Neural Coding of Green Flash in Retinal Bipolar Pathways

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Abstract

What visual information do the graded potentials among retinal bipolar pathways actually transmit from photoreceptors to ganglion cells? The answer does not exist. Even the graded electric signals have not been understood completely. Here, this paper tries to analyze the encoding mechanisms of graded signals among the parallel bipolar pathways in response to brief green flash. The typical ON, OFF and ON-OFF bipolar cells simultaneously abstracted vectors from green flash stimulus with *sine*-like functions on their dendritic plane. Atypical bipolar cell had the synchronously monopolar response in contrast to the bipolar responses of typical bipolar cells, they also annotated green flash with facilitated stochastic (asynchronous and rate-coded) responses. Some complex ON-OFF bipolar cells with large voltage-gated Na^+ current could generate high-frequent asynchronous responses, others had synchronous ON-OFF responses to green flash. The green flash was synchronously and asynchronously represented by the multiple-dimension signaling space among the parallel bipolar pathways. These results unraveled the multiple-dimension neural codes for brief green flash, demonstrated

the superior encoding capability of parallel bipolar pathways, and suggested the electrophysiological mechanisms of vision such as color space.

Key words: retinal bipolar cells, signaling space, *sine* function, stochastic, vector, bipolar currents.

There are multiple bipolar pathways to encode visual information with graded potentials. The graded potentials are the basic neural codes. The multiple bipolar pathways construct the visual signaling space. The color space exposes the complexity of visual signaling space. The ON and OFF pathways were identified forty years ago.[1] The ON bipolar cells depolarize in responses to center illumination but hyperpolarize to surround illumination. These ON responses are mediated by sign-inverting metabotropic glutamate receptors (mGluR6) on the dendritic plane [2, 3]. By contrast, the OFF bipolar cells hyperpolarize to center illumination but depolarize to surround stimulation, and their responses are driven by sign-preserving ionotropic glutamate receptors (AMPA and kainate receptors). The ON and OFF channels with bipolar responsibilities provides good "position" encoding on the bipolar cells' dendritic plane. Their synchronous responses provide spatial preciseness. The time preciseness does not matter so much when all the responses are synchronous in the spatial encoding of ON and OFF pathways, though time is essential factors in visual signaling space. The space and time can be processed separately through other channels than ON and OFF channels. In fact, the multiple parallel pathways exist in retinal bipolar cells. For spatial encoding, there are independent ON, OFF and ON-OFF channels. There should be more channels for temporal encoding. For example, to sense a changing green spot on a white screen, green color pathways (specific ON, OFF and ON-OFF bipolar cell types following the green photoreceptors) encode the shape and color change in time while the anatomical topography of green pathways could include the position information in the visual field. The early retinal neurons need also abstract the temporal

information which will activate the autonomic responses. This paper analyzes the parallel processing of green flash stimulus among multiple bipolar pathways. Multiple spatial and temporal channels with independent encoding patterns are identified. The electrical responsibilities to green flash presented a good example of the whole encoding raster of retinal bipolar pathways.

The light responses of the retinal bipolar cells and their intrinsic electrophysiological properties were studied in salamander retina. Diverse bipolar cell groups with distinct electrophysiological and morphological properties were identified. Their responsibilities to brief green flash were recorded with whole-cell patch clamp method. In addition to ON, OFF and ON-OFF bipolar cells, more bipolar groups were involved in the green-flash responses. Another possible code system exploiting asynchronously stochastic electric signals among different bipolar cell groups was observed. The typical bipolar cells had typical ON, OFF and ON-OFF light responses to green flash stimulus. ON, OFF and ON-OFF light responses were composed of inward and outward conductances (bipolar conductances). Their bipolar conductances were synchronously related to the stimulus. Vector stimuli (changing stimuli on the dendritic plane) vs *sine* (or *sine*-like) function (0~π) matched the precisely spatial encoding in typical bipolar cell. The typical ON, OFF and ON-OFF bipolar cells abstracted the unique stimulus properties with their independent *sine*-like functions, respectively. The atypical bipolar cells had synchronously monopolar response to the green flash stimulus like horizontal cell, they encoded the fourth signaling channel. In addition, the atypical bipolar cells responded to complex stimuli with facilitated stochastic signals (higher frequency or rate code). This group of bipolar cells like visceral sensory neurons had temporally delayed effect in representing the green flash. There were another group of complex bipolar cells. They had amacrine-like morphology and large intrinsic voltage-gated Na^+ current. Some complex bipolar cells always showed the high-frequent asynchronous currents in light response like typical amacrine cells while some complex

bipolar cells had synchronous ON-OFF responses. The independent stochastic signals (rate code) functioned in the atypical and complex bipolar cell groups in response to green flash. Their facilitated responses enabled temporal co-ordinate (or temporal effect in the bipolar cells) in information abstracting of retinal bipolar pathways. A whole image of multiple-dimension signaling space among retinal bipolar pathways emerged in response to green flash stimulus.

