Light Signaling in Scotopic Conditions in the Rabbit, Mouse and Rat Retina: A Physiological and Anatomical Study

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1Discipline of Physiology, School of Medical Sciences and Institute for Biomedical Research, The University of Sydney, Australia; 2Department of Neuroanatomy, Max Planck Institute for Brain Research, Frankfurt am Main, Germany; and 3Ophthalmology and Visual Science, University of Texas Medical School at Houston, Houston, Texas

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Protti, Dario A., Nicolas Flores-Herr, Wei Li, Stephen C. Massey, and Heinz Wässle. Light signaling in scotopic conditions in the rabbit, mouse and rat retina: a physiological and anatomical study. J Neurophysiol 93: 3479–3488, 2005. First published December 15, 2004; doi:10.1152/jn.00839.2004. In the dark, light signals are conventionally routed through the following circuit: rods synapse onto rod bipolar (RB) cells, which in turn contact all amacrine cells. All cells segregate the light signal into the ON and OFF pathways by making electrical synapses with ON cone bipolar (CB) cells and glycinergic inhibitory chemical synapses with OFF CB cells. These bipolar cells synapse onto their respective ganglion cells, which transfer ON and OFF signals to the visual centers of the brain. Two alternative pathways have recently been postulated for the signal transfer in scotopic conditions: 1) electrical coupling between rods and cones, and 2) a circuit independent of cone photoreceptors, implying direct contacts between rods and OFF CB cells. Anatomical evidence supports the existence of both these circuits. To investigate the contribution of these alternative pathways to scotopic vision in the mammalian retina, we have performed patch-clamp recordings from ganglion cells in the dark-adapted retina of the rabbit, mouse, and rat. Approximately one-half of the ganglion cells in the rabbit retina received OFF signals through a circuit that was independent of RB cells. This was shown by their persistence in the presence of the glutamate agonist 2-amino-4-phosphonobutyric acid (APB), which blocks rod→RB cell signaling. Consistent with this result, strychnine, a glycine receptor antagonist, was unable to abolish these OFF responses. In addition, we were able to show that some OFF cone bipolar dendrites terminate at rod spherules and make potential contacts. In the mouse retina, however, there seems to be a very low proportion of OFF signals carried by an APB-resistant pathway. No ganglion cells in the rat retina displayed APB- and strychnine-resistant responses. Our data support signaling through flat contacts between rods and OFF CB cells as the alternative route, but suggest that the significance of this pathway differs between species.

INTRODUCTION

Night time (scotopic) vision relies on rod photoreceptors that are responsive to extremely low light intensities, ranging from a single to a few photons. Rods hyperpolarize in response to light stimulation and light-evoked signals follow the well-described scotopic pathway (Bloomfield and Dacheux 2001; Daw et al. 1990). In the mammalian retina, rods synapse onto a unique type of bipolar cell, the ON-depolarizing rod bipolar (RB) cell. These cells do not make direct contact with ganglion cells (GCs). Instead they contact a bistratified narrow field amacrine cell, the AII amacrine cell, which splits the incoming signal to feed the ON and OFF channels of the visual system. All cells make electrical synapses with ON cone bipolar (CB) cells via gap junctions and inhibitory chemical synapses with OFF CB cells, using glycine as their neurotransmitter (see Fig. 1). The axon terminals of ON and OFF CB cells stratify in sublamina b and a, respectively, of the inner plexiform layer to synapse onto their appropriate ganglion cells.

Compelling physiological and anatomical evidence has corroborated this signaling pathway. Previous work has shown that 2-amino-4-phosphonobutyric acid (APB), an agonist at mGlur 6 receptors, blocks RB and ON CB responses (Slaughter and Miller 1981). Müller et al. (1988) have shown in the intact cat retina that APB abolishes light responses in scotopic conditions from all ON and OFF brisk sustained (X) and brisk transient (Y) GCs by acting at the rod→RB cell synapse and thereby blocking the signal transmission. Correspondingly, it was also found that strychnine, a glycine receptor antagonist, abolished scotopic light responses of OFF GCs by blocking AII→OFF CB cell synapses without substantially affecting the activity of ON GCs.

