Simultaneous Contribution of Two Rod Pathways to AII Amacrine and Cone Bipolar Cell Light Responses

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Trexler, E. Brady, Wei Li, and Stephen C. Massey. Simultaneous contribution of two rod pathways to AII amacrine and cone bipolar cell light responses. J Neurophysiol 93: 1476–1485, 2005. First published November 3, 2004; doi:10.1152/jn.00597.2004. Rod signals traverse several synapses en route to cone bipolar cells. In one pathway, rods communicate directly with cones via gap junctions. In a second pathway, signals flow rods–rods–rods–AII amacrine–cone bipolars. The relative contribution of each pathway to retinal signaling is not well understood. Here we have examined this question from the perspective of the AII amacrine. AII amacrine cells form bidirectional electrical synapses with ON cone bipolar cells. Consequently, as ON cone bipolar cells are activated by outer plexiform inputs, they too should contribute to the AII response. Rod bipolar inputs to AII amacrine cells were blocked by AMPA receptor antagonists, revealing a smaller, non-AMPA component of the light response. This small residual response did not reverse between -70 and +70 mV and was blocked by carbenoxolone, suggesting that the current arose in ON cone bipolar cells and was transmitted to AII amacrine cells via gap junctions. The residual component was sensitive to stimuli below cone threshold and was prolonged for bright stimuli, demonstrating that it was rod driven. Because the rod bipolar–AII pathway was blocked, the rod-driven residual current likely was generated via the rod–cone pathway activation of ON cone bipolar cells. Thus for a large range of intensities, rod signals reach the inner retina by both rod bipolar–AII and rod–cone coupling pathways.

INTRODUCTION

In the mammalian retina, bipolar cells are divided into rod and cone classes based on the photoreceptor type that provides their major presynaptic input. Only cone bipolar cells synapse with ganglion cells, the output cells of the retina. Rod bipolars do not synapse with ganglion cells (Strettoi et al. 1990). Therefore rod signals must first enter cone bipolar cells, and there are three well-defined anatomical pathways by which this occurs (for review, see Bloomfield and Dacheux 2001; Sharpe and Stockman 1999). The first utilizes synapses in the order of rod–rod bipolar–AII amacrine cells (Dacheux and Raviola 1986; Famiglietti and Kolb 1975; Kolb and Famiglietti 1974; Sterling et al. 1988; Strettoi et al. 1992). AII amacrine cells then relay the rod bipolar signals to ON cone bipolar cells via gap junctions (Veruki and Hartveit 2002b) and to OFF cone bipolar cells via inhibitory glycinergic synapses (Muller et al. 1988; Strettoi et al. 1992, 1994). In the second pathway, signals flow from rods directly to cones via gap junctions between the two photoreceptor types (DeVries and Baylor 1995; Nelson 1977; Raviola and Gilula 1973; Schneeweis and Schnapf 1995, 1999; Smith et al. 1986). The third pathway involves direct glutamate release from rods onto OFF cone bipolar cells (Hack et al. 1999; Li et al. 2004; Soucy et al. 1998; Tsukamoto et al. 2001).

In the first two pathways, gap junctions are a necessary conduit for rod signals. Cx36 is a neuronal connexin that has been localized in the retina to AII amacrine cells (Feigenspan et al. 2001; Mills et al. 2001) and to photoreceptors (Feigenspan et al. 2004). Recently, an elegant study from Deans et al. (2002) demonstrated by recording from ON center ganglion cells in Cx36 knockout mice that both the pathways described were abolished. That is, coupling between rods and cones and between AII amacrine cells and ON cone bipolar cells is necessary for transmission of rod signals to ON ganglion cells. These data confirm that there are multiple, perhaps redundant, pathways for rod signals, yet how these signals are routed depending on light intensity is not known. To address this question, we have examined the AII amacrine light response and the relative contributions of rod bipolar and ON cone bipolar cells that synapse with AII amacrine cells. Evidence from both physiological and tracer coupling indicates that the AII/ON cone bipolar gap junctions are open and allow for bidirectional communication over a large range of light intensities (Trexler et al. 2001; Veruki and Hartveit 2002b; Xin and Bloomfield 1999). In this manuscript, we take advantage of the fact that in the absence of the rod bipolar inputs to AII amacrine cells, the only other rod input to ON cone bipolar cells is via rod–cone coupling. Thus rod bipolar and ON cone bipolar inputs to AII amacrine cells represent the operation of two different rod pathways. We demonstrate that both pathways operate simultaneously over greater than a 3 log unit range, both substantially contributing to AII amacrine cell bipolar light responses.