Diversity of retinal bipolar cell and their blurry boundary from other retinal interneurons. 542 neurons were recorded and stained in the inner nuclear layer (INL) of salamander retinal slices. The Lucifer yellow in electrode solutions unraveled the unexpected diversity of retinal bipolar cells (Fig S1). The characteristic morphology of typical bipolar cells is symmetric bipolarity: matched dendritic and terminal field in the outter plexiform layer (OPL) and inner plexiform layer (IPL), respectively. Their terminals displayed the characteristic stratification in the IPL.[4] One group of complex bipolar cells, found near the proximal margin of the inner nuclear layer (INL), displayed some properties of amacrine cells (n = 35). They all generated large-amplitude voltage-gated Na^+ currents unlike typical bipolar cells which have either small or no voltage-gated Na^+ conductances. These complex bipolar cells had multiple primary amacrine-like processes in both OPL and IPL. Their processes occupy the entire IPL, including sublaminae *a* and *b* with the widest dendritic fields, so these cells could be ON-OFF bipolar cells. Another group of atypical bipolar cells was located near the distal margin of the INL (n = 35). These OFF bipolar cells displayed the thick processes in both OPL and IPL. Atypical bipolar cells had the light responses like those of horizontal cells. Some horizontal cells had primary or secondary centripetal processes while some amacrine cells had single primary centrifugal processes into the INL. There were intermediate neurons between bipolar cell and amacrine, horizontal cell (n = 32 and 15). These results revealed a previously unknown aspect of retinal bipolar cell diversity and suggest that the boundary between bipolar cells and other retinal interneurons is sometimes blurry.

Atypical bipolar cell's responses depended on monopolar current: synchronously outward conductance. Bipolar cells were voltage clamped at -70 mV (the reversal potential for excitatory conductance) to record inward currents, and at 0 mV (the reversal potential for inhibitory conductance) to record outward currents. Inward and outward responses to green flash (525 nm, irradiance was ~0.7 μw/cm^{2,} for 1 sec) were recorded at the two voltage holdings, respectively. The ON, OFF and ON-OFF bipolar cells displayed the synchronous ON, OFF and ON-OFF responses independently (Fig.1A, B, C). The ON, OFF and ON-OFF responses included both inward and outward conductances. One group of atypical bipolar cells (n = 35) showed distinct responses to green flash. They had only outward responses while light was ON. Their morphology was similar to horizontal cells and their terminals were located in the outer layer of inner plexiform layer (OFF bipolar cell) (Fig. S1). They exploited only monopolarity (outward currents) in synchronous responses (Fig. 1D.) also like horizontal cells. The monopolar responses had low amplitude (-32.43 ± 10.93 pA, at -70 mV; -24.86 ± 13.2 pA, at 0 mV, n =35, mean ± stand deviation, the same below. Intracellular solution included Cs^{-}.). The outward currents were assumed as antagonist to inward currents but not independent signaling pathway. The atypical bipolar cells depending on the outward currents were the strong proof that outward currents were independently neural signals in visual information processing beyond horizontal cells. Then for typical bipolar cells, they had synchronously bipolar currents (Fig. 1A, B, C, bottom). It is reasonable that both of inward and outward currents in the typical bipolar cells also functioned as independent signals.

Typical bipolar cells had both synchronously inward and outward conductances in response to green flash. Retinal bipolar cells displayed their characteristic ON, OFF responses to the center-surround light stimuli.[1] These responses include the information of specific stimulus position on the dendritic plane. But typical ON (Fig.1A, n = 30), OFF (Fig. 1B, n = 22) and ON-OFF (Fig. 1C, n =17) responses to full field green flash always happened. The