More recently, two alternative pathways for rod signals have been proposed. First, DeVries and Baylor (1995) showed that, in scotopic conditions, two types of GCs (OFF sluggish and ON-OFF direction-selective) in the rabbit retina were still responsive to light stimulation in the presence of APB. On the basis of anatomical (Raviola and Gilula 1973) and physiological evidence (Nelson 1977; Schneeweis and Schnapf 1995), DeVries and Baylor proposed that rod signals could flow to the cone pedicles via gap junctions and subsequently to the GCs through OFF CB cells (Fig. 1). Additionally, in connexin 36 knockout mice (Cx36 KO mice), extracellularly recorded scotopic ON responses of retinal GCs were suppressed, most likely due to lack of expression of functional gap junctions between rods and cones and between AII and CB cells, further supporting the existence of multiple rod pathways for night vision (Deans et al. 2002).

Second, rod-driven signals have been recorded in the presence of APB from OFF and ON-OFF GCs of normal and genetically derived coneless mouse retinas (Soucy et al. 1998). Based on these results, Soucy et al. suggested a second alternative circuit independent of cone photoreceptors, which implies a direct contact between rods and OFF CB cells (Fig. 1). Further support for this theory came from the localization of
the ionotropic glutamate receptors GluR1 and GluR2 to flat contacts between rods and putative OFF CB cells in the normal rodent retina (Hack et al. 1999), as well as from the recent demonstration of such contacts between a specific type of OFF cone bipolar cell, the CB2, and rod spherules in the mouse retina (Tsukamoto et al. 2001). In addition, GluR2 receptors were recently shown to be localized to dendrites of OFF CB retina (Tsukamoto et al. 2001). In addition, GluR2 receptors were recently shown to be localized to dendrites of OFF CB Cells contacting rod spherules in a large percentage of cells in the rabbit retina (Li et al. 2004).

To investigate whether these alternative pathways transmit rod signals through the mammalian retina, we examined the light responses of GCs in the rabbit, rat, and mouse retina under different light conditions and with specific pharmacological tools. We observed APB-resistant signals frequently in the rabbit, rarely in the mouse, and never in the rat retina. The high proportion of APB-resistant OFF signals in rabbit retina is supported by the high number of rod→OFF CB cell contacts observed anatomically. Our results favor the alternative circuit in which rod photoreceptors directly contact OFF CB cells, and they support the existence of this alternative pathway in rabbit as well as rodent retina.

**METHODS**

**Animals and tissue preparation**

Electrophysiological recordings were performed from retinal GCs in albino and pigmented rabbits (2–3.5 kg), mice from the strain C57Bl/6J (5–7 wk old) and Swiss rats (5–7 wk old). Light responses in the rabbit retina were recorded from both the whole mount preparation and slices, whereas in the case of mouse and rat retinas, only slices were used. For whole mount experiments, rabbits were dark-adapted for ≥5 h before they were killed by an overdose of pento-barbital sodium. The right eye was removed, and the retina was isolated under infrared illumination (>900 nm). A small piece (~3 × 4 mm) of the central part of the retina was placed photoreceptor side down in the recording chamber. Retinal vertical slices were prepared from rabbit, mouse, and rat retinas following previously described procedures (Protti et al. 1997). Rabbits were prepared as described above, whereas mice and rats were dark-adapted for 6–7 h and subsequently anesthetized with halothane and decapitated. All procedures were performed under infrared light and in accordance with the guidelines for animal experiments issued by the Federal Republic of Germany (Tierschutzgesetz). The eyes were rapidly enucleated and transferred to a dish containing Ames medium. The cornea was cut along the ora serrata, the lens and vitreous were removed, and the retina was separated from the sclera. The retina was cut into four pieces, one of which was embedded in 2% agar dissolved in Ames medium (kept at 38°C), and the resulting block was rapidly cooled. The block was transferred to a microslicer (Dosaka EM), and 200 μm-thick slices were cut. Slices were kept in Ames equilibrated with a mixture of 95% O2, 5% CO2 for 30 min before electrophysiological recording. The tissue could be maintained for ≤8 h in oxygenated Ames medium.

**Electrophysiological recordings**

During recordings, the tissue was mounted in a recording chamber and continuously perfused with oxygenated Ames medium (35°C, pH = 7.4). Cells were viewed on a video monitor coupled to a CCD camera mounted on a Zeiss microscope (ACM, Zeiss, Oberkochen, Germany) equipped with a water immersion objective (×40/0.75 W). The Nomarski optics of the microscope were modified for infrared illumination (900 nm). GCs were identified by their electrophysiological properties and their morphology, namely large sodium currents (>1 nA) and a large soma. Patch-clamp recordings of cells in the GC layer were performed using an EPC-9 patch-clamp amplifier (Heka Electronic, Landau, Germany). The pipette solution contained Cs+ as the main cation and gluconate as the main anion. The intracellular solution consisted of (in mM) 100 CsGlu, 0.5 MgCl2, 10 Na-HEPES, 5 EGTA, 0.5 CaCl2, 3 Mg-ATP, 0.5 Na GTP, 5 tetrabutylammonium chloride (TBA), and 5 QX-314. TBA and QX-314 blocked voltage-gated potassium and sodium conductances, respectively, thus allowing the recording of light-evoked synaptic currents. The chloride concentration yielded an ECl = −55 mV. Series resistances, which ranged from 10 to 30 Mohm, were left uncompensated. Drugs were diluted in Ames medium and perfused at ~3 ml/min. Ames medium, strychnine, and all other chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany). To block scotopic OFF responses in GCs, a racemic mixture of both d- and L forms of APB (Tocris, Cookson, Bristol, UK) at a concentration of 100 μM was used (see also Jin and Brunken 1996; Soucy et al. 1998).
Visual stimuli