METHODS

Preparation of retinas

Described in detail previously (Massey and Mills 1999), the isolation of the rabbit retina is briefly summarized as follows. Under a protocol approved by the Institutional Animal Welfare Committee, adult New Zealand Albino white rabbits (1.5–3 kg) were deeply anesthetized with urethane (loading dose, 1.5 g/kg ip), and the orbit was infused with 2% lidocaine hydrochloride before enucleation. The eye was then removed and hemisected. The inferior portion of the eyecup was cut into strips and attached to filter paper, vitreal side down. The scleral and choroidal tissues were removed and the retinas were bathed in a modified Ames medium (see following text). Retinas were then stored at 10°C for later recording. Slices were cut on a vertical slicer to varying thickness (120–200 μm).

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and transferred to the recording chamber. Experiments measuring light responses were performed on retinas from rabbits that were dark adapted for 1–2 h prior to enucleation; surgery, isolation of the retina, and preparation of slices were done under dim red light.

**Electrophysiology**

A modified Ames solution was used for storing retinas, bath perfusion of slices, and puffer application of drugs. The core salts, which are shared by the three solutions mentioned, consisted of (in mM) 115 NaCl, 3.1 KCl, 1.24 MgCl₂, 2 CaCl₂, 6 glucose, 2 succinate, 1 malate, and 1 lactate. For the storage solution, 10 mM HEPES, 12 mM NaHCO₃, and 1 mM pyruvate were added and pH was adjusted to 7.4 with NaOH. For bath perfusion, 24 mM NaHCO₃ and 1 mM pyruvate were added and the solution was bubbled with 95% O₂-5% CO₂. The perfusate was heated to 37°C with an inline heater (Warner Instruments, Hamden, CT).

Patch pipette filling solution consisted of (in mM) 110 potassium gluconate, 10 CsCl, 10 NaCl, 10 HEPES, 10 EGTA, 2 MgCl₂, 5 K’ATP, and 0.5 Na’GTP. The pH was adjusted to 7.2 with KOH. Lucifer yellow (0.5 mg/ml) or 0.1 mM Alexa-568 (Molecular Probes, Eugene, OR) were included in the patch solutions to confirm cell identity by epifluorescent visualization after recording. All chemicals and pharmacological agents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Drugs were dissolved in core salts or triple-barrel puffer pipettes. A single barrel would contain two or more drugs for testing the effects of simultaneous application rather than perfusion from multiple barrels.

Patch electrodes were probed on a Flaming-Brown horizontal puller from Sutter Instruments (Novato, CA) and fire polished on a Narishige MF-83 microforge (East Meadow, NY) to a resistance of 7–9 MΩ (in KCl pipette solutions, pipette resistances were 5–7 MΩ due to the higher mobility of chloride relative to gluconate). Seals in the cell attached configuration ranged from 8 to 20 GΩ. On obtaining a whole cell patch, series resistance was estimated from the peak of the capacitive transients due to a square wave voltage pulse and ranged from 7.4 with KOH. The perfusate was heated to 37°C with an inline heater (Warner Instruments, Hamden, CT).

**Results**

Rod-dominated light responses of all amacrines and bipolar cells

Rods differ from cones in their activation threshold and response kinetics, features commonly used to distinguish between rod and cone inputs to a given neuron. Individual rods respond to single photon absorptions, whereas the threshold for cone responses is much higher. We made estimates of the threshold for cone responses in our preparation based on studies performed in macaque. Cones in macaque respond to photon fluxes corresponding to photoisomerization rates as low as 50 s⁻¹ (Schnapf et al. 1990; Schneeweis and Schnapf 1999). Taking the cone collecting area as 0.6 μm² (Schneeweis and Schnapf 1999) and the near maximal absorption of 525 nm photons by green cones in rabbit (Nuboer 1971), a photon flux of >80 photons μm⁻² s⁻¹ would be necessary to excite cones in our rabbit retina slices. This corresponds to a rod photoisomerization rate of ~30 R⁺ · rod⁻¹ · s⁻¹, in agreement with the cone threshold intensity determined for ON-center ganglion cells in mouse retina (Deans et al. 2002). In addition, a signature of rod photoreceptors is a long-lasting afterpotential following exposure to bright stimuli (Euler and Masland 2000; Nelson 1977; Svaetichin 1956).

