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Morphological and physiological analysis of type-5 and other bipolar cells in the Mouse Retina

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Graphical Abstract

A neurobiotin injected, immunolabeled XBC bipolar cell and type 5 bipolar cell stratifying near the ON-ChAT (choline acetyltransferase) band in the mouse retina. The graph shows step-pulse evoked Na⁺ currents from voltage clamp recordings.

Retinal bipolar cells are the second-order neurons in the visual system, which initiate image feature-based, multiple neural streams. Among more than ten types of bipolar cells, type-5 cells are thought to play a role in motion detection pathways. Multiple subsets of type-5 cells have been reported; however, detailed characteristics of each subset have not yet been elucidated. Here, we found that they exhibit distinct morphological features as well as unique voltage-gated channel expression. We have conducted electrophysiological and immunohistochemical analysis of retinal bipolar cells. We defined type-5 cells by their axon terminal ramification in the inner plexiform layer (IPL) between the border of ON/OFF sublaminae and the ON ChAT band. We found three subsets of type-5 cells: XBCs had the widest axon terminals that stratified at a close approximation of the ON ChAT band as well as exhibiting large voltage-gated Na⁺ channel activity, type-5-1 cells had compact terminals and no Na⁺ channel activity, and type-5-2 cells

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contained umbrella-shaped terminals as well as large voltage-gated Na⁺ channel activity. HCN currents were also evoked in all type-5 bipolar cells. We found that XBCs and type-5-2 cells exhibited larger HCN currents than type-5-1 cells. Furthermore, the former two types showed stronger HCN1 expression than the latter. Our previous observations (Ichinose et al., 2014) match the current study: low temporal tuning cells that we named 5S corresponded to 5-1 in this study, while high temporal tuning 5f cells from the previous study corresponded to 5-2 cells. Taken together, we found three subsets of type-5 bipolar cells based on their morphologies and physiological features.

Keywords

patch clamp; voltage-gated Na⁺ channel; HCN1 channel; ChAT band; bipolar cells

Introduction

Bipolar cells receive visual inputs from photoreceptors, and relay them to retinal third-order neurons, ganglion cells, which exit the retina. Bipolar cells not only pass on visual signals, but also play a role in filtering diverse features of visual inputs. More than 10 morphological types of bipolar cells have been characterized, which is indicative of diverse neural parallel pathways (Euler and Wässle, 1995, Wu et al., 2000, Ghosh et al., 2004, Pignatelli and Strettoi, 2004, Wässle et al., 2009). Physiological diversity of bipolar cells has also been reported (Awatramani and Slaughter, 2000, DeVries, 2000, Euler and Masland, 2000, Wu et al., 2000, Ichinose et al., 2014). These lines of evidence indicate that bipolar cells filter complex images by encoding distinct aspects of visual signaling among each type.

In the mouse retina, functions of each bipolar cell type have been gradually unveiled. Scotopic (dim) visual signaling is mediated by rod bipolar cells as well as type-3 and type-4 OFF cone bipolar cells whereas photopic (bright) signaling is carried by cone bipolar cells (Mataruga et al., 2007, Haverkamp et al., 2008). Dichromatic color vision is mediated both by type-1 and type-9 bipolar cells (Haverkamp et al., 2005, Breuninger et al., 2011). Type-5 ON cone bipolar cells exhibit high temporal tuning, change-sensitive natures (Borghuis et al., 2013, Euler et al., 2014, Ichinose et al., 2014) and provide inputs to ON direction selective (DS) ganglion cells (Yonehara et al., 2013), indicating that they contribute to motion detection pathways.

Multiple subsets of type-5 bipolar cells have been suggested (Ghosh et al., 2004, Fyk-Kolodziej and Pourcho, 2007, Helmstaedter et al., 2013). Helmstaedter et al. (2013) recently reported a new type of bipolar cell, the XBC type; by earlier characterization standards (Ghosh et al., 2004), this cell is another set of type 5 bipolar cells and is considered as such for our purposes. We reported that multiple subsets of type-5 bipolar cells respond to light with distinct temporal features (Ichinose et al., 2014). In the current study, we analyzed type-5 cells in terms of morphology and voltage-gated channel expression. Because they are transient and high temporal tuning cells, we focused on voltage-gated Na⁺ channels and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. Both channels are known to shape transient, excitatory postsynaptic responses (Gonzalez-Burgos and

Barrionuevo, 2001, Ichinose et al., 2005, Cangiano et al., 2007, Barrow and Wu, 2009). We found that voltage-gated Na⁺ channels are expressed only in high-temporal tuning type-5 cells, while HCN1 channels show higher expression in these cells than in lower temporal tuning type-5 cells.

Experimental Procedures

Retinal preparation

Animal protocols were approved by the Institutional Animal Care and Use Committee at Wayne State University. The experimental techniques were similar to those described previously (Ichinose and Lukasiewicz, 2012, Ichinose et al., 2014). Briefly, mice (28–60 d of age; male, C57BL/6J strain; The Jackson Laboratory) were dark-adapted overnight and then euthanized using carbon dioxide and pneumothorax. Using a dissecting microscope, the cornea and the lens were quickly removed and the retina was isolated. The retina slab was placed on a piece of filter membrane (HABG01300, Millipore, Billerica, MA) and cut into slice preparations (250 µm thick) using a hand-made chopper. We used only the dorsal part of the retina. Retinal dissection and physiological recording procedures were performed in dark adapted conditions under infrared illumination. The dissection medium was cooled and continuously oxygenated. The retinal preparations were stored in an oxygenated dark box at room temperature.