typical bipolar cells produced their typically synchronous conductances in response to green flash. The green flash stimuli could include increasing light intensity (one of multiple properties) on photoreceptors during the brief beginning period of light ON. The green photoreceptors' sensitivity changed as light was increasing and stable in time (adaptation). The bipolar dendritic plane received changing input from photoreceptors during the stimulus period. The light responses in retinal bipolar cell showed the changing spatial stimuli in time on their dendritic plane. That may partially compromised the temporal factor in green flash stimulus and the spatial factor in the center-surround stimulus. The responsive bipolar cells could belong to specific visual pathways following the activated green photoreceptors. The green flash always activated inward and outward conductances at precisely the same time (Fig. 1A, B, C, bottom) in typical bipolar cells including ON, OFF and ON-OFF types. The peak amplitudes of inward and outward currents varied among different bipolar cells (ON: 70.11 ± 74.69 pA, at -70 mV; -30.4 ± 19.2 pA, at 0 mV, n = 30; OFF: 156.46 ± 133.15 pA, at -70 mV; -53.83 ± 63.19 pA, at 0 mV, n = 22; ON-OFF: 195 ± 78 pA, 31.1 ± 63 pA, at -70 mV; -54.9 ± 35.2 pA, -44.6 ± 29.3 pA at 0 mV, n = 17). The response currents usually appeared like *sine*-wave (180° or π). The response at any time point could be the sum of inward and outward currents in current clamp recording, which showed graded current with possibly different polarity. The responses to different stimuli (or different position of dendritic field at any time point) during the stimulus period formed electric wave of alternating current (AC, *sine*-like function in trigonometry, 0~π). One vector stimulus at a specific position on the dendritic plane was one point in *sine*-like function. So the recordings under green flash stimulus could be explained with series of vector stimuli in their dendritic field of bipolar cells (sum of many synaptic inputs in time). The vector stimuli and the responsive *sine*-like function matched well to translate the stimulus at each point on the dendritic plane into each point in *sine*-like function. The inward and outward conductances at all positions on the dendritic plane determined the characteristic *sine*-like function of each bipolar cell (Fig. 1A, B, C, middle). The main information encoded in the ON,

OFF and ON-OFF responses was the changing stimulus (vector) on the dendritic plane. The *sine*-like responsive curve showed the series of vector stimuli on the dendritic plane. The vector information was displayed live by the alternating conductance. Similar to center-surround stimulus, the time factor was not very important as the *sine* wave was changing (or photoreceptor sensitivity was changing and their inputs into bipolar cell were changing) during the green flash. The phase of *sine* function (codifying vector stimuli) mattered much more in the synchronously signaling. The encoding pattern of *sine* function enabled the similar ON, OFF and ON-OFF responses to green flash stimulus as to center-surround stimulus. There were the synchronized ON, OFF and ON-OFF responses with two-phase order in response to green flash among the typical bipolar pathways. Each point on the dendritic plane could encode vectors and all points were integrated according to its specific *sine*-like function within single bipolar cell. The amplitude of inward and outward currents should be decided by the relationship of stimulus vector and intrinsic vector. The circle stimulus on the plane would be encoded as a cycle of *sine*-like function (2π). That is the case in the center-surround stimuli. The responsive recordings showed the stimulus vectors on the dendritic plane for each typical bipolar pathway. With irregular dendritic field of most bipolar cells,[5] half cycle (π) of *sine*-like function was common for green flash stimulus. The atypical bipolar cells encoded the fourth channel of synchronized monopolar signal. The ON, OFF, ON-OFF, and monopolar bipolar cells formed the four-dimensional signaling system in visual encoding. Each channel was independent and did not form the corresponding three-dimension structures of outside physical space with other channels. The ON-OFF responses could codify the sharp edge or shape with two *sine*-like waves out of phase (~180°, ~π). The ON, OFF, ON-OFF bipolar channels formed the unique signaling space (Fig. 1A, B, C, upper). The independently inward and outward conductances were crucial for the vector encoding in retinal bipolar cells. The glutamate receptors or receptor cascades should include at least two conductances: outward and inward conductances on the dendritic plane. The anatomical position of each bipolar cell in visual parallel pathways could

provide the relative position information of stimulus in the visual field. Bipolar cell pathways worked with parallel electrical signal channels. Multiple stimulus properties (spatial properties on the dendritic plane) were abstracted synchronously through two-phase alternating currents among the multiple bipolar pathways. Changing (vector) was the content of visual signals in retinal bipolar cell.

