Excitatory Synaptic Inputs to Mouse On-Off Direction-Selective Retinal Ganglion Cells Lack Direction Tuning

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Direction selectivity represents a fundamental visual computation. In mammalian retina, On-Off direction-selective ganglion cells (DSGCs) respond strongly to motion in a preferred direction and weakly to motion in the opposite, null direction. Electrical recordings suggested three direction-selective (DS) synaptic mechanisms: DS GABA release during null-direction motion from starburst amacrine cells (SACs) and DS acetylcholine and glutamate release during preferred direction motion from SACs and bipolar cells. However, evidence for DS acetylcholine and glutamate release has been inconsistent and at least one bipolar cell type that contacts another DSGC (On-type) lacks DS release. Here, whole-cell recordings in mouse retina showed that cholinergic input to On-Off DSGCs lacked DS, whereas the remaining (glutamatergic) input showed apparent DS. Fluorescence measurements with the glutamate biosensor intensity-based glutamate-sensing fluorescent reporter (iGluSnFR) conditionally expressed in On-Off DSGCs showed that glutamate release in both On- and Off-layer dendrites lacked DS, whereas simultaneously recorded excitatory currents showed apparent DS. With GABA-A receptors blocked, both iGluSnFR signals and excitatory currents lacked DS. Our measurements rule out DS release from bipolar cells onto On-Off DSGCs and support a theoretical model suggesting that apparent DS excitation in voltage-clamp recordings results from inadequate voltage control of DSGC dendrites during null-direction inhibition. SAC GABA release is the apparent sole source of DS input onto On-Off DSGCs.

Key words: direction selectivity; glutamate sensor; mouse retina; retinal ganglion cell; synaptic mechanism; two-photon imaging

Introduction

Direction selectivity represents a fundamental visual computation and arises first in the retina (Barlow and Levick, 1965; Vaney et al., 2012). A direction-selective ganglion cell (DSGC) fires strongly to motion in the preferred direction but weakly to motion in the opposite null direction. Three DSGC classes respond to light (On DSGCs), dark (Off DSGCs), or both light and dark moving objects (On-Off DSGCs; Oyster and Barlow, 1967; Kim et al., 2008; Vaney et al., 2012).

The mechanism of direction selectivity (DS) has been studied most extensively in On DSGCs and On-Off DSGCs. Both types receive input from starburst amacrine cells (SACs), which comprise On and Off subtypes (Fig. 1A). SACs release both GABA and

acetylcholine from distal regions of their radiating dendrites (Brandon, 1987; O'Malley and Masland, 1989; Famiglietti, 1991; O'Malley et al., 1992) and are required for the DS response of DSGCs (Yoshida et al., 2001). In On SACs, individual release sites are DS, preferring centrifugal motion (soma→tip; Euler et al., 2002; Yonehara et al., 2013). These sites selectively wire to DSGCs such that SAC dendrites that point in a particular direction (e.g., leftward) connect with a DSGC that prefers the opposite direction (e.g., rightward; Fried et al., 2002; Lee et al., 2010; Briggman et al., 2011; Wei et al., 2011; Beier et al., 2013; Yonehara et al., 2013). This connectivity results in inhibition tuned to the DSGC's null direction (Fig. 1B; Fried et al., 2002, 2005; Taylor and Vaney, 2002; Sivyer et al., 2010).

DSGCs also apparently receive excitation tuned to their preferred direction (Vaney et al., 2012). However, conclusive evidence for the underlying mechanism is lacking. Pharmacological experiments provide some evidence for tuned cholinergic input to DSGCs, but the SAC wiring for tuned cholinergic excitation would require connections opposite to those that explain tuned GABAergic inhibition (Fig. 1B; Fried et al., 2005; Lee et al., 2010). Furthermore, blocking cholinergic synapses does not eliminate DS in DSGCs (He and Masland, 1997; Kittila and Massey, 1997). Whole-cell voltage-clamp recordings suggested that bipolar cell glutamate release provides a second source of tuned excitation (Fried et al., 2002, 2005; Taylor and Vaney, 2002). However, this tuning could instead be explained by an artifact of voltage-clamp

Received Nov. 30, 2013; revised Feb. 6, 2014; accepted Feb. 11, 2014.