Whole-cell recordings

Whole-cell patch recordings were made from bipolar cell somas in retinal slices by viewing them with an upright microscope (Slicescope Pro 2000, Scientifica) equipped with a CCD camera (Retiga-2000R, Q-Imaging, Canada). Whole cell voltage steps were applied to evoke voltage-gated Na⁺ currents and HCN currents. All recordings were made at 30–34°C. Liquid junction potentials were corrected after each recording. Electrodes were pulled from borosilicate glass (1B150F-4; World Precision Instruments) with a P1000 Micropipette Puller (Sutter Instruments) and had resistances of 8 – 12 MΩ. Clampex and Multi Clamp 700B (Molecular Devices) were used to generate waveforms, acquire data, and control LED light stimuli (Cool LED). The data was digitized and stored with a personal computer using Axon Digidata 1440A (Molecular Devices). Responses were filtered at 1 kHz with the four-pole Bessel filter on the Multiclamp 700B and sampled at 2–5 kHz.

Solution and drugs

Retinal dissections were performed in HEPES-buffered extracellular Ringer's solution containing the following (in mM): 115 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.0 MgCl₂, 10 HEPES, 28 glucose, adjusted to pH 7.36 by NaOH. Physiological recordings were performed in Ames' medium buffered with NaHCO₃ (294 mOsm) (Sigma). Ames' medium was continuously bubbled with 95% O₂ and 5% CO₂; the pH was 7.4 at 30°C. The intracellular solution contained the following (in mM): 111 Cs-gluconate, 1.0 CaCl₂, 10 HEPES, 10 EGTA, 10 NaCl, 1.0 MgCl₂, 5 ATP-Mg, and 1.0 GTP-Na, adjusted to pH 7.2 with CsOH (269 mOsm). For some experiments, we used K-gluconate (111 mM) instead of Cs-gluconate. Tetrodotoxin (TTX) was obtained from Alomone Laboratory (#T-500, Jerusalem, Israel) and ZD7288 was from Tocris-R&D Systems (#1000, Bristol, UK).

Morphological identification

A fluorescent dye, sulforhodamine B (0.005%, Sigma), and neurobiotin (0.5 %, Vector Lab) were included in the pipette; these dyes did not affect the physiological recordings (Ichinose et al., 2014). Immediately after electrophysiological recordings, sulforhodamine B images were captured using the CCD camera. To visualize neurobiotin staining, the slice preparation was fixed with 4% paraformaldehyde for 30 min, incubated with streptavidin-conjugated Alexa 488 (1:200, Life Technologies) and anti-ChAT antibody (1:200, Millipore) overnight, and then incubated with the secondary antibody for 2 hours at room temperature. The preparation was viewed with a confocal microscope (Leica, TCS SP2 or SP8). We determined bipolar cell types according to the description of Ghosh et al. (2004) and Ichinose et al. (2014). Type-5 bipolar cells were identified if their axon terminals ramified between the border of ON/OFF sublaminae (40% of the IPL depth) and ON ChAT band (~60% of the IPL depth).

Immunohistochemistry

Retinal thin sections or neurobiotin-filled thick sections were used for immunohistochemistry. For making thin sections, the retinal eyecups were fixed with 4% paraformaldehyde in 0.1 M PB, pH 7.4, for 30 min at room temperature. After fixation, retinas were separated from the sclera, rinsed several times in 0.1 M PB, and cryoprotected in 30% sucrose overnight at 4 °C. The tissue was embedded in Tissue Freezing Medium (EMS) and was sectioned vertically at 14–16 µm with a Microm HM 525 cryostat (Thermo Scientific). Cryosections were collected on histo-bond slides (Fisher Scientific).

Retinal thin sections were washed several times in 0.1 M PBS and blocked in a solution containing 3% normal donkey serum (NDS) and 0.5% Triton-X in PBS for 1 hour at room temperature. Primary antibodies were diluted in 1% NDS and 0.5% Triton-X in PBS. HCN4 (Neuro Mab, 75–150) and PKA RIIβ (BD Biosciences, 610625) were used for labeling types 3A and 3B bipolar cells, respectively. Pan-Na⁺ channel antibody (Alomone Labs, ASC-003) and HCN1 antibody (NeuroMab, 75–110) were used for channel expression and CtBP (Synaptic Systems, 192003) was for labeling the ribbon synapses. Detailed information regarding antibodies is summarized in Table 1. Sections were incubated with primary antibodies overnight at room temperature, followed by a mixture of secondary antibodies, conjugated with Alexa dyes (Life Technologies, Inc.) at room temperature for 2 hours. The preparation was viewed with a confocal microscope (Leica, TCS SP8) using a 63× oil, 63× water or 20× water immersion objective lens. The z-step for stack images was 0.3 µm. For double- or triple-labeled tissue, sequential scanning was used to eliminate crosstalk between channels and to better separate signals from each other.