All visual stimuli were achromatic and presented on the 13-in RGB color monitor of a Macintosh IICi computer. Images were generated by software kindly provided by Dr. Markus Meister (Harvard University, Boston, MA) and modified by Dr. Rowland Taylor (Neurological Sciences Institute, Oregon Health and Sciences University, Portland, OR). In both scotopic and mesopic/photopic conditions, two main stimulation patterns were used: 1) bright stimuli on a dark background (ON stimulus) and 2) dark stimuli on an illuminated background (OFF stimulus). Stimuli were focused onto the photoreceptors, and their luminance was adjusted from the software and by neutral density filters and ranged from $\sim 6.5 \times 10^{-7}$ to 0.65 cd/m². For whole mount experiments, we used optimal spots (50–200 μm diam) to elicited centered responses. For the slice experiments, full-field stimuli were employed.

Analysis

Peak current amplitude and latency of the stimulus-evoked responses were calculated using homemade routines (Igor Pro, WaveMetrics, Lake Oswego, OR). For the intensity-response function, peak amplitude of the currents were normalized and plotted versus the logarithm of the stimulus luminance. The values of $I/I_{\text{max}}$ were fitted with the Hill function: $I/I_{\text{max}} = L^{h}(L_{\text{max}} + L_{\text{opt}}^{h})$, where $I_{\text{max}}$ represents the maximal current amplitude, $L$ corresponds to the luminance (in cd/m²), $h$ represents the slope of the curve (i.e., the Hill coefficient), and $L_{\text{opt}}$ is the luminance evoking a half-maximal response (in analogy to the equations describing the intensity-response function of photo-receptors membrane potential; Normann and Werblin 1974).

Morphology and immunocytochemistry, rabbit retina

Bipolar cells were recognized in whole mount retina pieces prelabeled with 5 μM 4',6-diamino-2-phenylindole (DAPI, Molecular Probes, Eugene, OR) and impaled under visual control using pipettes tip filled with 4% Neurobiotin (Vector Laboratories, Burlingame, CA) and 1% Lucifer yellow-CH (Molecular Probes) in ddH₂O and backfilled with 3 M LiCl. The cells were injected with a biphasic current (+1.0 nA for 100 ms and −1.0 for 100 ms) for 1–2 min. After the last injection, the retina pieces were fixed in 4% paraformaldehyde for 20 min. The tissues were washed extensively with 0.1 M phosphate buffer (PB, pH 7.4) and blocked with 3% donkey serum in 0.1 M PB buffer (PB, pH 7.4) and blocked with 3% donkey serum/PBS/0.5% Triton X-100 over night. The tissues were washed extensively with 0.1 M phosphate and RB cells using APB was also studied in OFF responses originating from OFF and ON-OFF GCs. OFF responses were elicited either at the cessation of a square light pulse (ON stimulus) or at the presentation of a dark spot on an illuminated background (OFF stimulus). Scotopic OFF stimuli are shown by a black rectangle on a dark gray background, and mesopic/photopic stimuli by a black rectangle on a light gray background. The top trace in Fig. 2A shows a response elicited by an OFF stimulus recorded from an ON-OFF GC under scotopic conditions. Inward currents followed both transitions in light intensity. Due to increased latency and slow response kinetics in scotopic conditions, the ON and OFF part of the light response cannot always be clearly separated when using 400-ms stimuli. APB (100 μM) reversibly abolished the OFF component in 9 (7 OFF and 2 ON-OFF) of 21 cells that presented OFF responses. When using an intensity of 1.625 × 10⁻³ cd/m², stimulus-evoked currents were $-51.4 \pm 15$ (SD) pA (n = 7 cells). In four of these cells, currents in control conditions were $-51 \pm 17$ pA and were reduced by 96% in the presence of 100 μM APB, as shown in Fig. 2A (middle). However, in the presence of 100 μM APB, stimulus-evoked OFF currents were restored by increasing light intensity toward the cone activation levels, either by illuminating with brighter spots or by presenting a logical profile of the light responses proved to be equally valid for transient as well as for sustained responses; therefore no further classification has been done. GCs were held at a voltage of ~75 mV. Since light-evoked currents consist of a mixture of cationic (Na⁺/K⁺) and anionic (Cl⁻) currents, at this holding potential, the light-driven synaptic currents are inward. Light-evoked currents were elicited by using spots of different light intensities. Under very low light conditions (scotopic), the lowest intensities we tested occasionally failed to evoke light responses. Above a threshold of $6.5 \times 10^{-7}$ to 3.25 × 10⁻⁶ cd/m², light-evoked currents were consistently elicited, and intensity-response functions were measured. Under these conditions, light-evoked currents elicited by small spot diameters (50–200 μm) consisted of inward currents and represented responses of the receptive field center. The reversal potential of light-evoked currents was measured by setting the membrane potential to different values between −105 and 45 mV and presenting a spot of optimal size. We found that stimulus-evoked currents reversed at approximately −20 mV (data not shown), suggesting a predominance of excitatory input (as shown in detail in Flores-Herr et al. 2001).