Author contributions: S.J.H.P., I.-J.K., J.B.D., and B.G.B. designed research; S.J.H.P. performed research; L.L. contributed unpublished reagents/analytic tools; S.J.H.P. and J.B.D. analyzed data; S.J.H.P., L.L., J.B.D., and B.G.B. wrote the paper.

This work was supported by the National Institutes of Health (Grants EY019355, EY023038, and EY014454 to I.-J.K., B.G.B., and J.B.D.), the Howard Hughes Medical Institute (to L.L.), and an unrestricted grant from Research to Prevent Blindness to Yale University.

B.G.B. owns Borghuis Instruments, which sells the specialized syringe that was used for intravitreal virus injections in this study. The remaining authors declare no competing financial interests.

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DOI:10.1523/JNEUROSCI.5017-13.2014

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Figure 1. Models for On-Off DSGC synaptic inputs. **A**, Basic circuit for the On-Off DSGC. Bipolar cells (BCs) and SACs of both the On and Off subtypes synapse onto DSGC dendrites. **B**, Existing model of DS synapses. Three types of synaptic input onto a rightward-prefering (→) DSGC dendrite show DS. A rightward projecting SAC dendrite prefers rightward motion and releases acetylcholine (ACh); this dendrite would release GABA onto a leftward-prefering DSGC, which is not shown. A leftward projecting SAC dendrite prefers leftward motion and releases GABA. This SAC also releases GABA onto a bipolar terminal, inhibiting its glutamate (glu) release during leftward motion and consequently generating a rightward preference for glutamate release. Each synaptic connection (large colored arrow) has a direction preference indicated by a small black arrow. **C**, Proposed model of DS synapses. A leftward projecting SAC dendrite prefers leftward motion and releases GABA. Both leftward and rightward projecting SAC dendrites release ACh onto the DSGC so that the net input lacks tuning. ACh input from the rightward projecting SAC dendrite could be explained if ACh, but not GABA, acted over a relatively longer distance (i.e., spillover) without direct synaptic contact (Briggman et al., 2011; Vaney et al., 2012). The glutamate synapse lacks DS; no SAC input to the bipolar terminal is required, and any such input would lack net tuning. NP, No direction preference.

recording (Poleg-Polsky and Diamond, 2011). Consistent with this interpretation, glutamate release onto the On-type of DSGC, measured with the glutamate biosensor intensity-based glutamate-sensing fluorescent reporter (iGluSnFR) (Marvin et al., 2013), lacked DS (Yonehara et al., 2013).

Here, we evaluated the DS of acetylcholine and glutamate release onto mouse On-Off DSGCs using pharmacology and iGluSnFR imaging of both On- and Off-layer dendrites. Our results do not support DS tuning of either acetylcholine or glutamate release onto On-Off DSGCs and instead suggest that the sole DS input to these cells is SAC GABA release.

Materials and Methods

Mouse retinas (C57/B6 mice, 2–6 months of age, either sex) were prepared as described previously (Borghuis et al., 2011, 2013). All procedures were conducted in accordance with NIH guidelines under protocols approved by the Yale University Animal Care and Use Committee. Retinas were perfused (~6 ml/min) with oxygenated (95% O₂–5% CO₂) Ames medium (Sigma-Aldrich) at 32–34°C and recorded *in vitro* using a custom-built two-photon fluorescence microscope controlled with ScanImage software (Pologruto et al., 2003).

On-Off DSGC spike and whole-cell recordings were obtained as described previously (Borghuis et al., 2011, 2013). Whole-cell pipettes

Figure 2. Tuning of mouse On-Off DSGC excitatory conductance persists with nicotinic receptors blocked. **A**, Spike response to motion in a DSGC's preferred and null direction. **B**, Polar plot showing spike rate, averaged over 5 s, to the grating moving in eight directions (same cell as in A). Red arrow indicates the vector mean angle of the response (preferred direction). **C**, Excitatory and inhibitory conductances for preferred- and null-direction motion in the control condition and in the presence of hexamethonium (100 μM). **D**, Synaptic conductance calculated for excitation (bottom) and inhibition (top) for responses, averaged over 5 s, to preferred (P) and null (N) directions. Mean values (± SEM) for P and N directions and the difference (P – N) shown in green (n = 16 cells). **E**, The P – N difference remained constant in the presence of hexamethonium (i.e., points lie near the identity line).