Protein extraction and western blotting

Total protein was extracted from the cortex of an adult mouse and the cerebellum of a postnatal day 5 mouse (C57BL/6J, The Jackson Laboratory). Brain tissues were harvested and rinsed in ice-cold PBS for 3 times to remove blood before mincing into small pieces. Collected tissues were transferred to lysis buffer (Thermo) for homogenization, followed by incubation at 4°C for 20 min before centrifugation at 12,000 rpm for 20 min to harvest supernatant. Western blotting was used to detect the protein expression of sodium channels

and HCN1 channels. Antibodies used in western blotting included rabbit polyclonal anti-Pan NaV (Alomone, 1:500) and mouse monoclonal anti-HCN1 (NeuroMab, 1:500). Secondary antibodies included donkey anti-rabbit HRP-conjugated antibody, donkey anti-mouse HRP-conjugated antibody and HRP standard protein (all from Bio-Rad). SuperSignal® West Femto Stable Peroxide Solution and SuperSignal® West Femto Luminol/Enhancer Solution (ECL, all from Thermo) was applied to blotting membrane for protein detection. Images were captured using a ChemiDoc-It® 2 imaging system.

Data analysis

Image analysis was performed using AutoQuant X3 and Image-Pro Premier 3D software (Media Cybernetics). Stack images captured by confocal microscopy were usually not aligned because retinal slices were more or less tilted. Using 3D analysis tools, ChAT bands in stack images were realigned and neurobiotin-filled bipolar cells were analyzed. Widths of axon terminals and branching points were measured. The IPL depth was defined as 0% at the border of INL/IPL and as 100% at the border of IPL/ganglion cell layer. The depth of axon terminals in the IPL was measured and the location of axon terminals was calculated by being compared to the total IPL thickness. To measure the colocalization with ChAT band, axon terminals were turned into volume objects by iso-surface renderings (Image-Pro Premier 3D). The volume of terminals within and at a close approximation of the ChAT band was measured and the ratio was calculated relative to the total volume.

For Na⁺ and HCN1 channel staining, colocalization with bipolar cell processes was analyzed using 3D analysis tools. Cell markers and channel immunolabeling colocalization was initially examined by rotating the 3D images and by analyzing series of single optical sections (0.3 µm thickness) from collected *z*-stack images.

We also used mathematical analysis. We performed two-dimensional cross correlation coefficient analysis using a custom Matlab (MathWorks, Natick, MA, USA) code (TI wrote) (Soto et al., 2011, Zinchuk et al., 2011). Images of XBC or type-5-1 terminals and HCN1 were excised (4 × 4 µm). Single digital sections (0.3 µm) of two colors are separated into two grayscale images. Background noise levels were reduced to 0 levels. The 2D correlation coefficient program compares two images using the equation:

$$R(i, j) = \frac{C(i, j)}{\sqrt{C(i, i)C(j, j)}}$$

Where R is the correlation coefficient and c is the covariance of the relevant channels. The maximum of correlation coefficient is 1. The correlation coefficient of images was compared to that of images when one channel was rotated 180° to control for random colocalization. The correlation coefficient was plotted in a 3D mesh graph using Matlab.

To evoke voltage-gated Na⁺ currents, whole cell voltage step pulses (−90 to +60 mV, 10 mV steps) were applied from the baseline voltage at −70 mV. To evoke HCN currents, hyperpolarization steps (−60 to −140 mV, −10 mV steps) were applied followed by a −73 mV test pulse to evoke tail currents (Figure 5A). The existence of Na⁺ currents was

determined if a transient inward current was evoked within a few millisecond of the step pulse. Peak amplitude (pA) and charge transfer (area under responses, pA×ms) were measured using Clampfit (Molecular Devices). The decay phase of HCN currents was fit with a standard double exponential equation: $f(t) = A_1 e^{-\frac{t}{\tau_1}} + A_2 e^{-\frac{t}{\tau_2}} + C$ where A is the amplitude and τ is the time constant. The fast time constant (τ) was analyzed. Values are presented as mean $\pm$ SEM. An unpaired *t* test was conducted and differences were considered significant if $p < 0.05$.

Results

Type-5 cell morphology and Na⁺ channel activity

We analyzed type-5 ON bipolar cells whose axon terminals ramified between the border of ON/OFF sublaminae and the ON ChAT band (Figure 1A–1B). Figure 1C–1E show neurobiotin filled, type-5 bipolar cell terminals and ChAT bands. XBC was first reported by Helmstaedter et al. (2013), and was clearly distinguished from other type-5 cells. Their axon terminals were wide and stratified in mono-layer (Figure 1A & 1C, Table 2). Terminals showed close approximation to the ON ChAT band ($87.9 \pm 5\%$ colocalization). Other type-5 cell terminals were narrower and less colocalized with the ChAT band ($p < 0.01$ both for terminal width and ChAT colocalization, other type-5 cell, $N=30$).