We first tested the ability of 100 μM APB to abolish ON light responses in scotopic and mesopic conditions. APB is an agonist at the metabotropic glutamate receptor type 6, which blocks the light-evoked responses of all ON-depolarizing bipolar cells, including RB cells. As expected, ON responses of ON and ON-OFF cells were completely blocked in all cases (data not shown). This blockade persisted over a wide range of light intensities. After removal of APB from the bath, light-responsive responses were fully recovered (data not shown).

The effect of abolishing synaptic transmission between rods and RB cells using APB was also studied in OFF responses originating from OFF and ON-OFF GCs. OFF responses were elicited either at the cessation of a square light pulse (ON stimulus) or at the presentation of a dark spot on an illuminated background (OFF stimulus). Scotopic OFF stimuli are shown by a black rectangle on a dark gray background, and mesopic/photopic stimuli by a black rectangle on a light gray background.
FIG. 2. Effect of 2-amino-4-phosphonobutyric acid (APB) and strychnine on light-induced currents of rabbit retinal ganglion cells in scotopic and mesopic conditions. Scotopic OFF stimuli are shown as black rectangles on dark gray background; mesopic photopic stimuli as black rectangles on light gray background. A: ON-OFF ganglion cell stimulated for 400 ms with an OFF stimulus (background luminance = 3.25 × 10⁻³ cd/m²). Responses in control conditions (top) and in the presence of 100 µM APB (middle) were recorded using this dark stimulus. Afterward, background illumination was increased to 3.25 × 10⁻¹ cd/m² and the OFF response was rescued despite the presence of 100 µM APB (bottom). B: ON-OFF ganglion cell that was also stimulated with an OFF stimulus. Control responses were recorded (top) before application of 2 µM strychnine (middle) under a background intensity of 3.25 × 10⁻³ cd/m². When background intensity was increased (3.25 × 10⁻¹ cd/m²), OFF responses were recovered. C: responses from an OFF ganglion cell evoked with a dark stimulus on an illuminated background (luminance = 1.625 × 10⁻³ cd/m²) in control conditions (top) and in the presence of 100 µM APB (bottom). Note that APB did not block the OFF response. D: responses from an OFF ganglion cell were evoked with an OFF stimulus in control conditions (top) and after perfusion with 2 µM strychnine (bottom); note again that the OFF response is not abolished. Background illumination was 1.625 × 10⁻³ cd/m². All recordings were done with cells voltage clamped at \( V_{\text{hold}} = -75 \text{ mV} \).

dark spot on a brighter background (Fig. 2A, bottom). This response under bright conventional conditions indicates direct cone→OFF CB→OFF GC cell signaling, which is expected to be APB-resistant.

In the same set of cells, in agreement with the classical signaling pathway for rod vision, 2 µM strychnine also effectively blocked OFF responses, without affecting the ON component of ON-OFF cells (3 of 3 cells). A representative light-evoked response from an ON-OFF GC elicited with an OFF stimulus is shown in Fig. 2B (control, top). Under scotopic conditions, the OFF component of the response was blocked on perfusion with strychnine (2 µM) without substantially affecting the ON component (Fig. 2B, middle). After adapting the retina to 10,000-fold higher background intensity, strychnine failed to block OFF light responses (Fig. 2B, bottom). Again, this bright stimulus produces a conventional response via OFF CB cells.