Atypical bipolar cells displayed facilitated stochastic signals. In contrast to the synchronous light response, ON, OFF, ON-OFF bipolar cells and atypical bipolar cells could show the spontaneous inhibitory/excitatory postsynaptic currents (sI/EPSCs) at resting condition. These sI/EPSCs were stochastic (Fig. 2A). The function of stochastic sI/EPSCs remained a mystery. Most of atypical bipolar cells could respond to complex stimuli (n = 27) in a completely different signaling pattern from synchronous response. One of the best complex stimuli was the repeated stimuli (x3) of high frequency pulse (100 Hz) and combined brief green flash (50 ms) (Fig. 2B). This stimulus condition evoked facilitated outward IPSCs (at 0 mV), but not evoke any EPSCs (at -70 mV). The facilitation could happen with < 100 ms delay and last for a few seconds according to the stimulus condition. The facilitated outward currents happened stochastically after stimulating (Fig. 2C, * marks two events). These responses were delayed and unpredictable after stimuli, unlike the ON, OFF synchronous responses in typical bipolar cells. The facilitated events could be completely blocked by glycine or GABA antagonists (10 μM strychnine, or 20 μM TPMPA and 10 μM SR95533, or 10 μM picrotoxin) respectively in different cell. So the atypical bipolar cells included at least two types, one expressed glycine receptors, another expressed GABA receptors. The facilitation increased the frequency of IPSCs but not the amplitude. They displayed as wide burst events (1.82 ± 0.76 s, 70.63 ± 20.64 pA, n = 63, for GABA currents; 0.65 ± 0.34 s, 250.3 ± 120.77 pA, n = 43, for glycine currents) after stimuli (Fig. 2C, top). The facilitated signals were rate-coded. The facilitated stochastic events were independent on the synchronous responses in atypical bipolar cells. GABA and

glycine antagonists could not block the synchronous light responses (n = 10, P > 0.05, t-test) (Fig. 2C, gray). The delayed responses displayed the time effect of green flash stimulus in these bipolar pathways and were similar to the responses of visceral sensory neurons.

Some complex bipolar cells always had asynchronous responses. Some bipolar cells showed the amacrine-like morphology and large voltage-gated Na^+ currents (n = 35, Fig. S1). The asynchronous responses like amacrine were more common in these complex bipolar cells after green flash. The asynchronous responses included EPSCs (Fig. 4A) and/or IPSCs. The asynchronous IPSCs could be blocked by glycine antagonist (10 μM strychnine, n = 23) or GABA antagonists (or 20 μM TPMPA and 10 μM SR95533, or 10 μM picrotoxin, n =12). The asynchronous EPSCs could be blocked by 20 μM DNQX. Asynchronous light responses displayed as higher frequent E/IPSCs (wide burst currents) while some complex bipolar cells showed the typical synchronous ON-OFF responses (Fig. 4B). These asynchronous responses were similar to the light responses of amacrine cells. The facilitated inward and outward stochastic events encoded another vector was easy to understand, because the opposite stochastic events were not perfect antagonists one for one. The inward and outward stochastic events were independent, they could not eliminate or balanced each other easily. These stochastic rate codes should encode another co-ordinate in the visual signaling space of retinal bipolar cells. This signal channel with delayed responses was involved in the physiological status of activated bipolar cells. The temporal effect (clock signals) should be embedded in the asynchronous responses.

The type-specific expressions of ion channels (ligand- and voltage-gated) identified the diversity of retinal bipolar cells. The diversity of bipolar cells constituted the parallel visual pathways. Each pathway encoded the specific spatial and/or temporal properties of green flash. All the pathways formed the visual signaling space. Some pathways shared the common encoding pattern. The ON, OFF and ON-OFF bipolar cells and some complex bipolar cells

encoded the vector stimulus with two-phase order ACs (*sine* fuctions). The atypical bipolar cells like horizontal cells encoded non-vectors. The stochastic pattern (rate code) should be linked to the autonomous system with its delayed responses and temporal factors. The green flash was represented with at least seven signaling channels among retinal bipolar pathways. The neural codes are already in retinal bipolar cells even before the output neurons-retinal ganglion cells. All useful visual informations were precisely translated into specific neural codes among the parallel bipolar pathways. As an extrapolation, the simple arithmetic operation could be encoded by the dendritic plane of ON-OFF retinal bipolar cells (edge detectors) as figure 5.

Figure 1. Parallely encoding of green flash formed neural signaling space distinct from physical space. A, The signaling space for ON, OFF, and ON-OFF bipolar cells. B, C, D. Typical ON, OFF and ON-OFF bipolar cells encoded vectors with bipolar responses to green flash stimulus (*sine* function, upper; voltage clamp recording, lower). E. Atypical bipolar cells had monopolar responses to green flash.