(6–10 MΩ) contained the following (in mM): 120 Cs-methanesulfonate, 5 TEA-Cl, 10 HEPES, 10 BAPTA, 3 NaCl, 2 QX-314-Cl, 4 ATP-Mg, 0.4 GTP-Na₂, and 10 phosphocreatine-Tris, pH 7.3, 280 mOsm, with red fluorophores (Alexa Fluor 594 biocytin or Alexa Fluor 568, 10 μM). Excitatory and inhibitory currents were recorded at holding potentials near E_{Cl} (–67 mV) and E_{cation} (+10 mV) after correcting for the liquid junction potential (–9 mV) and converted to conductances by dividing by the ±70 mV driving force. Series resistance (~20–40 MΩ) was compensated by 50–60%.

On-Off DSGCs were targeted based on soma size (~15 μm diameter) in retinas with unlabeled DSGCs, or by targeting cells expressing green fluorescent protein (GFP) in a transgenic line (thyrotropin-releasing hormone receptor [TRHR]-GFP; Rivlin-Etzion et al., 2011). Cell identity was confirmed by light response and dendritic morphology. The stimulus was a drifting grating (1 Hz temporal frequency; 0.85 or 1 mm period;

Figure 3. iGluSnFR signals on DSGC dendrites lack directional tuning. **A**, Two-photon images show selective, Cre-dependent expression of iGluSnFR in the CART-Cre retina. The image plane was at the On-layer dendrites of a DSGC filled with Alexa Fluor 594 (magenta). Box indicates the region in **B1**. **B1**, Fluorescence signals from a selected ROI (combination of subregions outlined with dashed lines) over trials. **B2**, Same as **B1** for Off-layer ROI. **C**, Polar plot of the spike response for cell in **A** and **B**. **D**, Top, Average iGluSnFR signals for the ROI in **B1** showed apparent DS. **E**, Same as **D** for the ROI in **B2**. **F**, Excitatory conductance was larger for preferred-direction motion compared with null-direction motion (i.e., points below the identity line). Recordings during imaging of On (magenta) and Off (black) layer dendrites are shown separately. Average response is shown in green. Error bars for individual points indicate $\pm$ SD across trials; error bars for the mean indicate $\pm$ SEM of the population (25 ROIs across $n = 14$ cells). **G**, Same as **F** for iGluSnFR signal amplitudes. Amplitudes were similar in the preferred and null directions.

100% contrast; ~ 31 or $36^\circ/\text{s}$) windowed in an aperture (0.43- or 0.55 mm), presented through the microscope condenser [mean: $\sim 0.5\text{--}1.0 \times 10^4$ photoisomerization (R^*) cone $^{-1}$ sec $^{-1}$; Borghuis et al., 2011; Wang et al., 2011]. DS tuning was measured from responses to motion in eight directions.

Approximately $1.0 \mu\text{l}$ of AAV2/1.syn.FLEX.iGluSnFR in PBS (FLEX, Cre-dependent; $0.8\text{--}2.0 \times 10^{13}$ IU/ μl) was injected into the vitreous humor of CART-Cre transgenic mice (cocaine- and amphetamine-regulated transcript; Jackson Laboratory; Kay et al., 2011), which drives expression in On-Off DSGCs as well as several other cell types (Kim et al., 2008; Kay et al., 2011). The retina was harvested 14–28 d later. iGluSnFR signals were recorded with two-photon microscopy as described previously (Borghuis et al., 2013).

A cell's preferred direction was the vector mean angle of the polar plot for spike responses (Fig. 1B) or, in some cases, the opposite of the vector mean angle of the polar plot for inhibition. We used one-tailed Student's t tests to test for tuning in the preferred direction (excitation and glutamate release) or null direction (inhibition). Unless specified, data are

reported as mean $\pm$ SEM. For imaging data, the number of cells, rather than the number of ROIs, was used as n (i.e., independent samples) for calculating SEM and statistical p -values.