In response to whole-cell voltage steps, fast inward currents were evoked in XBCs (Figure 2A). Their I-V relationships (Figure 2B) and their tetrodotoxin sensitivity (Figure 2C) demonstrated that the inward currents were voltage-gated Na⁺ currents. The inward currents were detected in most XBCs (Table 3), indicating that XBCs express voltage-gated Na⁺ channels.

Compared to XBCs, other type-5 cell axon terminals ramified above the ON ChAT band and the branching point was higher than that of XBCs (Table 2, $p < 0.05$). Still, the majority of type-5 cells extended their terminals onto the ON ChAT band (Figure 1D & 1E). We also investigated whether they possessed Na⁺ channels. We found that a subset of non-XBC type-5 cells also exhibited Na⁺ currents in response to voltage steps (Figure 2D–2F). We named this subset of type-5 cells with Na⁺ channels type-5-2 (Figure 1D), and those cells without Na⁺ channels type-5-1 (Figure 1B.1E). Type-5-2 cells exhibited wider axon terminals than type-5-1 cells (Table 2, $p < 0.05$), and colocalized with the ON ChAT band less than type-5-1 did ($p < 0.01$). Taken together, these results suggest that type-5 cells consist of 3 subsets; XBCs, type-5-1 and type-5-2 cells.

Na⁺ channels are expressed only in a subset of cone bipolar cells as shown in the rat, goldfish, salamander, and ground squirrel retinas (Pan and Hu, 2000, Zenisek et al., 2001, Ichinose et al., 2005, Ichinose and Lukasiewicz, 2007, Cui and Pan, 2008, Saszik and DeVries, 2012). As bipolar cell types that express Na⁺ channels in the mouse retina have not been characterized, we examined all bipolar cell types using whole cell clamp recordings and morphological analysis. Table 3 shows that the fast inward current was recorded only in 3 types of bipolar cells; a subset of type-3, type-5-2, and XBCs. A couple of cells exhibited currents similar to Na⁺ channel activity, namely in type-2 and type-7 cells; however, the majority of cells in these types did not show rapid voltage-gated currents. Therefore, we

analyzed only three types. The peak amplitude of the fast inward currents was measured (Table 3). There was no significant difference between type-5-2 and XBC ($p = 0.07$) or between XBC and type-3 ($p > 0.1$); Type-5-2 and type-3 did however show significantly different Na^+ peak amplitudes ($p = 0.001$).

We further investigated Na^+ channel expression using immunohistochemical techniques. We first tested the Pan- Na^+ channel antibody we used. Western blotting was used to detect the presence of Na^+ channels in the mouse brain. The antiserum binds to multiple bands, including the 250kDa reported band for NaV1.1 on Western blot (Figure 3A). We also conducted a pre-adsorption test with this antibody. Pre-incubation with control peptide revealed minimal non-specific labeling in retina (Figure 3B–B'), while furthermore no labeling was detected when the primary antibody was omitted (Figure 3B''). Additionally, the antibody labeled nerve fibers and puncta in the NFL and IPL (Figure 3B, 3C), which is consistent with the previous reports (Van Wart et al., 2005). These tests indicate that the antibody we used targeted the voltage-gated Na^+ channels.

Two subsets of type-3 bipolar cells have been identified using HCN4 and PKARII β antibodies (Mataruga et al., 2007, Wässle et al., 2009). We examined whether one of these type-3 cells expressed Na^+ channels using these antibodies to distinguish each type. Anti- Na^+ channel antibody labeled nerve fibers and puncta in the IPL (Figure 3B–C). HCN4 labeled type-3A cells colocalized with Pan Na^+ channel antibody at the axon shaft (Figure 3C–3C'', $N = 3$ mice). However, PKARII β -stained type 3 cells did not colocalize with Na^+ channels (Figure 3D). Therefore, we conclude that the subset of type 3 cells with Na^+ currents was type 3A.

There are no specific antibodies for type 5 subsets available. We injected neurobiotin to individual bipolar cells, and when type 5 cells were labeled, immunostaining for Na^+ channels was conducted. Neurobiotin-filled XBCs were colocalized with Na^+ channels at the axon shaft ($n = 3$ XBCs, Figure 4A–4E). Also, neurobiotin-filled type 5 cells colocalized with Na^+ channels at the axon shaft ($n = 3$ type 5 cells, Figure 4F–4J). Taken together, we found that voltage-gated Na^+ channels were expressed in type 5-2 cells, XBCs, and HCN4-labeled type-3A cells.

HCN channels and type-5 bipolar cells

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels were originally found in heart pacemaker cells, which maintain pumping rhythms (Brown et al., 1979, Baker et al., 1997). In the retina, HCN1 through HCN4 channels have been detected (Ivanova et al., 2006, Fyk-Kolodziej and Pourcho, 2007), and have been shown to modify temporal tuning (Cangiano et al., 2007, Barrow and Wu, 2009). Because temporal tuning among subsets of type-5 cells differed (Ichinose et al., 2014), we examined how HCN currents were expressed in type-5 bipolar cells.

