. The symmetry index (SI), which estimates the precise spatial symmetry of a given component, was extracted. Calculation of the SI followed previous studies [42]. The SI was calculated for each cell using the following formula: SI=10 (B/A), where A is the spatial density of kainate immunopuncta on one direction of the DS-RGC and B is the spatial density of kainate immunopuncta on the dendritic arbor of symmetry partners. We calculated the SI of the dendrites for all directions. A SI value of 10 indicates a complete symmetry and a SI value of 0 indicates no symmetry.

III. Results

Morphological identification of DS-RGCs

The DS-RGCs injected with Lucifer yellow are shown in Figure 1. The cells in panels A-F were used to analyze the distribution of KA1 and KA2 in developing and adult mouse retinas, respectively (P5, P10, and Adult). There are many types of ganglion cells in developing and adult mouse retina [14, 45]. The DS-RGCs have unique morphological properties. The dendritic arbor of On-Off DS-RGCs has a honeycomb-like appearance and is bistratified: one portion narrowly stratifies within sublamina a of the IPL, whereas the other ramifies narrowly within sublamina b [56]. These cells also have a loop-forming pattern which is almost similar to the dendritic morphology of the On-Off DS-RGCs of the rabbit.

Immunoreactivity of vertical section

In vertical sections of the developing and adult mouse retina, immunoreactive puncta showed a distinctive pattern (Fig. 2). The panels A, B, and C show the distribution of P5 KA1, P10 KA1, and Adult KA1, respectively. The panels D, E, and F show the distribution of P5 KA2, P10 KA2, and Adult KA2, respectively. The immunofluorescence labeling was most prominent in the IPL. Some immunopuncta were also found in the outer plexiform layer (OPL) and the ganglion cell layer (GCL). The staining pattern is comparable to other mammalian retina. For example, GluR6/7 and KA2 were both present in the IPL and the OPL of rat and monkey retina [6, 19, 20, 37]. In the cat retina, GluR5 and GluR6 have been localized both in the IPL and the OPL [43]. In the immunocytochemical analysis of the mouse retina, GluR6/7 was found in the OPL as well as the IPL [22]. This is very similar to our present results. Our present results are also similar to our previous studies of the adult rabbit retina [30, 34]. In the present study, however, there are more immunopuncta in the adult retina than in the earlier developing retinas.

Immunoreactivity of On-Off sublamina of the IPL

In whole mounts of the retina (Fig. 3), the immunoreactive puncta were prominent in both the On and Off sublaminae of the IPL. In many areas the immunoreactive puncta densely surrounded unlabeled elements. These results were similar to previous studies of the adult rabbit retina [30, 34].

Triple labeling of DS-RGCs

We used a triple-labeling technique in order to show the clustering of kainate GluRs at the bipolar cell terminals. The input of the bipolar cell was identified by using antibodies against kinesin II which has been shown to be a marker of the ribbons both in the OPL and the IPL [41]. Figure 4 shows triple-labeling of dendrites against kinesin II and KA1 in developing and adult stages. When we double-labeled tissues for KA1 (Fig. 4B, G, and L, red puncta) and kinesin II (Fig. 4A, F, and K, blue puncta), KA1-immunoreactive puncta coincided with kinesin-labeled ribbons (Fig. 4C, H, and M, purple puncta). Figures 4D, I, and N show merged triple-labeled images of kainate II and KA1 on the dendrites of Lucifer yellow-injected DS-RGCs (Fig. 4D, I, and N, green) in developing and adult stages. The location of the double-labeled puncta of KA1 and kinesin II overlapping upon the dendrite (arrowheads in Fig. 4D, I, and N) is illustrated in Figures 4E, J, and O, respectively. Figure 5 shows triple-labeling of dendrites using kainate II and KA2 in developing and adult stages. When we double-labeled tissues for KA2 (Fig. 5B, G, and L, red puncta) and kinesin II (Fig. 5A, F, and K, blue puncta), KA2-immunoreactive puncta coincided with kinesin-labeled ribbons (Fig. 5C, H, and M, purple puncta). Figures 5D, I, and N show merged triple-labeled images of kainate II and KA2 on the dendrites of Lucifer yellow-injected DS-RGCs (Fig. 5D, I, and N, green) in developing and adult stages. The location of the double-labeled puncta of KA2 and kinesin II overlapping the dendrite (arrowheads in Fig. 5D, I, and N) is illustrated in Figures 5E, J, and O, respectively.