Figure 2. Facilitated asynchronous responses to complicated stimuli in atypical bipolar cells. A. Spontaneous IPSCs and EPSCs in resting atypical bipolar cell. B. The best stimuli for facilitation were the combination of pulses (100 Hz) and brief green flashes (50 ms). C. Facilitated light responses (L-IPSCs, *) in an atypical bipolar cell were stochastic and could be blocked by GABA antagonist (gray).

Figure 3. Asynchronous responses in complex bipolar cell with large voltage-gated Na^+ currents. A. A complex bipolar cell displayed stochastic responses with higher frequency. Spontaneous IPSCs and EPSCs (upper), light responses (lower). B. Another complex bipolar cell had typical ON-OFF responses.

Figure 4. The black number and symbols could be encoded by single ON-OFF bipolar cells according to vector signaling of typical bipolar cells.

Figure S1. A previously unknown aspect of retinal bipolar cell diversity and the blurry boundary between bipolar cells and other retinal interneurons. A. Typical amacrine cell. B. Intermediate cell on the amacrine cell side. C. Complex bipolar cell on the amacrine cell side. D. Typical ON-OFF bipolar cells. E. Atypical bipolar cell on the horizontal cell side. F. Intermediate cell on the horizontal cell side. G. Interplexiform cell on horizontal cell side. H. Typical horizontal cells.

Figure S2. The ion channels identified complex and atypical bipolar cells. A. The large voltage-gated Na^+ currents in amacrine cell (1 μM TTX, gray). B. The large voltage-gated Na^+ currents in complex bipolar cell (1 μM TTX, gray). C. Monopolar light responses in atypical bipolar cell. D. Monopolar light responses in horizontal cells.

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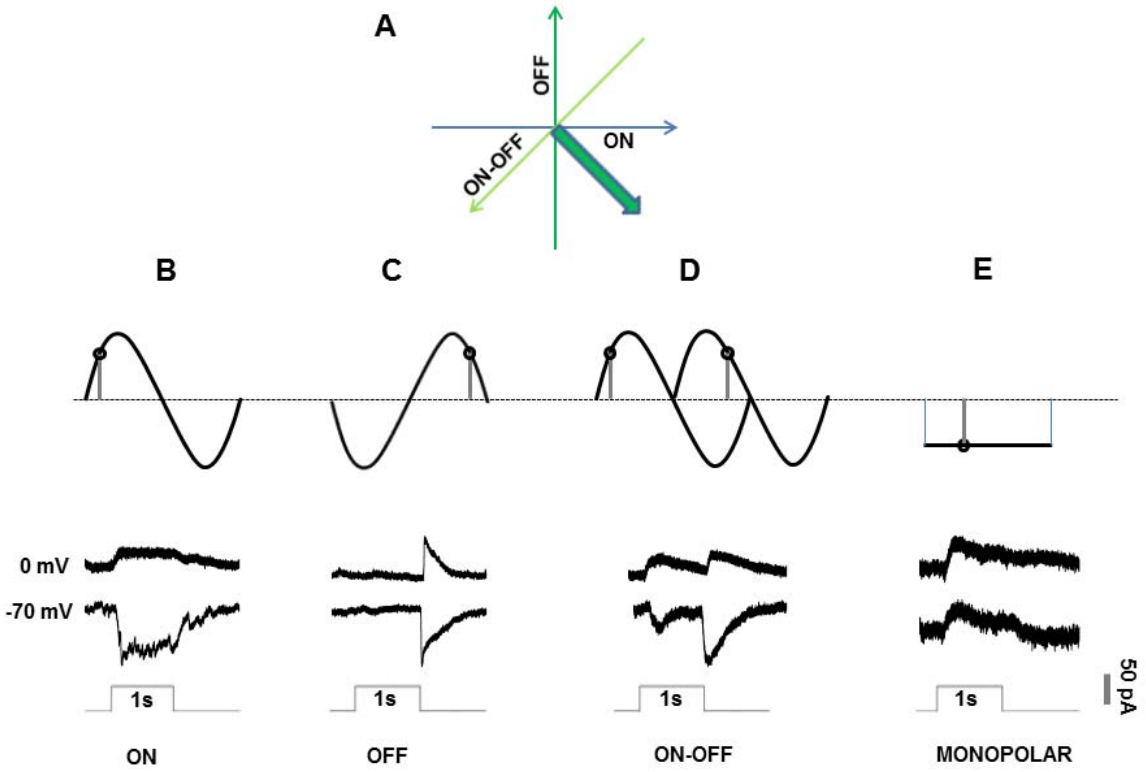