We applied whole-cell voltage steps from -60 mV to -140 mV, each followed by a step at -73 mV. The former evoked steady-state inward currents (asterisk) while the latter voltage step evoked tail currents (arrow) (Figure 5A). Both inward currents and tail currents were reduced by a HCN channel blocker, ZD7288 (100 μM) (Figure 5B). The peak amplitude of

tail currents was significantly reduced ($21 \pm 8\%$, $N=4$, $p<0.01$). However, the steady-state inward currents were reduced by only about 50%. This might be attributable to the contamination by other potassium currents and instantaneous currents (Khakh and Henderson, 1998, Horwitz et al., 2011). Therefore, we analyzed the tail currents.

Tail currents were evoked from all type-5 cells including XBCs (Figure 5C–E). The charge transfer of tail currents was increased when cells were increasingly hyperpolarized by a pre-pulse. Tail-currents of XBCs and type 5-2 cells were similar in size ($p>0.3$). However, type 5-1 cell tail currents were significantly smaller than those of the other two types ($p<0.05$). We also analyzed the decay phase kinetics of tail currents because HCN isoforms exhibit distinct current kinetics (Ivanova and Muller, 2006, Biel et al., 2009). We measured the time constant (τ) of all three type-5 cells (Figure 5G). The time constant varied slightly for different pre-pulses although no significant differences were detected among the 3 subsets. Taken together, the results suggest that all 3 subsets of type-5 cells exhibit the same type of HCN channels; however, the HCN current was significantly smaller in type-5-1 cells than in the other two subsets.

Finally, we examined if the HCN1 channel is expressed in type-5 cells using immunohistochemical methods. We initially used Western blotting to demonstrate that the antiserum binds to a single band of the reported 100kDa molecular weight in mouse cerebellum tissue (Figure 6A). Furthermore, the HCN1 antibody was shown to have no cross-reactivity with the HCN2 peptide (manufacturers technical information). Additionally, the antibody was characterized in both wild-type and HCN1 knock-out tissue (Pan et al., 2014) where the 100 kDa band was shown to disappear. Retinal slice staining in the knock-out animal revealed minimal non-specific labeling by the antibody. These facts indicate that the antibody we used targeted HCN1.

The HCN1 antibody labeled processes in the entire IPL, and bright immunoreactivity was recognized in the outer layer of the ON ChAT band where axon terminals of type-5 cells ramify (Figure 6B), which is consistent with previous reports (Muller et al., 2003, Cangiano et al., 2007). The bright HCN1 band was colocalized with a ribbon synaptic marker, CtBP2, suggesting that the HCN1 band is localized to bipolar cell axon terminals (Figure 6B'). We injected neurobiotin into type-5 cells and investigated the colocalization with the bright HCN1 immunolabeling. Axon terminals of XBCs and type-5-2 cells colocalized with bright HCN1 (Figure 6D–6E) (type 5-2 $n=5$, XBC $n=8$), which was verified with 2D correlation coefficient analysis (Figure 6F for type 5-2, Figure 6G for XBCs). By contrast, type-5-1 cells were not colocalized with the bright HCN1 (Figure 6C) ($n=5$). These results indicate that XBCs and type-5-2 cells express HCN1 channels with a higher density than those of type-5-1 cells.

Discussion

Voltage-gated Na^+ channels

Outer retinal neurons including bipolar cells are slow-potential neurons that respond to light with graded potential changes. However, a subset of cone bipolar cells exhibit TTX-sensitive Na^+ channel activities (Pan and Hu, 2000, Zenisek et al., 2001, Ichinose et al.,

2005) which even generate action potentials (Ma et al., 2005, Saszik and DeVries, 2012, Puthussery et al., 2013). These bipolar cells may play a key role in fast-changing visual signal transmission.

Na⁺ channel-expressing bipolar cells have been identified in the retina of various species. Cui and Pan (2008) demonstrated that type-3 cone bipolar cells and a subset of type-5 cells show Na⁺ currents in the rat retina. Saszik and DeVries (2012) showed that a subset of type-5 cells exhibit Na⁺ currents and spiking activities in the ground squirrel retina. Consistent with these reports, we found that a subset of type-5 bipolar cells (type 5-2, XBCs) and a subset of type-3 cells (type-3A) exhibit Na⁺ channel activities in the mouse retina. Na⁺ current sizes varied among cells but no differences were found between XBCs and type-5-2 (Table 3).

We previously demonstrated that XBCs and a subset of type-5 bipolar cells respond transiently to light stimuli (Ichinose et al., 2014). Na⁺ channel activities were observed in the same morphological subsets of bipolar cells, indicating that Na⁺ channels are the underlying mechanism of shaping transient light responses in these cells. Type-3A OFF bipolar cells also showed Na⁺ channel activity (Table 3) and exhibited transient light responses (Ichinose and Hellmer, 2015), consistent with the notion that Na⁺ channels shape transient light responses. Taken together, our results indicate that type-3A, type 5-2, and XBCs utilize Na⁺ currents to play a role in the transient signaling pathway.