Results

Cholinergic input to On-Off DSGCs lacks directional tuning

On-Off DSGC spike responses to a drifting grating showed clear tuning (Fig. 2A), with the preferred direction defined as the vector mean angle of the polar plot (Fig. 2B). In whole-cell recordings, inhibition was larger in the cell's null direction (Preferred – Null [$P - N$]: -2.43 ± 0.31 nS; $t = -7.97$; $p < 0.00001$), whereas excitation was larger in the preferred direction (0.31 ± 0.05 nS; $t = 6.33$; $p < 0.000001$; Fig. 2C; Weng et al., 2005; Rivlin-Etzion et al., 2011). After blocking nicotinic acetylcholine receptors with the antagonist hexamethonium ($100 \mu\text{M}$), inhibition remained direction tuned (-2.22 ± 0.28 nS; $t = -7.93$; $p < 0.000001$), as did excitation (0.26 ± 0.04

Figure 4. Apparent tuning in excitation can be explained by inhibition of the DSGC. **A**, iGluSnFR signals, averaged over four trials, in On and Off ROIs in a control condition and after adding hexamethonium (100 μ M) and gabazine (50 μ M). **B**, Excitatory conductance measured simultaneously with the iGluSnFR signals in **A**. **C**, iGluSnFR signal (peak-to-peak amplitude of F1:4 fit) for On (magenta) and Off (black) ROIs in response to preferred (*P*) and null (*N*) directions in each condition. Mean values ($\pm$ SEM) for *P* and *N* directions and the difference (*P* – *N*), shown in green (7–9 ROIs in each condition, across *n* = 4 cells). The iGluSnFR response lacked directional tuning in each condition. **D**, Same as **C** for excitatory conductance (response averaged over 5 s) measured simultaneously with iGluSnFR signals in **C**. The excitatory conductance showed apparent directional tuning in the control condition and in the presence of hexamethonium, but lacked tuning in the presence of gabazine.

nS; $t = 6.34$; $p < 0.00001$). Hexamethonium did not significantly reduce the *P* – *N* difference for inhibition (-0.21 ± 0.23 nS; $t = -0.95$; $p = 0.82$) or excitation (0.051 ± 0.0385 nS; $t = 1.32$; $p = 0.103$; Fig. 2E). Therefore, DS tuning of excitatory conductance appeared to depend on bipolar cell glutamate release.

Glutamate release onto On-Off DSGC dendrites lacks DS tuning

We measured DS tuning of iGluSnFR (Borghuis et al., 2013; Marvin et al., 2013) conditionally expressed on the DSGC dendrites using the CART-Cre mouse (see Materials and Methods). Identified DSGCs showed typical DS tuning (Fig. 3C) based on null and preferred responses ($\text{Response}_{\text{Null}}$, $\text{Response}_{\text{Pref}}$), and their DS index ($[\text{Response}_{\text{Pref}} - \text{Response}_{\text{Null}}] / [\text{Response}_{\text{Pref}} + \text{Response}_{\text{Null}}]$) of 0.65 ± 0.05 ($n = 14$) was similar to cells from TRHR and wild-type retinas (0.73 ± 0.03 ; $n = 38$). A red dye in the pipette helped define ROIs on the recorded DSGC's dendrites (Fig. 3B). Conditional iGluSnFR expression in the CART-Cre line was relatively sparse. An ROI could be drawn that included the recorded

cell's dendritic segments (approximate length 3–20 μ m) without other cells' processes for several micrometers above and below the image plane (Fig. 3A,B); this minimized voxel averaging (Borghuis et al., 2013).

iGluSnFR responses modulated primarily at the stimulus frequency and were quantified as the peak-to-peak amplitude of a fit based on the sum of the first four Fourier harmonics of the stimulus frequency (F1:4 fit; Fig. 3D,E). Glutamate release was less rectified in the On layer (i.e., there was tonic release that could be suppressed) compared with the Off layer (Fig. 3D,E; Borghuis et al., 2013). In both the On and Off layers, iGluSnFR response amplitude (F1:4 fit) was nearly identical for preferred and null directions ($P - N$, -0.073 ± 0.04 ; $t = -1.76$; $p = 0.95$) even though the simultaneously recorded excitatory conductance was larger for the preferred direction ($P - N$, 0.35 ± 0.07 nS, $t = 4.87$; $p = 0.00015$; Fig. 3F,G). Therefore, fluorescence measurements provided no evidence for directional tuning of glutamate release onto On-Off DSGC dendrites.