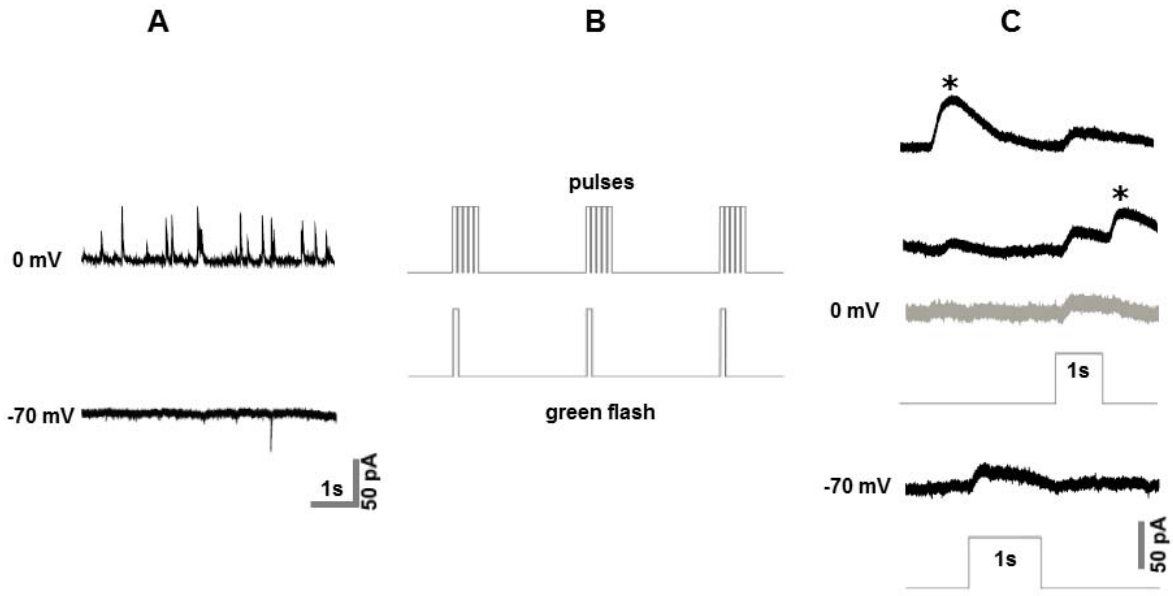

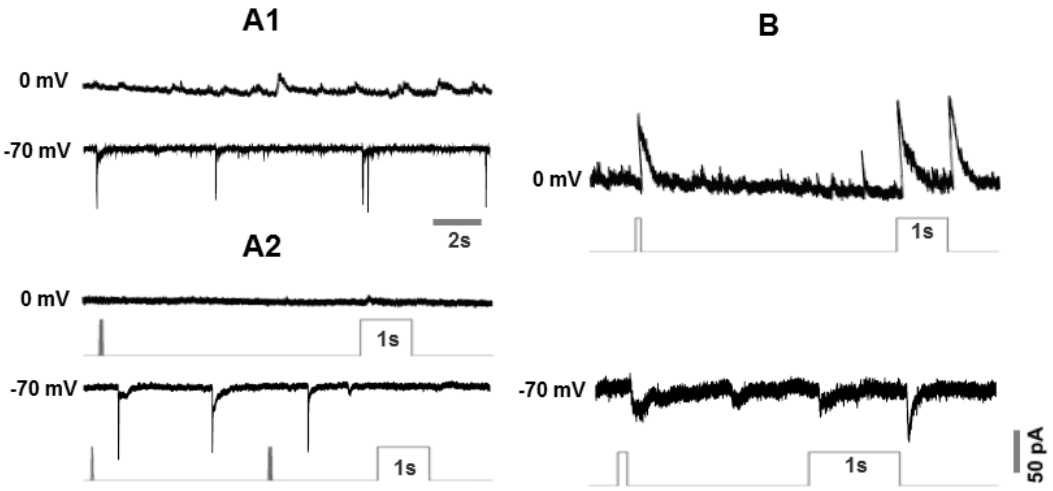