Examples of rod driven light responses from three different cell types are displayed in Fig. 1. In Fig. 1A, a family of averaged light responses is shown for an All amacrine cell. The flash duration was 100 ms, and the hallmarks of rod input are evident. The All responds to flashes as dim as 0.4 R⁺, rod⁻¹ · s⁻¹, much lower than the calculated cone threshold of 30 R⁺ · rod⁻¹ · s⁻¹. Rise times quicken with increasing flash intensity, and the All reaches its maximum response amplitude with a flash intensity of 10 R⁺ · rod⁻¹ · s⁻¹. Above this value, the peak of the response actually decreases slightly, but its duration increases. Later portions of the depolarizing response are of lower amplitude than the peak, and for the two brightest flashes, there is a secondary depolarization that appears to rebound from the decay of the first. A hyperpolarizing OFF response follows the depolarizing portion of the waveform and increases in magnitude and duration with increasing intensity.

Next, we examined light responses of the two bipolar cells that provide inputs to All amacrines. A family of ON cone bipolar flash responses is depicted in Fig. 1B. For this cell, 50-ms flashes were used, and each trace represents the average of 8–10 responses. The lowest flash response shown is for 1 R⁺ · rod⁻¹ · s⁻¹, which is lower than cone threshold. Although the flash duration was halved relative to the All in Fig. 1A, the maximal response amplitude is reached with a
The response amplitude was reached with 10 R°. Increasing the intensity prolongs the response but does not increase its amplitude. As with the AII, the depolarizing response is followed by a hyperpolarizing rebound the amplitude of which increases with flash strength.

In Fig. 1C, responses to 100-ms flashes were recorded for a rod bipolar. The nine traces are averages of nine or more flashes. Responses were obtained for flashes as dim as 0.4 R°. Although its origin is unknown, there is a small “shoulder” that occurs with intermediate intensities in rodents (Euler and Masland 2000; Field and Rieke 2002) that also appears in rabbit rod bipolar responses. The maximum response amplitude was reached with 10 R°. With intensities greater than those that produce the shoulder (≥100 R°), the rod bipolar responses reached a plateau, and the duration of this plateau increased with further increases in flash intensity.

In Fig. 1D, the responses of the rod bipolar in Fig. 1C, the AII in Fig. 1A, and an ON cone bipolar, different from that in Fig. 1B, are aligned. The flash durations are all 100 ms at 200 R° intensity to for comparison of response kinetics. All three cell types exhibit a light response that lasts much longer than the stimulus. Furthermore, the AII and ON cone bipolar responses are much faster, reaching their peak within 40 ms. For all cell types, the response is followed by an OFF response of the opposite polarity. To the right of each trace are micrographs of representative cells identified by their morphology under epifluorescent illumination.

FIG. 1. AII amacrines, rod bipolars and ON cone bipolar cells responses to increasing flash intensities. Families of light responses (averages of ≥9 flashes each) were elicited by flashes of 525 nm light. For each cell in A–C, the flash intensities (given in R° ⋅ rod−1 ⋅ s−1) are displayed to the left of each trace. A: a family of AII amacrine responses to 100-ms flashes demonstrates that the AII depolarizing response is prolonged with higher intensities. B: a family of light responses was elicited from an ON cone bipolar cell. Flash duration was 50 ms. C: a family of rod bipolar cell responses to 100-ms flashes. Note the decrease in the delay and the speeding of the rise time of the initial depolarizing response with increasing intensity. With increasing intensity, a smaller “shoulder” (*) appears at 10, 20, and 40 R° ⋅ rod−1 ⋅ s−1. Also with increasing intensity, a large afterhyperpolarization develops. As discussed in RESULTS, the threshold for cone responses is 30 R° ⋅ rod−1 ⋅ s−1. Therefore the recordings shown here represent rod dominated responses at lower intensities. Although cones may contribute at the higher intensities, the prolonged, saturated responses are a hallmark of rod afterpotentials, indicating substantial rod input even above cone threshold. D: responses from 3 different cell types to 100-ms flashes of 200 R° ⋅ rod−1 ⋅ s−1 intensity are aligned to illustrate differences in kinetics and waveform. The time of the flash is denoted by the long rectangle that overlaps the voltage traces for comparison of response kinetics. From the top are a rod bipolar (from C), an AII (from A), and an ON cone bipolar (different cell than B). The rod bipolar response does not reach its peak amplitude until after the 100-ms stimulus is extinguished. The AII and ON cone bipolar responses are much faster, reaching their peak within 40 ms. For all cell types, the response is followed by an OFF response of the opposite polarity. To the right of each trace are micrographs of representative cells identified by their morphology under epifluorescent illumination.
All light responses consist of two distinct components