Distribution of kainate receptor subunits

We reconstructed the dendritic arbors using a computer screen. Mapping of the apparent distribution of kainate receptor subunit is shown in Figure 6. The three panels in the first column show the distribution of P5 KA1 (A), P10 KA1 (E) and Adult KA1 (I) kainate receptor subunits (red dots) on the On dendritic arbors (black line). The three panels in the second column show the distribution of P5 KA1 (B), P10 KA1 (F) and Adult KA1 (J) kainate receptor subunits (blue dots) on the Off dendritic arbors (green line). The three panels in the third column show the distribution of P5 KA2 (C), P10 KA2 (G) and Adult KA2 (K) kainate receptor subunits (red dots) on the On dendritic arbors (black line). The three panels in the fourth column show the distribution of P5 KA2 (D), P10 KA2 (H) and Adult KA2 (L) kainate receptor subunits (blue dots) on the Off dendritic arbors (green line). Despite their variation in size,
Fig. 1.

Fig. 2.
morphology, and different PN period, the kainate receptor subunits are distributed around the dendritic arbors in a seemingly regular pattern [27, 33].

**Densities of kainate receptor subunits on the On-Off dendritic arbors**

We calculated the kainate GluR immunopuncta on dendrites for each of the eight cardinal directions, that is, along the possible preferred-null directions. Immunopuncta were

![Fluorescence confocal micrographs of midperipheral mouse retinal whole mounts immunolabeled with antibodies against KA1 in P5 (A, D), KA1 in P10 (B, E), KA1 in Adult (C, F), KA2 in P5 (G, J), KA2 in P10 (H, K), and KA2 in Adult (I, L). Panels (A), (B), (C), (G), (H), and (I) were taken from On layers, while panels (D), (E), (F), (J), (K), and (L) were taken from Off layers. Strong punctate immunoreactivity is present in both the On and Off layers. Bar=10 µm.

**Fig. 3.**

**Fig. 1.** Direction-selective ganglion cells injected with Lucifer yellow. In these micrographs, taken at low magnification, both dendritic arbors are visible. Cells in panels (A), (B), (C), (D), (E), and (F) were used to analyze the distribution of kainate glutamate receptor subtypes KA1 and KA2 immunoreactivity, respectively. Bar=20 µm.

**Fig. 2.** Fluorescence confocal micrographs of vertical vibratome sections through midperipheral mouse retina immunolabeled with antibodies against kainate glutamate receptor subtypes KA1 in P5 (A), KA1 in P10 (B), KA1 in Adult (C), KA2 in P5 (D), KA2 in P10 (E), and KA2 in Adult (F). OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Bar=20 µm.
Fig. 4. Fluorescence confocal micrographs of retinal whole mounts immunolabeled with antibodies against kinesin II (A, F, K) and KA1 (B, G, L) in developing and adult stages. (C) Superimposed image of kinesin II and KA1 in P5. (H) Superimposed image of kinesin II and KA1 in P10. (M) Superimposed image of kinesin II and KA1 in Adult. Kainate GluR immunoreactive puncta coincide with kinesin-labeled ribbons. Arrowheads indicate double labeled puncta of kinesin II and kainate overlapping upon the dendrites. (D, I, N) Merged images of dendrite, kinesin II immunoreactivity, and kainate GluRs immunoreactivity. (E, J, O) Tracing of dendrite and co-localized kinesin II and kainate puncta. Bar=5 µm.