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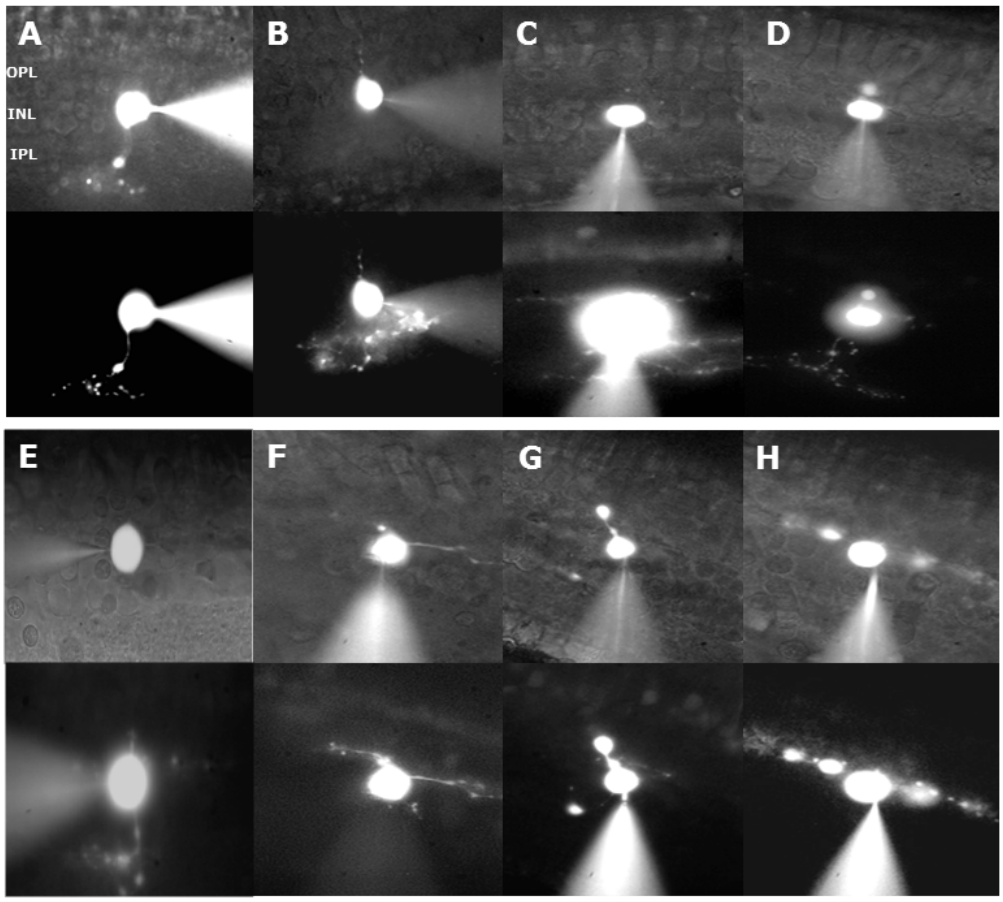