The AII functions as a relay for rod bipolar signals, communicating with ON cone bipolar cells via gap junctions (Strettoi et al. 1992; Veruki and Hartveit 2002b). However, there are two possible pathways for rod signals to reach ON cone bipolar cells. In addition to input from AII s, ON cone bipolars can receive rod signals directly from cones because of rod-cone gap junctional coupling (Raviola and Gilula 1973; Smith et al. 1986; Zhang and Wu 2004). One would expect that as ON bipolar cells are activated via synaptic pathways in the outer plexiform layer, an ON cone bipolar contribution to the AII light response would arise. Thus two components of the AII light response should be evident: rod bipolar input via glutamate receptors and ON cone bipolar input via gap junctions. Record-}

ging light responses from AII s in the presence of AMPA antagonists would eliminate rod bipolar input (Cohen and Miller 1999; Ghosh et al. 2001; Li et al. 2002; Morkve et al. 2002; Qin and Pourcho 1999; Singer and Diamond 2003), effectively isolating the ON cone bipolar component, if it exists.

We recorded the current responses of AII amacrine cells to full field monochromatic (525 nm) stimuli in the absence and presence of AMPA antagonists. Figure 2 depicts responses to flash intensities of 0.5, 1, 100, and 1,000 R* · rod⁻¹ · s⁻¹. AII s were held at −50 mV, the reversal potential for Cl⁻ conductances, to isolate excitatory input. For these intensities, application of 20 μM 1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI) was the antagonist, whereas C was recorded from a different AII using 20 μM 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoline-7-sulfonamide (NBQX). Flash intensities are displayed above each record. The AII current response to light flashes indicates that not all synaptic input is blocked with NBQX or GYKI. All spontaneous excitatory postsynaptic currents (EPSCs) are abolished in AMPA antagonists, but a distinct light response remains. This residual component likely reflects direct input from ON cone bipolars to AII amacrinies via gap junctions. The peak of the control traces, representing rod bipolar input to AII s, grows larger with increasing intensity. The amplitude of the residual light response in the presence of AMPA antagonists was variable. The inset in (B) shows the AMPA and gap junction components of the light response normalized to their peak. The rising phase of both components overlaps. D: shown is an example of AII amacrine responses to a photopic stimulus (black bar, 100 ms, 1,000 R* · rod⁻¹ · s⁻¹). The black trace is an average of 10 responses in control perfusate, and the gray trace is an average of 8 responses recorded in 20 μM NBQX. Note that the light response in both conditions lasts for almost 1 s, indicative of long-lasting rod afterpotentials.

**FIG. 2.** A component of the AII light response is resistant to AMPA antagonists. The black rectangles mark the durations of the flashes. Black traces are controls and gray traces were recorded in the presence of antagonist. Averages of 2 to 9 flashes are displayed. A and B are from the same cell and 20 μM 1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI) was the antagonist, whereas C was recorded from a different AII using 20 μM 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoline-7-sulfonamide (NBQX). Flash intensities are displayed above each record. The AII current response to light flashes indicates that not all synaptic input is blocked with NBQX or GYKI. All spontaneous excitatory postsynaptic currents (EPSCs) are abolished in AMPA antagonists, but a distinct light response remains. This residual component likely reflects direct input from ON cone bipolars to AII amacrinies via gap junctions. The peak of the control traces, representing rod bipolar input to AII s, grows larger with increasing intensity. The amplitude of the residual light response in the presence of AMPA antagonists was variable. The inset in (B) shows the AMPA and gap junction components of the light response normalized to their peak. The rising phase of both components overlaps.

A: shown is an example of AII amacrine responses to a photopic stimulus (black bar, 100 ms, 1,000 R* · rod⁻¹ · s⁻¹). The black trace is an average of 10 responses in control perfusate, and the gray trace is an average of 8 responses recorded in 20 μM NBQX. Note that the light response in both conditions lasts for almost 1 s, indicative of long-lasting rod afterpotentials.
The space clamp through gap junctions would be too poor, thus the current should not reverse. Accordingly, we found that the current did not reverse between $-70$ and $+70$ mV (Fig. 3A). If the residual current was due to incomplete block of glutamate receptors, we would expect it to reverse at $0$ mV.