Previously, excitatory current and iGluSnFR signals were correlated in alpha ganglion cells (Borghuis et al., 2013), whereas in the On-Off DSGCs recorded here, these measures lacked correspondence. To explain this, we first applied hexamethonium (100 μ M) to isolate the glutamatergic component of excitation. Under control conditions, the excitatory conductance showed DS tuning ($P - N$, 0.50 ± 0.072 nS; $t = 6.90$; $p = 0.003$), but iGluSnFR signals (i.e., F1:4 fit) did not ($P - N$, -0.11 ± 0.14 ; $t = -0.80$; $p = 0.76$; Fig. 4, left). Hexamethonium reduced the excitatory conductance but the apparent DS tuning persisted ($P - N$, 0.42 ± 0.064 nS; $t = 6.44$; $p = 0.004$). Moreover, hexamethonium did not suppress the simultaneously recorded iGluSnFR signals (Fig. 4, middle) and these signals remained untuned ($P - N$, -0.14 ± 0.10 ; $t = -1.40$; $p = 0.87$).

When we subsequently blocked GABA-A inhibition with SR95531 (gabazine; 50 μ M), both excitatory conductance (Fried et al., 2005) and fluorescence responses lacked DS tuning (Fig. 4, right). $P - N$ was -0.0065 ± 0.039 nS ($t = -0.166$; $p = 0.56$) for excitatory conductances and -0.07 ± 0.05 ($t = -1.31$; $p = 0.86$) for fluorescence responses. Therefore, direction tuning originally observed in excitatory conductance apparently does not reflect DS glutamate release, but rather reflects an inability to accurately measure excitatory conductance in the presence of strong inhibition during null-direction motion.

Discussion

We evaluated the synaptic mechanism for DS in mouse On-Off DSGCs. Apparent DS of excitatory conductance remained constant in the presence of a nicotinic antagonist, suggesting that glutamate release, but not Ach release, was DS (Fig. 2). In some cases, glutamate conductance was apparently suppressed below a baseline level during null-direction motion (Fig. 4D). However, iGluSnFR signals on DSGC dendrites lacked DS (Figs. 3, 4) and blocking GABAergic inhibition caused the excitatory conductance to lose its selectivity (Fried et al., 2005), which is consistent with the iGluSnFR signals measured simultaneously (Fig. 4). These results do not support DS tuning of excitatory input to On-Off DSGCs and suggest that any apparent tuning of excitation is caused by inaccurate control of the DSGC dendrites, particularly in the presence of strong inhibition during null-direction motion (Poleg-Polsky and Diamond, 2011). Although the excitatory conductance could similarly impair measurements of inhibition, excitation was several times smaller than inhibition (Fig. 2), so its effect would be minor. Inhibition is therefore genuinely tuned.

Our data support a model in which the sole source of directionally tuned synaptic input to On-Off DSGCs is GABA release by SACs (Fig. 1C; Yonehara et al., 2013). This inhibition would counteract the depolarizing response to glutamatergic inputs from bipolar cells during the DSGC's null direction (Taylor et al., 2000).

Evidence for apparent tuning of excitation in DSGCs

The previously proposed mechanism for DS glutamate release depended on local SAC input to subregions of a bipolar terminal (Fig. 1B; Vaney et al., 2012), but anatomical evidence for these connections has been equivocal (Famiglietti, 1991; Helmstaedter et al., 2013). Pharmacological evidence did not support a role for feedback inhibition onto bipolar terminals (Massey et al., 1997). On-type DSGCs lacked DS tuning in iGluSnFR signals despite apparent tuning in the excitatory currents measured under voltage-clamp conditions (Yonehara et al., 2013). Further, the Ca^{2+} response of the On DSGC's presynaptic type 5a or 5b On bipolar cells lacked tuning. Accordingly, our results for the On-Off DSGC suggest that all bipolar inputs to On-Off DSGCs lack DS tuning, including Off bipolar types that release onto Off-layer dendrites (Figs. 3, 4).