Fig. 5. Fluorescence confocal micrographs of retinal whole mounts immunolabeled with antibodies against kinesin II (A, F, K) and KA2 (B, G, L) in developing and adult stages. (C) Superimposed image of kinesin II and KA2 in P5. (H) Superimposed image of kinesin II and KA2 in P10. (M) Superimposed image of kinesin II and KA2 in Adult. Kainate GluR immunoreactive puncta coincide with kinesin-labeled ribbons. Arrowheads indicate double labeled puncta of kinesin II and kainate overlapping upon the dendrites. (D, I, N) Merged images of dendrite, kinesin II immunoreactivity, and kainate GluRs immunoreactivity. (E, J, O) Tracing of dendrite and co-localized kinesin II and kainate puncta. Bar=5 µm.
Synaptic Pattern of Kainate Receptors

included in the histogram for a given cardinal direction within 45° (Fig. 7A). Each histogram indicates the average densities of immunoreactive puncta/μm dendrites calculated at 10 μm intervals from the soma to the terminal of the dendrites within each cardinal direction. The density of the kainate GluR immunopuncta was also expressed in terms of the average density of immunoreactive puncta/total length of μm dendrites within each cardinal direction (Fig. 7B–G, central graphs). In general, the distribution of puncta does not show an asymmetrical pattern.

Symmetry Index (SI)

The SI estimates the precise spatial symmetry of a given component [42]. We calculated the SI for each cell to measure how evenly the spatial density of kainate immunopuncta is distributed throughout the dendritic arbors of the DS-RGCs. Figure 8 shows the SI for a spatial density of kainate immunopuncta on the On-Off dendritic arbors of DS-RGCs. The SI of immunopuncta was 6.53 (On dendritic arbors) and 8.13 (Off dendritic arbors) in P5 KA1, 6.40 (On dendritic arbors) and 5.92 (Off dendritic arbors) in P10 KA1, 8.45 (On dendritic arbors) and 8.46 (Off dendritic arbors) in Adult KA1, 7.25 (On dendritic arbors) and 7.90 (Off dendritic arbors) in P5 KA2, 6.34 (On dendritic arbors) and 7.75 (Off dendritic arbors) in P10 KA2, and 8.43 (On dendritic arbors) and 7.45 (Off dendritic arbors) in Adult KA2 (Fig. 8). There were no obvious differences in the SI among different developing and adult stages.

IV. Discussion

In the present study, we analyzed the distributional pattern of KA1 and KA2 kainate receptors on On-Off dendritic arbors in developing and adult mouse DS-RGCs. The distribution of these receptor subunits on the dendritic arbor reveals no asymmetrical pattern in all periods of P5, P10, and Adult.

In the present study, we revealed that the kainate-immunoreactive puncta coincided with kinesin-immunoreactive ribbons in the bipolar terminals. We analyzed the puncta using a confocal microscope. We understand that true synapses can only be identified by electron microscopy. Although each of the synapses can certainly be identified using electron microscopy, the method has its limitations: the analysis of the whole dendritic arbor using thin serial sections is an exceedingly demanding task and practically
Fig. 7. Densities of P5 KA1 (B), P5 KA2 (C), P10 KA1 (D), P10 KA2 (E), Adult KA1 (F), and Adult KA2 (G) subunit immunopuncta on the On and Off dendritic arbors of direction-selective retinal ganglion cells. (A) Method for calculating separately the distribution of kainate subunit immunopuncta for each of the eight cardinal directions along the possible preferred-null axes. Central graphs (B–G) show the average density of immunoreactive puncta/total length of dendrites (µm) within each cardinal direction.
impossible. Thus, analysis at the light microscope level with high resolution confocal microscopy is a practical approach and for better analysis we triple labeled the samples in the present study. Previous studies showed that the immunoreactive puncta was attributable to the synaptic sites [20, 28, 30, 34, 37].

DS-RGCs are found in most vertebrates [2] and many invertebrates [18]. Particularly, DS-RGCs are well studied in the rabbit retina by using intracellular electrophysiological recordings, pharmacological manipulation, and anatomical methods [3, 23, 50, 55]. In the survey of adult mouse retinal ganglion cells, two subtypes of bistratified RGc were found [45]. Especially, the Rg2 type exhibits a remarkable resemblance to the dendritic morphology of the rabbit DS-RGCs [3]. This cell exhibits almost identical properties to rabbit DS-RGCs as identified by physiological and pharmacological methods [52]. In the survey of developing mouse retinal ganglion cells, DS-RGCs have been found in the mouse retina 3 days postnatally [11, 14, 44]. In agreement with previous physiological and pharmacological studies on the mouse, we could morphologically identify DS-RGCs in the early stage (P5) mouse retina in the present study.