HCN channel activity

HCN currents (hyperpolarization-activated currents; I_h) were initially found in sinoatrial node cells of the heart. HCN currents are cationic currents carried both by Na⁺ and K⁺, and are activated by hyperpolarizing voltage steps negative to -55 mV (Biel et al., 2009). It should be a key component for cell repolarization after signaling, and is thought to play a key role in pacemaker activity in the heart (Brown et al., 1979). In the retina, HCN currents support neural responses to flicker light stimuli (Cangiano et al., 2007, Knop et al., 2008, Barrow and Wu, 2009). By hastening the repolarization of cells after signaling, they allow a quicker return to a threshold where voltage-gated cation channels can contribute to the next signal. Therefore, high temporal tuning cells might utilize HCN currents to maintain their high frequency voltage changes.

Four isoforms of HCN channels are known. In the retina, HCN1, 2, and 4 are expressed in subsets of bipolar cells while HCN3 is expressed mainly in cone photoreceptor pedicles (Muller et al., 2003). Although immunoreactivity of HCN1, 2, and 4 are detected throughout the IPL in the rodent retinas (Figure 6A for HCN1) (Muller et al., 2003, Ivanova and Muller, 2006, Mataruga et al., 2007), each HCN isoform signal is strong in particular layers of IPL. Strong HCN1 immunoreactivity has been seen immediately above ON ChAT band where type-5 bipolar cells ramify (Figure 6) (Muller et al., 2003, Cangiano et al., 2007). HCN2 immunoreactivity is strong near the border of IPL/GCL where rod bipolar cells and type-8 cells ramify (Muller et al., 2003, Ivanova and Muller, 2006, Cangiano et al., 2007). HCN4 bright reactivity is observed in the inner IPL near OFF ChAT band where type-3A cells ramify (Mataruga et al., 2007). In the present study, we labeled single type-5 bipolar cells by

neurobiotin injection technique and found that XBCs and type-5-2 cells, but not type-5-1 cells colocalized with strong HCN1 immunoreactivity.

Tail-current analysis revealed that all three subsets exhibited HCN channel activity. However, their charge transfer differed. XBCs and type-5-2 cells showed significantly higher charge transfer than type-5-1 cells' HCN currents, indicating that HCN activity played a greater role in temporal tuning in these subsets. While our immunological results indicate that HCN1 plays a role in these cells, it was not possible to rule out contamination of HCN2 or HCN4 currents in these cells or to distinguish the contributions of each type to overall HCN activity.

Type-5 and other bipolar cell types

Bipolar cells are thought to initiate diverse visual signal pathways, and the various types are the key elements of different signaling pathways (Euler et al., 2014). In the mouse retina, thirteen types of bipolar cells have been elucidated by their morphology, marker expressions, connectomic study, and physiological studies (Euler and Wässle, 1995, Wu et al., 2000, Pignatelli and Strettoi, 2004, Wässle, 2004, Wässle et al., 2009, Helmstaedter et al., 2013, Ichinose et al., 2014). However, subsets of type-5 bipolar cells have not been clearly characterized because of a lack of markers (Wässle et al., 2009). Therefore, we combined morphological and electrophysiological methods to analyze type-5 cells in the present study.

Because XBCs and type 5-2 cells are high temporal tuning cells, we thought that excitatory voltage-gated channels might be expressed in these subsets. As we expected, Na⁺ channel activity was detected in these subsets, but not in type-5-1 cells (Table 2 & 3). Similarly, HCN currents were significantly higher in these subsets than in type-5-1 cells (Figure 5). These observations divide classic type-5 cells into two subsets. In addition, XBCs are morphologically distinct from other type-5 cells in terms of axon terminal widths and mono-stratification patterns (Figure 1). Therefore, 3 subsets of type-5 cells were identified. In our previous study (2014), we categorized two subsets of type-5 cells: type-5F cells were high temporal tuning whereas type-5S cells were low temporal tuning. Our records showed that type 5S cells correspond to type-5-1 cells, and type 5F cells to type-5-2 cells, indicating that Na⁺ channels and HCN1 channels shape high temporal tuning.

How do type-5 cells give rise to visual signal processing pathways? Type-5 bipolar cells provide synaptic inputs to direction selective (DS) ganglion cells (Yonehara et al., 2013, Chen et al., 2014). Although individual subset contribution is not clear, their figures seem to show all subsets of type-5 cells that we detected may play a role. However, a connectomic study indicated that XBCs hardly make direct synaptic contact with ganglion cells, contributing instead to ganglion cell signaling via amacrine cells (Helmstaedter et al., 2013). Furthermore, ON-OFF DSGCs respond to faster visual stimuli while ON DSGCs respond to slower visual stimuli (Sivyer et al., 2010), and ONDS ganglion cells play a role in image stabilization by contributing to a vestibulocentric coordinate system (Sabbah et al., 2015). Taken together, the different temporal properties of Type 5-1, 5-2, and XBC we found might contribute differentially to separate motion pathways.

Conclusion

Type-5 ON bipolar cells were analyzed using morphological and physiological methods. XBCs are morphologically distinct from other type-5 bipolar cells. TTX-sensitive Na⁺ currents were detected in XBCs and type-5-2 cells. HCN-evoked currents were detected in all type-5 cells; however, HCN currents were significantly larger in XBCs and type-5-2 cells than in type-5-1 cells. Taken together, we found that high temporal tuning properties of XBCs and type-5-2 cells may arise from Na⁺ channels and HCN channels in these cell types.