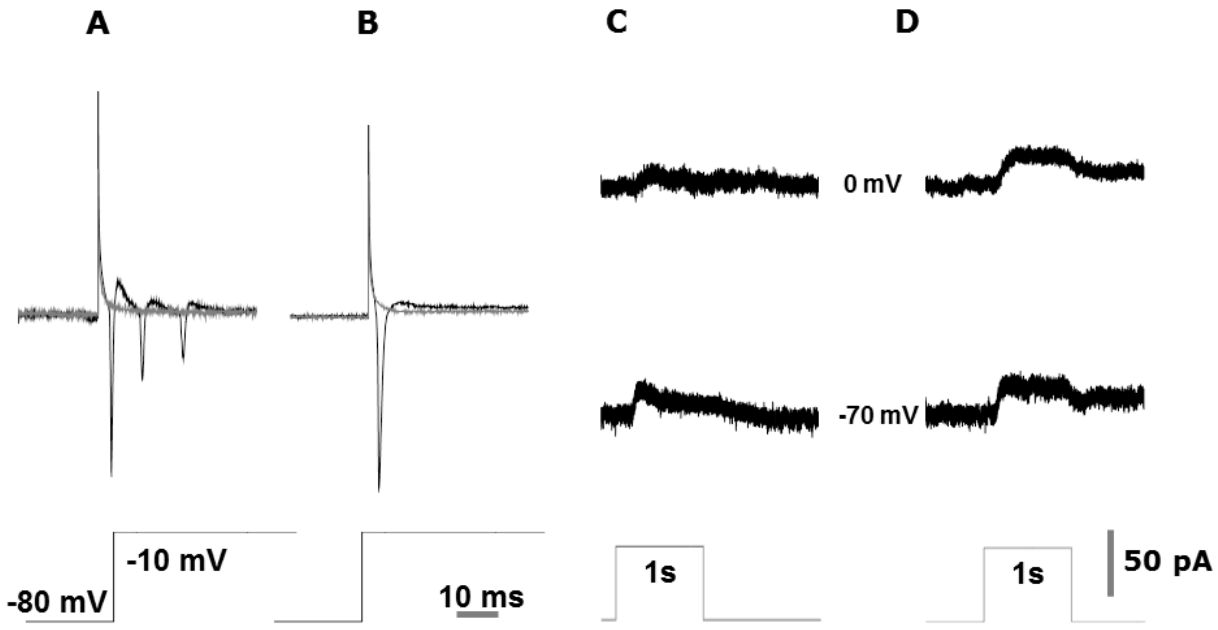

1. Materials and Methods

Retinal slices (210 μm thick) were prepared from Larval tiger salamanders (*Ambystomatigrinum*) obtained from Kons Direct (Germantown, WI, USA) and Charles Sullivan (Nashville, TN, USA). They were kept in tanks maintained at 4°C on a 12 h dark–light cycle. Experiments were performed on tissue slices of retina from tiger salamander in accordance with National Institutes of Health and University Animal Care guidelines. The retinal slice preparation was described in detail previously by Wu (Wu, 1987) and modified by Awatramani and Slaughter (Awatramani & Slaughter, 2001). Briefly, retinas were isolated and sliced in Ringer solution continuously bubbled with 100% O_2 and containing the following (in mM): 111 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 dextrose and 5 Hepes, buffered to pH 7.8 with NaOH. Recorded cells were first identified by their morphology of somata located in the inner nuclear layer (INL), and their dendrites directly adjacent to or in the outer plexiform layer (OPL), and were further revealed by fluorescent visualization and taking videos, using internal solution that included Alexa 488 hydrazide (50 μM) or Lucifer Yellow (1%).

Once in the microscope recording chamber, retinal slices were continuously superfused with 100% O_2 at a rate of 1–2 ml/min. Patch electrodes contained the following (in mM): 120 CsMeSO4, 10 CsCl, 2 $MgCl_2$, 5 EGTA and 5 Hepes, and buffered to pH 7.4 with CsOH. All experiments were performed at room temperature. All drugs were obtained from Sigma-Aldrich and Tocris Bioscience, except TTX (Alomone Labs), Alexa 488 and Lucifer Yellow (Invitrogen).

Unless otherwise indicated, bipolar cells were voltage clamped at 0 mV (the reversal potential for excitatory inputs) or -70 mV (the reversal potential for inhibitory inputs) to record inhibitory and excitatory responses.

Data were not corrected for junctional potential. The calculated chloride reversal potential is −66 mV. In addition, the pipette solution contained 4 mM ATP, 20 mM phospho-creatine and 50 U ml^{-1}creatine phosphokinase to maintain intracellular ATP. Tetrodotoxin (TTX, 1 μM) was

employed to block voltage-gated Na^+ currents. Voltage ramps and steps were used to monitor voltage-gated channels of all recorded cells. The open tip resistance of the electrodes was 8–11 MΩ. The access resistance, ranging from 10 to 20 MΩ, produced a voltage error usually less than 5 mV and was not corrected. Electrophysiological data were collected with a DIGIDATA 1322A (Axon Instrument, USA), Pclamp 10 software and a Dell Dimension 8300 computer. The analog signals were filtered at 5 kHz. Data were analyzed with Igor Pro 5.03 software (WaveMetricsInc, Lake Oswego, OR, USA), Clampfit 10.3 and Microsoft Excel software, PANASONIC Video and kworld convertor. The pictures were taken from preview offline of videos after recording, then processed with ImageJ software.

1.1. Light stimulation

The animals were not dark adapted though they were kept in the dark overnight. The slices were kept in the dark box for 30 minutes before recording. In light response experiments, a full-field green light-emitting diode LED (525 nm, irradiance was ~0.7 μw/cm^2) were used to stimulate retinal neurons. The light duration was usually ≤2 s. The interval time of two light stimuli was 5 minutes. All light-response experiments were performed on a dark background in a light-tight Faraday cage.

1.2. Drugs

Glycine, strychnine, picrotoxin, tetraethylammonium (TEA)-Cl, tetrodotoxin (TTX), D-AP5, AP-4, TPMPA, and SR95533 were obtained from Sigma Chemical (St Louis, MO, USA). All other drugs were applied extracellularly, unless otherwise specified. A DAD12 air-driven superfusion system (ALA Scientific Instrument, NY, USA) was used in experiments on retinal slices. This provided fast exchange (~100 ms) over an area of about 300 μm. Control Ringer solution was applied to the cell or tissue, then exchanged with drugs dissolved in the control

Ringer solution, and after an effect was observed the drug was removed by exchange with the control Ringer solution.