Further evidence that the residual component comes through gap junctions with ON cone bipolars comes from the ability to interpret carefully due to its effects on photoreceptor quantal sensitivity (Verwei et al. 2003) and Ca$^{2+}$ channels in photoreceptors (Vessey et al. 2004) as well as its lack of reversibility. However, the carbenoxolone blockade taken together with the lack of reversal of the residual current suggests that the residual current arises due to coupling with ON cone bipolars and that the AII light response is the sum of inputs from rod bipolars and ON cone bipolars. Because AII are coupled to each other and some or all of the neighboring ON cone bipolars, the NBQX-insensitive component represents the average response of the AII/ON cone bipolar network in the absence of rod bipolar input. In the absence of rod bipolar input to AII, the remaining rod input to ON cone bipolars most likely comes from rod–cone coupling. Thus the residual current allows for an estimation of the magnitude of the contribution of rod–cone coupling to inner retina responses (see DISCUSSION).

The experiments described in the preceding text support the conclusion that the light response of AII is generated by two different rod pathways via two different bipolar cells. In the following sections, we examine each bipolar cell’s input to the AII in greater detail.

Synaptic transmission between rod bipolars and AII

Rod bipolar cells and AII amacrine cells in slices of rabbit retina were identified by morphology, and synaptically connected pairs were found with a high level of success. AII amacrine cells were identified by a characteristically small soma and stout primary dendrite that descends into the inner plexiform layer (IPL) (Kolb and Nelson 1983; Massey and Mills 1999; Strettoi et al. 1992) (Fig. 4A). In some cases, lobular appendages in sublamina 2 and 3 of the IPL, as well as fine dendritic processes in sublaminas 4 and 5, could be visualized. Rod bipolar cells had large somas (10–12 μm) near the top of the inner nuclear layer (INL) with axons that branched into three or four bulbous terminals in sublamina 5 of the IPL. Rod bipolar cells could be distinguished from other bipolar cells by their larger soma and the depth and size of the axon terminals. Patch pipettes included fluorescent dyes, and after recording, cells were imaged using epifluorescence to confirm their identity (Fig. 4B).

Pairs of rod bipolar and AII amacrine cells were tested for synaptic communication by stepping the rod bipolar from a holding potential of $-70$ to $-30$ mV. EPSCs were isolated from inhibitory currents by recording from AII at a holding potential of $-50$ mV, the reversal potential for chloride conductances (Fig. 4C). Slices were perfused with $1 \mu$M TTX to block Na$^+$ spikes in the coupled AII network (Boos et al. 1993; Veruki and Hartveit 2002a; Veruki et al. 2003), isolating the glutamatergic EPSCs. Analysis of averaged EPSCs revealed an inward current with a large transient and a much smaller sustained component (Fig. 1C) (see also Singer and Diamond 2003). We determined pertinent kinetic parameters of the AII postsynaptic response from averages of $9$ to $>20$ repeated rod bipolar stimulations in 10 separate pairs. The average EPSCs followed the onset of rod bipolar depolarization after a delay of $1.65 \pm 0.33$ (SE) ms ($n = 10$ pairs) and rose to a peak within $1.28 \pm 0.43$ ms (10–90%). They decayed with an exponential time course ($\tau = 2.06 \pm 0.34$ ms) to $4 \pm 2.2\%$ of the peak.
current. Peak synaptic currents ranged from 42 to 1.1 nA at a holding potential of \(-50 \text{ mV}\) (373 \(\pm\) 340 pA, \(n = 18\) pairs).

The transient component of the averaged EPSC decay slowed to \(\tau = 9 \text{ ms}\) in cyclothiazide from \(\tau = 2 \text{ ms}\) in control conditions (Fig. 4D) (Singer and Diamond 2003), and the sustained component was slightly increased in cyclothiazide, likely due to the slowing of the decay of individual exocytotic events (Fig. 4D, inset) (Veruki et al. 2003). The amplitude of the sustained relative to the peak amplitude in control (4\%) or cyclothiazide (7\%) conditions suggests that the transient component of the EPSC is not due solely to receptor desensitization but a decrease in vesicle release rate.

We compared the effects of the specific antagonists NBQX and GYKI-53655. NBQX blocks AMPA and kainate receptor subtypes, whereas GYKI is specific for AMPA receptors alone (Łukasiewicz et al. 1997). Puffer application of 20 \(\mu\text{M}\) NBQX reversibly abolished the evoked EPSCs as well as all spontaneous EPSCs in 11 of 11 pairs (not shown). In separate experiments (\(n = 5\)), 10–20 \(\mu\text{M}\) GYKI was applied (Fig. 4E).

We saw complete blockade of evoked and spontaneous EPSCs with GYKI. These data taken together with the effects of cyclothiazide indicate that AMPA receptors are the sole current carriers of rod bipolar to AII synaptic transmission at \(-50 \text{ mV}\).