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Abbreviations

ChAT	choline acetyltransferase
IPL	Inner plexiform layer
OPL	Outer plexiform layer
INL	inner nuclear layer
GCL	ganglion cell layer
NFL	neurofiber layer
CBC	cone bipolar cell
DSGC	direction selective ganglion cell

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Highlights

- We show 3 subsets of type-5 ON bipolar cells in the mouse retina by morphological and physiological analysis
- We demonstrate strong and weak HCN1 expression across subsets of type-5 ON bipolar cells
- We demonstrate Na⁺ current in 3 types of retinal bipolar cells including type-5 cells
- Combined with previous work, we show that Na⁺ and HCN currents play a role in temporal tuning of visual signaling

Figure 1.

Type-5 ON bipolar cells and ChAT bands. Neurobiotin was injected in single cells and labeled along with ChAT band using immunohistochemistry. Confocal-captured images were aligned to the ON ChAT band and rotated to show the widest terminals. (A) A XBC cell whose axon terminals were wide and ramify near the ON ChAT band. (B) A type-5 cell whose axon terminals were narrower than XBCs' and ramify near the ON ChAT band. (C) Axon terminals of two XBCs, which were wide and thin. (D) Axon terminals of two type-5-2 cells looked like umbrellas. (E) Axon terminals of two type-5-1 cells were compact. Scale bar in (B) is also for (A). Scale bar in (F) is also for (D) and (E).

Figure 2.

Na⁺ currents in type-5 cells. (A) Fast inward currents were evoked in response to voltage steps recorded in a XBC. (B) Peak amplitudes were plotted as a function of testing voltages from 3 XBCs. (C) The inward current was TTX-sensitive. The inset shows inward currents in control and TTX (1 μ M) solution. The main panel shows the inward currents revealed by subtracting recordings of TTX application from control currents ($n=3$ XBCs). (D) Fast inward currents were evoked in response to voltage steps in a type-5-2 cell. (E) Peak amplitudes were plotted as a function of voltage step potentials from 3 type-5-2 cells. (F) A digital subtraction of the current-voltage function during TTX application from a control function revealing a TTX sensitive component. The inset shows the current in control and TTX solution ($n=3$ Type-5-2s).

Figure 3.

Type 3A bipolar cells colocalized with Na⁺ channel immunolabeling. (A) Western blot of Anti-Pan Na⁺ antibody (polyclonal) against mouse cerebellum tissue shows multiple bands including one for the reported molecular weight for Na⁺ channel at ~250 kDa. (B) Pan Na⁺ channel immunostaining revealed ganglion cell axon bundles (NFL) and puncta in the IPL. The arrow denotes Na⁺ staining of neural fibers. (B') Control staining via pre-incubation with 10µg control peptide per µg antibody abolishes typical staining of retinal layers. (B'') Omit-primary antibody staining. (C) Pan Na⁺ channel immunostaining. The arrow denotes Na⁺ staining of neural fibers. (C') HCN4 antibody labeled type-3A bipolar cells. (C'') Pan Na⁺ channel immunostaining colocalized with type-3A axon shafts. (D) PKARIIB labeled type-3B bipolar cells. Pan Na⁺ channel staining did not colocalize with any parts of these cells.

Figure 4.

Types 5-2 and XBC bipolar cells colocalized with Na^+ channel immunolabeling. (A) Neurobiotin-injected XBC was further labeled with Pan Na^+ channel antibody. An arrowhead indicates colocalization with Na^+ channels ($n=3$ XBCs). The soma was removed when patch pipette was retracted (dotted circle). (B) High power view of the XBC- Na^+ channel colocalization in (A). Immunoreactivity only with Pan Na^+ antibody is shown in the right panels. (B1–B3) Single scan images of Pan- Na^+ colocalization with the XBC axon shaft from the image (B). The z-depth between each image is $0.3\mu\text{m}$. (C) Neurobiotin was injected in a type-5-2 bipolar cell which was later labeled with Pan Na^+ channel antibody. An arrowhead indicates colocalization with Na^+ channels. ($n=3$ type-5-2 cells). The soma was removed when patch pipette was detached (dotted circle). (D) High power view of the type-5-2- Na^+ channel colocalization in (C). (D') The image (C) was rotated 90° . Na^+ channel was still colocalized. (D1–D3) Single scan images of Pan- Na^+ colocalization with the type-5-2 axon shaft. The z-depth between each image is $0.6\mu\text{m}$. The images from (C,D) were rotated via 3D imaging to reveal the morphology of the entire cell. The single scan images are not rotated and show the tilt of the cell in the slice tissue. Stack size: (A) 83 stack images ($0.3\mu\text{m}$ thick each, total $25\mu\text{m}$ thick). (B) 12 stack images (total $3.6\mu\text{m}$ thick). (F) 45 stack images ($0.6\mu\text{m}$ thick each, total $27\mu\text{m}$ thick). (G) 7 stack images (total $4.2\mu\text{m}$ thick).

Figure 5.