However, in the remaining 12 cells (4 OFF and 8 ON-OFF), OFF responses, although partly reduced in amplitude, always persisted in the presence of 100 µM APB. Figure 2C represents a typical APB-resistant light-evoked current recorded in scotopic conditions and shows that, although the amplitude of the light-evoked currents was reduced by application of APB, a substantial inward current still persisted. Slower kinetics of the light response after application of 100 µM APB (as in Fig. 2C) was not seen in all cells tested. In the remaining 3 of 7 cells for which currents were measured in response to the same stimulus intensity, the mean amplitude of light-evoked currents in control conditions was \(-52 ± 16 \text{ pA} (n = 3)\) and it was only reduced by 7% by 100 µM APB. This strengthens the idea that 100 µM APB blocks the light responses of ON CB and RB cells without substantially affecting OFF CB cell signaling. Consistent with this result, in this group of cells, 2 µM strychnine exhibited only partial blocking effects in scotopic conditions with stimulus-evoked currents being reduced to 58% of their control amplitude (3 cells), implying that these responses might be independent of the RB cell-AII amacrine cell circuit. A representative recording of the strychnine effect on OFF responses is shown in Fig. 2D, in which presentation of an OFF stimulus elicited an inward current (top), and application of strychnine partly, but not fully, blocked this response (bottom). In summary, these experiments indicate the presence of rod input to OFF GCs, independent of RB cells.

**Rod contacts of OFF cone bipolar cells**

We set out to investigate the anatomical substrate of the alternative rod pathway in the rabbit retina, as previously described in the rodent retina (Hack et al. 1999; Tsukamoto et al. 2001). This was accomplished by dye-injecting single OFF CB cells with Neurobiotin and Lucifer yellow and double labeling the tissue with an antibody against mGluR6. Each whole mount retina was examined with a confocal microscope. The mGluR6 antibody labeled the dendritic tips of RB cells and ON CB cells, as previously reported (Vardi et al. 2000). Each rod spherule was identified as two bright dots, one for each of the two rod bipolar dendrites forming the central pair...
in a tetrad of processes that invaginate the rod terminal (Rao-Mirotznik et al. 1995). Cones were identified by multiple ON CB cells contacting one cone pedicle forming a lightly labeled cluster of fine dendritic tips. This pattern of mGluR6 labeling provides a map for the locations of rods and cones.

When viewed in whole mount, the dendritic tree of each OFF CB cell formed a typical pattern of branching dendrites with terminal clusters. As shown in Fig. 3, most of the dendritic tips contact cone pedicles, and almost every cone within the dendritic field is contacted (Fig. 3). Occasionally, the terminals of two separate dendrites meet at a single cone pedicle. As might be expected for OFF CB cells, the dendritic tips were not double labeled for mGluR6.

While most OFF CB cell dendrites terminate at cone pedicles, we also found examples that clearly terminate at rod spherules, identified by the presence of two brightly stained RB cell dendritic tips. Two examples are highlighted by arrows in Fig. 3B. We have intentionally been very conservative in identifying rod contacts from OFF CB cells. The dendrites of OFF CB cells certainly pass close to many rod spherules as they traverse the outer plexiform layer to make contact with the relatively sparse cone array. However, we cannot assign these as rod contacts, as opposed to passing coincidence, without the presence of a third synaptic marker. Instead, we have chosen to look for the terminal contacts of OFF CB dendrites. If an OFF CB dendrite terminates in an area where there are no cones but an obvious rod spherule is present, we have counted this as a rod contact. At such contacts, the OFF CB dendrites are never double labeled for mGluR6 but always occur basolateral to the rod bipolar dendritic tips at the base of the rod spherule (Fig. 3B). We are aware that this approach may lead to a substantial underestimate of rod contacts, but these examples are very likely to represent rod input to OFF CB cells. We have observed this connectivity pattern in several OFF CB cells, showing the existence of a putative locus for signal transmission from rods directly to OFF CB cells.

Light responses in the mouse retina

Light-evoked currents were recorded from cells in the GC layer in mouse retinal slices. Cells were identified as GCs using the same criteria as described for rabbit GCs. The light stimulus consisted of a full-field pulse (ganzfeld). Typical light responses at a holding potential of $-75$ mV consisted of inward currents that reversed between $-55$ and $-35$ mV, suggesting that there was also a strong inhibitory input presumably originating from the surround illumination.