**Electrical synapses between AIIIs and ON cone bipolars**

Next we examined synaptic transmission between AIIIs and ON cone bipolar cells. Anatomical studies have shown that AIIIs and ON cone bipolars are connected by gap junctions (Strettoi et al. 1994), and tracer injection studies in whole-mount retina have shown that the two heterologous cell types form an extensively coupled network (Hampson et al. 1992; Mills and Massey 1995, 1998). ON cone bipolars could be identified and distinguished from rod bipolars due to their IPL axon projection depth, which was not as deep as that of rod bipolar axons (Ghosh et al. 2004; MacNeil et al. 2004), and terminal branching pattern, which involved a larger number of processes with smaller endings. In Fig. 5A, a fluorescence image of an AII–ON cone bipolar pair is overlaid on a dim DIC image of a slice. The bipolar cell ramified in the middle of sublamina b of the IPL, and its axon terminals spread out near the AII dendrites. Gap junction plaques labeled with an antibody specific for Cx36 have been found at this level (Feigenspan et al. 2001; Mills et al. 2001), suggesting that pairs with proper overlap should exhibit electrical coupling.

The presence of electrical coupling was tested as shown in Fig. 5B. Both cells were held at \(-50 \text{ mV}\), and either the ON cone bipolar or the AII was stepped to a different holding potential to create a transjunctional voltage. Rectangular displacements of the holding current in the nonstepped cell correspond to the changes in holding potential of the other cell. The nature of the synaptic currents, including the opposite...
direction of the current in the stepped and nonstepped cells in each pair as well as the lack of any transmission delay (compared with 1.65-ms delay at the rod bipolar–to–AII synapse) indicate that they are the result of gap junction coupling, not chemical synaptic transmission (Bennett 1977). Furthermore, the synaptic currents persisted in the presence of antagonists of chemical synaptic transmission (Bennett 1977), indicative of direct electrical communication via gap junctions. A view of the transjunctional voltage in an expanded time scale is shown in Fig. 5B: the ON cone bipolar was pulsed, generating rectangular displacements of the AII holding current. There were spontaneous EPSCs throughout the recording. B2: application of 20 μM NBQX, 20 μM picrotoxin, and 2 μM strychnine were used to block fast chemical synaptic transmission, yet pulses to the ON cone bipolar resulted in rectangular displacements of the AII holding current, indicative of direct electrical communication via gap junctions. C: a view of the transjunctional current (Ij) at the onset of Vj (marked by the vertical line) is shown in an expanded time scale. The transjunctional current recorded in the AII rises rapidly with no delay, indicative of an electrical synapse. D: the current voltage relation of another pair is shown. In this example, the AII was pulsed, and transjunctional currents (Ij) were recorded in the ON cone bipolar. The slope conductance of 569 pS was determined from the linear regression shown by the solid black line (R = 0.99753).

FIG. 5. Paired recordings show synaptic communication between AII and ON cone bipolar cells is via gap junctions. A: fluorescent images of an ON cone bipolar (green) and an AII (red) are overlaid on a DIC image of the slice. The bipolar cell ramiﬁes in sublamina b of the IPL near the AII dendrites. B: both cells were held at −50 mV, and voltages were applied to the ON cone bipolar to generate a transjunctional voltage (Vj). B1: the ON cone bipolar was pulsed, generating rectangular displacements of the AII holding current. There were spontaneous EPSCs throughout the recording. B2: application of 20 μM NBQX, 20 μM picrotoxin, and 2 μM strychnine were used to block fast chemical synaptic transmission, yet pulses to the ON cone bipolar resulted in rectangular displacements of the AII holding current, indicative of direct electrical communication via gap junctions. C: a view of the transjunctional current (Ij) at the onset of Vj (marked by the vertical line) is shown in an expanded time scale. The transjunctional current recorded in the AII rises rapidly with no delay, indicative of an electrical synapse. D: the current voltage relation of another pair is shown. In this example, the AII was pulsed, and transjunctional currents (Ij) were recorded in the ON cone bipolar. The slope conductance of 569 pS was determined from the linear regression shown by the solid black line (R = 0.99753).