The DS-RGCs receive powerful excitatory input from the cone bipolar cell [40]. The role for bipolar cells in the direction-selective mechanism is also supported by immunocytochemical and pharmacological evidence. Antagonists to kainate receptors block direction selectivity in the mouse retina [52]. This result showed that the blocking of direction selectivity can occur by interrupting the kainate cone bipolar synapse in the DS-RGCs [17]. Thus, identifying the synaptic pattern of kainate receptors upon the entire On-Off dendritic arbors of DS-RGCs is indispensable for understanding the mechanism of direction selectivity. In the present study, we first identified the synaptic pattern of KA1 and KA2 not only in the developing retina (P5 and P10) but also in the adult retina on the entire On-Off dendritic arbors of DS-RGCs.

Significant attempts have been made to identify the mechanism of direction selectivity. However, at present, it is the center of controversy. There is conflicting evidence whether the directional decision is made presynaptically or postsynaptically [16, 46–48, 50]. In most direction-selective models, the excitatory and inhibitory synapses are arranged on the dendritic arbor of DS-RGCs according to precise spatial rules, since it is hypothesized that the onset of excitation precedes that of inhibition when the stimulus moves in the preferred direction [5, 16, 23, 47, 48, 50]. Our research group showed that there were no specific patterns on the dendritic arbor of DS-RGCs, which would predict direction selectivity in the rabbit retina [28]. We also showed that there is no evidence of asymmetrical synaptic patterns in both AMPA and kainate GluRs on the dendritic arbors of DS-RGCs in the adult rabbit retina [30, 34]. In very recent studies, postsynaptic glutamate input into DS-RGCs in the rabbit was regular and symmetrical [27, 33]. In agreement with these recent studies, we showed that there were no specific asymmetrical patterns on the dendritic arbors of DS-RGCs in developing and adult mouse retinas.

There is also conflicting evidence whether SACs are required or not for directional discrimination [7, 10, 23, 35, 39, 51, 57, 58]. When blocking the excitatory input from the SACs, DS-RGCs still evoke a direction selectivity response [23, 31]. On the other hand, DS-RGCs lose their ability when SACs are completely eliminated by using immunotoxin-mediated cell targeting method [21]. Direction selectivity also persisted after laser ablation of SACs [23]. On the other hand, On-Off ganglion cells in SACs knockout mice no longer show direction selectivity [58]. Lee et al. demonstrated that Ach-GABA corelease enables the SACs to encode both motion sensitivity and direction selectivity [35]. In very recent studies, it was demonstrated that asymmetric inhibitory inputs from SACs are essential for making directional selectivity in rabbit DS-RGCs [7, 51, 57]. However, the conflicting evidence of presynaptic and postsynaptic mechanisms, the controversial role of SACs for direction selectivity, and the extreme diversity among amacrine cells open various possibilities for the synaptic circuitry of DS-RGCs that generates the directional signals and that needs to be identified in future studies.

Light is a significant element for the development of the visual system. Many studies have shown a relation between light and the physiological property of the retina [36, 53, 54]. The earliest record of a direction-selective response is the opening of the eyes both in the rabbit and the mouse [8, 15, 23, 59]. In recent studies, it has been
suggested that the direction-selective circuitry is independent of light [8, 15]. Similarly, Chen et al. found that the dendritic morphology and synaptic input pattern were not affected by light deprivation [9]. In the present study, we attempted to identify whether there are differences in synaptic patterns between developing and adult stages on the dendritic arbor of DS-RGCs. Our results indicate that there is already a kainate-mediated synapse on the dendritic arbors of DS-RGCs in the early stage (P5) and that the synaptic patterns are similar in all stages. In agreement with our results, retinal ganglion cells expressed mRNA for kainate glutamate receptor subunits on day 5 postnatally [26].

In conclusion, the synaptic distributional pattern of KA1 and KA2 receptor subunits may be an important clue in understanding the basis for the differential processing of direction-selective signals in the retina. The present results showed that their presence on the entire dendritic arbor of DS-RGCs is without any evidence of asymmetry, which would predict direction selectivity. The results may suggest that direction selectivity lies in the neuronal circuitry afferent to the DS-RGC itself. However, the conflicting evidence of presynaptic and postsynaptic models, the controversial task of SACs, and the excessive diversity of amacrine cells open various possibilities for mechanism.

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VI. References

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