As in the rabbit retina, APB reversibly abolished OFF responses at all intensities tested. We studied light responses from 18 ganglion cells (10 OFF and 8 ON-OFF) that presented OFF responses in scotopic conditions. In 17 of them, $100 \mu M$ APB completely abolished OFF responses at low light intensities. Representative responses are shown in Fig. 4. In ON-OFF GCs, inward currents were elicited at both onset and offset of the stimulus (Fig. 4A, top). These responses were completely blocked after bath application of $100 \mu M$ APB (Fig. 4A, middle). An increase of stimulus intensity (2–3 log units brighter) rescued OFF responses still in the presence of APB, whereas ON responses remained hindered due to blockade of signal transfer at the photoreceptor–ON depolarizing bipolar cell synapse. OFF GCs (Fig. 4B) exhibited a similar behavior: responses to an OFF stimulus were abolished by $100 \mu M$ APB (middle) but were recovered by using a brighter background (bottom). The effect of $2 \mu M$ strychnine on OFF responses recorded from ON-OFF and OFF GCs was also tested. In ON-OFF cells, strychnine abolished the OFF component of light-evoked currents in response to scotopic stimulation without affecting the ON responses (Fig. 4C, top and middle). Using a brighter uniform illumination as background in the presence of strychnine, application of an OFF stimulus resulted in recovery of the OFF response and an increase in the amplitude of the ON response (bottom). Figure 4D shows the effect of strychnine on light-evoked responses of OFF GCs. OFF responses were evoked by presentation of an OFF stimulus (top) and were abolished by $2 \mu M$ strychnine (middle). Using brighter background intensity during perfusion with strychnine rescued OFF responses (bottom). The ON response is most likely inhibitory chloride input to the cell that was not present or masked by spontaneous membrane fluctuations at lower light intensities. Strychnine fully abolished the OFF responses in seven of seven cells (5 ON-OFF and 2 OFF).

**FIG. 3.** Synaptic contacts established by OFF-cone bipolar cells with rod and cone terminals. A: neurobiotin-injected Ba3 bipolar cell (green) was double labeled with antibody to mGluR6 (red), which labels rod bipolar dendritic tips in the form of bright doublets within the rod spherules. ON cone bipolar dendritic tips form dimmer, larger clusters beneath each cone pedicle. B: in the zoomed image of the area indicated by the square in A, the cone pedicles are outlined by circles. In this region, most of the dendritic tips from this Ba3 cell contact cones. However, several branches terminate at the rod spherules as indicated by arrows. Scale bar in both A and B is 5 μm.
FIG. 4. Effect of APB and strychnine on light-induced currents of mouse retinal ganglion cells in scotopic and mesopic conditions. A: control light responses (top) from this ON-OFF ganglion cell were elicited by a dim light stimulus (full field; $6.5 \times 10^{-5}$ cd/m$^2$) for 400 ms (indicated by a dark gray rectangle) on a dark background (black area) before 100 $\mu$M APB was added to the perfusion medium, blocking all responses (middle). OFF responses, but not ON responses, were recovered (bottom) by increasing stimulus intensity to 0.65 cd/m$^2$ (light gray rectangle). B: responses from an OFF ganglion cell evoked with an OFF stimulus (top) in scotopic conditions and in the presence of 100 $\mu$M APB (middle; background intensity: $5.25 \times 10^{-3}$). Increasing background intensity to 0.65 cd/m$^2$ recovered the OFF responses (bottom). C: light responses evoked with an OFF stimulus from an ON-OFF ganglion cell in scotopic conditions, control response (top) and response in the presence of 2 $\mu$M strychnine (middle) are shown. The response elicited by the appearance of the dark stimulus (OFF response) is blocked by the application of strychnine. In contrast, the subsequent ON response is not blocked. Afterward, the background intensity in the presence of strychnine was increased by 4 log units, and OFF responses were fully recovered (bottom). D: responses were elicited from this OFF ganglion cell using an OFF stimulus. Control recordings (top) and in the presence of 2 $\mu$M strychnine (middle) were obtained with a background intensity of $(6.5 \times 10^{-6}$ cd/m$^2$). Bottom: background intensity was increased to $6.5 \times 10^{-4}$ cd/m$^2$, and the OFF response was recovered. $V_{\text{hold}} = -75$ mV for all recordings.