Our recordings suggest an absence of net DS tuning in the cholinergic inputs to On-Off DSGCs (Fig. 2). Paired recordings of On SACs and On-Off DSGCs in rabbit suggested a lack of selective wiring for cholinergic synapses: SACs on a DSGC's preferred and null side showed similar functional connectivity (Lee et al., 2010). Extracellular recordings of light responses in rabbit did not support a role for acetylcholine release in the DS response (He and Masland, 1997; Kittila and Massey, 1997), whereas whole-cell recordings showed an apparent DS component of the acetylcholine input, with excitation strongest in the DSGC's preferred direction (Fried et al., 2005; Lee et al., 2010). We now question the accuracy of the whole-cell recordings, given that similar apparent tuning of glutamate conductance was not confirmed by iGluSnFR imaging (Figs. 3, 4; Yonehara et al., 2013) and could be explained by the same artifact of voltage-clamp measurements (Poleg-Polsky and Diamond, 2011). Additional analysis of the DS tuning of acetylcholine release would be greatly facilitated by optical sensors for acetylcholine.

Technical considerations with iGluSnFR measurements

Two-photon imaging has a resolution in the z-axis of $\sim 2.5 \mu\text{m}$ (full-width at half maximum; Borghuis et al., 2013). Accordingly, each voxel averages the focused dendrite with dendrites above and below the focal plane. To address this concern, we conditionally expressed iGluSnFR in the CART-Cre retina, where expression is much sparser than when labeling broadly with non-Cre-dependent iGluSnFR (Borghuis et al., 2013). We took care to measure only ROIs that lacked fluorescence above and below the focal plane to minimize voxel averaging.

A possible limitation of iGluSnFR imaging relates to glutamate spillover from bipolar terminals that are not presynaptic to the DSGC. Measurements from glial cell membranes showed that iGluSnFR detects spillover (Borghuis et al., 2013). However, release from adjacent On and Off bipolar cell terminals can be distinguished in the central inner plexiform layer, demonstrating that spillover is limited to $\sim 2 \mu\text{m}$ (Borghuis et al., 2013). Even if iGluSnFR signals on a DSGC's dendrite were mediated by synaptic release from DS-tuned bipolar terminals combined with extrasynaptic release from untuned terminals, the combination should have been biased in the preferred direction of the recorded cell and this was not the case (Fig. 3).

Model of synaptic mechanism for direction selectivity

We propose a parsimonious model for the synaptic basis of DS in On-Off DSGCs (Fig. 1C). For each DSGC, excitation comprises glutamate and acetylcholine release from bipolar terminals and SAC dendrites, respectively. Both inputs report changes in local contrast. The glutamate release lacks directional tuning (Figs. 3, 4; Yonehara et al., 2013). The acetylcholine release is directionally tuned locally, because the starburst dendrite's Ca^{2+} signal shows DS and acetylcholine release is Ca^{2+} dependent (Euler et al., 2002; Lee et al., 2010; Yonehara et al., 2013). However, acetylcholine release integrated across the DSGC arbor is not biased to dendrites pointing in any particular direction (demonstrated for On SACs and assumed to also be true for Off SACs; Lee et al., 2010), resulting in a net signal that lacks DS. Finally, a DSGC receives inhibition from SAC dendrites that release GABA most strongly in the DSGC's null direction. The mechanisms for a SAC's direction-tuned GABA release and selective wiring with DSGCs have been established previously (Euler et al., 2002; Fried et al., 2002; Briggman et al., 2011; Vaney et al., 2012). Finally, this synaptic mechanism combines with additional network and cell-intrinsic mechanisms in the On-Off DSGC to further enhance the selectivity in the firing response (Oesch et al., 2005; Schachter et al., 2010; Trenholm et al., 2011; Sivyer and Williams, 2013).

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