Discusssion

In this manuscript, we have demonstrated that light responses of AII s and rod and ON cone bipolars are driven by rods, based on the criteria that the neurons respond to stimuli below cone threshold and that responses to bright stimuli last much longer than the stimulus due to rod afterpotentials. Next, we show that the AII light response consists of two distinct components. One is totally blocked by AMPA receptor antagonists, consistent with rod bipolar input. The second does not reverse between −70 and +70 mV and is abolished by the gap junction blocker, carbenoxolone. These experiments suggest that the residual current is generated in ON cone bipolars and propagated to AII s via gap junctions. Paired recordings demonstrate that rod bipolar–AII synaptic transmission is indeed mediated solely by AMPA receptors and that AII s and ON cone bipolars are well coupled in our slices. Thus, the two components of the AII light response represent activation of two different bipolar cells via two different rod pathways, rod bipolar–AII and rod–cone coupling. In the following text, we extrapolate the relative contribution of each pathway in AII s to their respective contributions in ON cone bipolars, demonstrating that both pathways contribute to inner retina responses over a large range of intensities.

Rod bipolar–AII amacrine synapse

Using simultaneous patch-clamp recordings from both the pre- and postsynaptic cells, we were able to demonstrate that AMPA receptor antagonists totally blocked spontaneous and evoked glutamatergic input to AII s (Veruki et al. 2003). Rod bipolar input to AII s was completely blocked by puffer applied 20 μM NBQX or 20 μM GYKI-53655. Singer and Diamond (2003) found incomplete block of rod bipolar input to AII s in rat using 25 μM GYKI-54266 to block AMPA receptors. The differences in our findings might be explained by the eightfold higher efficacy of GYKI-53655 relative to -54266 in cultured rat hippocampal neurons (Donevan et al. 1994) and retinal ganglion cells (Luokasiewicz et al. 1997). The IC₅₀ of GYKI-53655 is ~1 μM.

The present study, as well as Singer and Diamond (2003), provide evidence that contradicts previous reports in the literature regarding transmission at the rod bipolar–AII synapse. Here we have shown that in addition to total block of synaptic transmission in rod bipolar–AII pairs, the light response of AII s in dark-adapted retina are substantially attenuated by AMPA antagonists. However, in a study of AII amacrine with intracellular recordings, AMPA antagonists totally blocked spontaneous and evoked glutamatergic input to AII s (Veruki et al. 2003). Synaptic currents persisted in the presence of antagonists of chemical synaptic transmission (Bennett 1977), indicative of direct electrical communication via gap junctions. A view of the transjunctional voltage in an expanded time scale is shown in Fig. 5B: the ON cone bipolar was pulsed, generating rectangular displacements of the AII holding current. There were spontaneous EPSCs throughout the recording. B2: application of 20 μM NBQX, 20 μM picrotoxin, and 2 μM strychnine were used to block fast chemical synaptic transmission, yet pulses to the ON cone bipolar resulted in rectangular displacements of the AII holding current, indicative of direct electrical communication via gap junctions. C: a view of the transjunctional current (Ij) at the onset of Vj (marked by the vertical line) is shown in an expanded time scale. The transjunctional current recorded in the AII rises rapidly with no delay, indicative of an electrical synapse. D: the current voltage relation of another pair is shown. In this example, the AII was pulsed, and transjunctional currents (Ij) were recorded in the ON cone bipolar. The slope conductance of 569 pS was determined from the linear regression shown by the solid black line (R = 0.99753).

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bipolar, and we have shown that cone bipolars downstream of AIIIs are also faster than rod bipolars. Perhaps the initial transient component of vesicle release from rod bipolars is related to the speeding of responses, or the large initial volley of release might be required to faithfully transmit small depolarizations in a rod bipolar that result from a single photon absorption by one of its presynaptic rods (Field and Rieke 2002). However, the slow rate of rod bipolar depolarization by light might not evoke the same transient release as a voltage step. In fact, the output of a rod bipolar might be linear with respect to its slow light response, based on the following argument.

Rods themselves respond sluggishly to photon absorption and likely account for a majority of the rod bipolar’s slowness at low intensities. The slowness of the rods is also visible in the ON cone bipolar component of the AII light response (Fig. 2), and, by analogy, this current likely reflects the voltage waveform of the rod bipolar (Fig. 2B, inset, gray trace). Therefore in a single AII recording, we can visualize the light responses of ON cone bipolars rod bipolars in addition to measuring the glutamate release from the rod bipolar. In a comparison of the bipolar waveform to the glutamate release (Fig. 2B, inset), the rising phase of the two overlap, suggesting that the vesicle release machinery of the rod bipolar is linearly dependent on the membrane voltage, at least initially. The glutamate release does decline over time, whereas the rod bipolar voltage remains steady. Perhaps the decline in release rate is due to the significant GABA input that rod bipolar terminals receive from S1 and S2 amacrine cells (Zhang et al. 2002). The decrease in vesicle release rate might represent desensitization of the vesicle fusion machinery to a sustained increase in [Ca$^{2+}$], (Hsu et al. 1996), or it might reflect a decrease in the number of vesicles available for fusion (Heidelberger et al. 1994; Heinemann et al. 1994; Neher and Zucker 1993; Thomas et al. 1993). Nevertheless, because the rod bipolar waveform and the glutamate current on the AII have the same time course initially, the speeding of responses of cells downstream from the rod bipolar (Fig. 1D) must arise from elsewhere than the rod bipolar–AII synapse. It is more likely that the quickening of voltage responses in AIIIs and ON and OFF cone bipolars is the result of Na$^+$ spikes in all amacrines (Boos et al. 1993; Veruki and Hartveit 2002a).