The peak amplitude of OFF responses originating from OFF and ON-OFF GCs was measured, normalized, and plotted as a function of stimulus intensity. Intensity-response curves for OFF responses in control conditions and in the presence of pharmacological agents are shown in Fig. 5. In control conditions, OFF responses were elicited by using minimal intensities in the scotopic range. Application of both APB and strychnine abolished responses at low light levels. However, when the light intensity was raised to about three log units above the scotopic threshold, in the presence of either APB or strychnine, OFF responses could be evoked. Further increases of the light intensity resulted in increasing responses. Changes in the shape of the intensity-response curve observed on drug application could be attributed to higher light intensities needed to elicit responses and, in the case of strychnine, to removal of glycine-ergic inhibition.

The one cell where the OFF responses were not fully blocked by APB was an ON-OFF GC. Figure 6A shows the effect of 100 $\mu$M APB on this cell. Inward currents evoked at light onset were completely abolished on application of APB (left). Under these conditions, however, an inward current was still elicited at light offset, indicating the presence of an APB-resistant OFF component. When OFF stimuli were used (Fig. 6B), responses were partly reduced by APB, but not abolished. Note that the ON component of the response is fully blocked (right). Normalized peak current amplitude is plotted as a function of stimulus strength for the OFF responses of this cell in Fig. 6C; the sensitivity profile in the presence of APB matches almost perfectly with the control responses, providing evidence for an APB-resistant OFF component in scotopic conditions. In summary, APB-resistant responses were found in a very small proportion of GCs in the mouse retina.

FIG. 5. Intensity-response function for OFF responses from mouse ON-OFF and OFF ganglion cells in control conditions and in the presence of 100 $\mu$M APB and 2 $\mu$M strychnine. Peak amplitudes were normalized and plotted against the log of light intensity. Symbols represent the average of different cells, and bars are their corresponding SE (control: $n = 5$, APB: $n = 5$, strychnine: $n = 4$). Lines show the fit of the experimental points to the Hill function: $I/I_{\text{max}} = L^h/(L^h + L_{50}^h)$, where $I_{\text{max}}$ was the maximal current amplitude, $L$ was the luminance, $h$ represents the slope of the curve (i.e., the Hill coefficient), and $L_{50}$ the luminance evoking a half-maximal response.
Light responses in the rat retina

Light-evoked currents were also recorded from cells in the GC layer of rat retinal slices. Cells were identified according to the criteria used for rabbit and mouse GCs. As in the previous sections, light responses were inward currents and reversed between $-55$ and $-35$ mV.

As previously described for the rabbit and mouse retinas, 100 $\mu$M APB completely and reversibly abolished light-evoked ON responses recorded from all GCs at all intensity levels, and this blockade was reversible. Light-evoked OFF responses were recorded from a total of 20 (11 OFF and 9 ON-OFF) GCs in scotopic conditions. In all cases, stimulus-evoked OFF currents were fully blocked by 100 $\mu$M APB, an effect that was reversible. Figure 7A shows the response of an OFF GC in scotopic conditions (top) that is completely blocked by 100 $\mu$M APB (middle). However, using brighter background illumination in the presence of APB allowed OFF responses to recover (bottom). This indicates input mediated by OFF CB cells in the photopic range.

The effect of strychnine was also tested in 10 of the 20 cells. Strychnine efficiently blocked OFF responses in scotopic conditions in all 10 cells tested (6OFF and 4 ON-OFF GCs). Figure 7B shows the action of strychnine on an ON-OFF GC. An inward current was evoked with an OFF stimulus (top), and strychnine fully blocked this response. Note that the ON response is enlarged, presumably by removal of glycinergic inhibition (middle). A further increase of the background illumination during perfusion with strychnine made it possible to obtain OFF responses once more (bottom). Again, these represent direct photopic input from OFF CB cells.

Analysis of the intensity-response relationship confirms the blocking action of APB for OFF responses at low intensities. When higher stimulus intensities were applied during the application of APB, light responses could be generated via direct input from OFF CB cells. Further increases in light intensity produced larger responses (Fig. 8). These results suggest that, in the rat retina, the majority of rod level signals are processed via the RB pathway, which is sensitive to APB. We were unable to detect alternative rod-driven pathways in this sample of GCs.

**FIG. 6.** Light-evoked responses and intensity-response function of an APB-resistant ON-OFF ganglion cell of the mouse retina in scotopic conditions. A: left: light responses elicited with a light stimulus ($6.5 \times 10^{-3}$ cd/m²) in control conditions and in the presence of 100 $\mu$M APB. Right: responses elicited with an OFF stimulus on a dimly illuminated background ($6.5 \times 10^{-6}$ cd/m²) in control conditions and after addition of 100 $\mu$M APB. $V_{\text{hold}} = -75$ mV for all recordings. B: intensity-response function for OFF responses from this ganglion cell in control conditions and in the presence of 100 $\mu$M APB. Peak amplitudes were normalized and plotted against the log of light intensity. Lines show the fit of the experimental points to the Hill function. Fitting function and parameters are described in METHODS and in Fig. 5.