HCN currents were detected in all type-5 cells, but their sizes differed. (A) Inward currents were evoked in response to a series of hyperpolarization. Steady-state currents (*) and tail-currents (arrow) were evoked. (B) 100 μ M ZD7228 reduced the inward currents. Steady-state currents were reduced in half. The residual currents might be contamination of other currents. The tail currents were reduced to $21.0 \pm 8\%$ ($n=4$, $p<0.01$), suggesting that tail currents were mostly evoked by HCN channels. (C) Tail currents evoked in a Type 5-1 cell. (D) Tail currents evoked in a Type-5-2 cell. (E) Tail currents evoked in a XBC cell. (F) Charge transfer of tail currents was compared among 3 subsets. Currents in Type-5-1 cells were significantly smaller at a pre-pulse of -100 mV and below potentials (*: $p<0.05$ vs. other subsets, $n=6$ Type-5-1 cells, $n=6$ Type-5-2 cells, $n=5$ XBCs). (G) Decay phases of tail currents were fit with exponential curves and the time constant of the initial phase was analyzed. At a prepulse of -100 mV and below potentials, no differences were observed. This indicated that all subsets possess the same types of HCN channels.

Figure 6.

HCN1 immunoreactivity and type-5 cell terminals. (A) Western blot of mouse anti-HCN1 antibody against mouse cortex. The arrow shows the reported molecular weight for HCN1 at ~100 kDa. (B) HCN1 labeled the entire IPL. Bright immunoreactivity was observed right above the ON ChAT band where type-5 cells terminate. (B') The bright HCN1 and ChAT staining was magnified. The bright HCN1 signaling was colocalized with CtBP2 antibody, a ribbon synapse marker, indicating that the bright HCN1 colocalized with bipolar cell terminals. (C) Neurobiotin was injected to a Type-5-1 cell, which was not colocalized with HCN1 bright signaling (n=5). (D) Neurobiotin was injected to a Type-5-2 cell, which was colocalized with HCN1 bright signaling (n=5). (E) Neurobiotin was injected to a XBC. HCN1 bright signaling also colocalized with its terminals (n=8). Immunohistochemical studies shown in (B) and (B') were conducted using thin sections (14 μ m thick). Panels in (C) through (E) were performed in thick slice sections (250 μ m thick). Lower panels of (C) through (E) were captured images of a few digital sections (~1 μ m thick). (F) 2D cross-correlation coefficient analysis was performed between type 5-2 axon terminals and HCN1 staining using single digital sections (0.3 μ m thick). Terminals of type 5-2 cells were colocalized with HCN1 strong staining (n=20 images, left). The colocalization was not random because when the HCN1 image was rotated 180°, the correlation coefficient was reduced (right panel). (G) 2D cross-correlation coefficient analysis also revealed that XBC axon terminals and HCN1 staining colocalized (n=10 images, left). The colocalization was not random because when the HCN1 image was rotated 180°, the correlation coefficient was reduced (right panel).

Table 1

Primary antibodies

Antibody	Immunogen	Source, Cat. #, Species	RRID**	Dilution
HCN1 clone N70/28	Rat HCN1 amino acids 778–910	NeuroMab, 75–110 Mouse monoclonal	AB_2115181	1:200
HCN4	Rat HCN4 amino acids 1019–1108	NeuroMab, 75–150 Mouse monoclonal	AB_2248534	1:200
Pan NaV	Rat NaV1.1* amino acids 1501–1518	Alomone, ASC-003 Rabbit polyclonal		1:500
CtBP2	Synthetic Rat Ribeye amino acids 974–988	Synaptic Systems, 192003 Rabbit polyclonal	AB_1210392	1:10,000
PKA RII β	Human PKA RII β amino acids 1–418	BD Biosciences, 610625 Mouse monoclonal	AB_397957	1:3000

* NaV- α epitope is conserved across isoforms and species

** The RRID provided for antibodies is a Research Resource Identifier number which links the antibody to the NIH sponsored NIF (Neuroscience Information Framework) database.

Table 2

	Type 5-1	Type 5-2	XBC
N	20	10	6
Na ⁺ currents	No	Yes	Yes
Branching point (IPL %)	43.8 ± 0.5 [*]	43.1 ± 0.9 [*]	45.8 ± 0.8
Axon widths (μm)	16.7 ± 0.9 ^{*,#}	22.8 ± 2.0 [*]	36.4 ± 4.0
ON ChAT band colocalization (%)	57.9 ± 0.1 ^{*,#}	34.0 ± 0.8 [*]	87.9 ± 0.1

^{*}: p<0.05 vs. XBCs

[#]: p<0.05 vs. type-5-2

Table 3

	N	I _{Na} cells	(%)	current
Type-1	7	0	0	
Type-2	17	1	6	
Type-3A	15	15	100	−145 ± 13 pA (n=4)
Type-3B	7	0	0	
Type-4	9	0	0	
Type-5-1	17	0	0	
Type-5-2	12	12	100	−314 ± 18 pA (n=3)
XBC	16	13	81	−196 ± 54 pA (n=3)
Type-6	20	0	0	
Type-7	13	1	8	
Type-8	5	0	0	
RBC	20	0	0	