Further work is necessary to address this issue.

**ON cone bipolar–AII synapse**

In our rabbit retina slices, we have shown that AIIIs and ON cone bipolars are coupled by gap junctions with an average conductance of 500 pS. This value is lower than the 1.2-nS average reported for paired recordings of AII–ON cone bipolar synapses in rat (Veruki and Hartveit 2002b). Pipette solutions and series resistance values are very similar for recordings in that study and those reported here. Perhaps the disparity in the average values obtained for coupling conductance represent a true species difference. A species-related difference in coupling strength is supported by comparison of recordings of AII–AII pairs. We have determined the average conductance to be $\sim$260 ± 107 pS ($n = 6$ pairs), which is smaller than the 700-pS value reported for rat (Veruki and Hartveit 2002a). For both gap junctions, AII–AII and AII–ON cone bipolar, the conductance values for rabbit are $\sim$40% of those found in rat.

Although our mean gap junction conductance values differ, there are similarities in our findings regarding the large range of conductances around the mean. The large range may reflect differences in the number of AII dendritic processes retained as a result of slicing, or the difference in coupling strengths between AIIIs and ON cone bipolars of different classes (Veruki and Hartveit 2002b). We did not classify our ON cone bipolars from paired recordings and therefore cannot speak to differences between types. However, we did notice a substantial variability in the ON cone bipolar component of the AII light response, which we attribute to different numbers of contacts between the recorded AII and neighboring ON cone bipolars. Although not tested exhaustively, we expect that for a given AII, increasing flash intensities should lead to larger ON cone bipolar contributions to the AII light response (see Fig. 2).

**Rod contributions to ON cone bipolar light responses**

In the absence of rod bipolar input, the AII receives rod input from ON cone bipolars, and the most likely route for the rod component of the ON cone bipolar light response involves rod–cone coupling. However, we must consider the possibility of direct contacts between rods and ON cone bipolars, as these connections have been demonstrated for OFF cone bipolars (Li et al. 2004; Soucy et al. 1998; Tsukamoto et al. 2001). Direct rod–ON cone bipolar contacts are unlikely considering the results from Deans et al. (2002). The complete loss of rod input to ON center ganglion cells in the Cx36 knockout mouse indicates that gap junctions are required for transmission of rod signals in the ON pathways. Our interpretation that rod signals in ON cone bipolars result from rod–cone coupling depends on the lack of direct ON cone bipolar to rod contacts in the rabbit as was shown in the mouse. Although unlikely, the influence of direct contacts cannot be eliminated at this time.

One of the most interesting findings in the study by Deans et al. (2002) relates to the separate populations of ganglion cells with different thresholds and different intensity response functions. One explanation for these results is that the different ganglion cell populations receive disproportionate input from the different rod pathways. Concomitantly, different classes of ON cone bipolar cells must receive disproportionate input as well. The results presented here suggest that the rod–rod bipolar–AII path and the rod–cone coupling path operate at similar intensities, down to 0.5 R* * rod$^{-1}$ * s$^{-1}$, and that ON cone bipolars get input from both pathways. However, there might be a population of ON cone bipolars that are not coupled to AIIIs. These cells would not contribute to the AII light response.

We can make predictions about the relative contributions of the rod bipolar–AII and rod–cone coupling pathways to ON cone bipolar responses based on data presented here. The values for the AII current with and without NBQX or GYKI can be translated to the values for coupled ON cone bipolars, using published values for input resistances and coupling coefficients (Veruki and Hartveit 2002b). In Fig. 6, a diagram of a cell pair is displayed together with arrows representing the flow of current injected into one cell. This simple circuit yields a system of equations

\[ I_T = I_1 + I_{1T-2} \]  
\[ V_1 = I_1 R_1 \]