**FIG. 7.** Effect of APB and strychnine on light-induced currents of rat retinal ganglion cells in scotopic and mesopic conditions. A: control light responses (top) from this OFF ganglion cell were evoked with an OFF stimulus on a dimly illuminated background ($6.5 \times 10^{-3}$ cd/m²) before adding 100 $\mu$M APB to the bathing solution (middle), after which responses were completely blocked. OFF responses were recovered after increasing background intensity to $6.5 \times 10^{-3}$ cd/m² (bottom). B: responses evoked with an OFF stimulus from an ON-OFF ganglion cell. Control responses (top) in scotopic conditions ($1.625 \times 10^{-5}$ cd/m²) and in the presence of 2 $\mu$M strychnine (middle) are shown. Note recovery of OFF responses (bottom) as background intensity in the presence of strychnine was increased by 4 log units. $V_{\text{hold}} = -75$ mV for all recordings.

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and AII amacrine cell. APB and strychnine. These data imply that both rod cells, and bars are their corresponding SE (control: parameters see METHODS and Fig. 5.

The APB- and strychnine-resistant responses cannot be attributed. The APB- and strychnine-resistant light-evoked responses were observed in a high proportion of OFF and ON-OFF GCs in the rabbit retina in a small proportion of cells in the mouse retina, and in no cells in the rat retina from our sample. GCs in the rabbit retina in a small proportion of cells in the whole mount rabbit retina establish not only contacts with cone pedicles but also some terminal contacts with rod spherules, as previously reported for mouse and rat retina (Hack et al. 1999; Tsukamoto et al. 2001). These terminal contacts cannot be confused with dendrites merely passing close to rod spherules on the way to a cone pedicle. Rather, they suggest the presence of direct basolateral contacts between OFF CB cell and rod spherules, as in the study by Li et al. (2004), in which these contacts were confirmed by colocalization with the glutamate receptor GluR2. Nevertheless, this type of contact could be the locus of direct synaptic transmission from rods to OFF CB cells in the rabbit retina.

Our electrophysiological results in the rabbit retina are very similar to the observations made by DeVries and Baylor (1995). However, while they suggested that APB-resistant signaling could occur via rod→cone gap junctions, we interpret these results in a different manner. Because it is very likely that single cones contact all types of CB cells (Boycott and Wässle 1991, 1999; Grünert et al. 1994), if rod signals were to spread to the cones and were subsequently transmitted to the CB cells, theoretically all OFF and ON-OFF GCs should exhibit APB-resistant OFF responses. However, this was not the case (DeVries and Baylor 1995; this study), and therefore we propose that these signals do not arise from rod signals spreading into cones but from direct rod→OFF CB cell contacts. Such contacts, at least in mouse retina, have been shown to involve only one type of OFF CB cell, and hence, it is highly likely that their signals reach only a subset of GCs. In rabbit retina, all three OFF CB cells establish dendritic contacts with rod spherules in a relatively high proportion (Li et al. 2004). These results are in agreement with Deans et al. (2002), who showed that, in scotopic conditions, GC ON responses from connexin 36 knockout mice, but not OFF responses, were suppressed. Because Cx36 KO mice most likely lack functional gap junctions between rods and cones, the persistence of light responses in OFF GCs suggests that rod→cone signaling does not play a major role for the OFF pathway at scotopic intensities.

Soucy et al. (1998) have also described an alternative pathway for scotopic vision in both normal and coneless mouse retina, which carries signals to all OFF and ON-OFF GCs. The anatomical substrate suggested to transmit these signals are flat contacts established by a particular type of OFF CB2 with rod spherules (Tsukamoto et al. 2001), and where ionotropic glutamate receptors have been localized (Hack et al. 1999). In the mouse retina, however, we only observed APB-resistant OFF responses in 1 of 18 cells. What possible explanations could account for this difference? First, we recorded synaptic currents by doing patch-clamp recordings in the slice preparation, whereas Soucy et al. (1998) recorded spike trains with a multielectrode array in the whole mount retina. If when doing multielectrode recordings, there were a bias toward recording from particular GC types that happened to be those cells receiving input from an APB-resistant pathway, the role of this pathway might have been overestimated. Along a similar line of reasoning, if GCs that receive input from CB2 have a particular morphology that made them unsuitable for our recordings, or if either CB2 or their postsynaptic GCs do not survive the slicing procedure, we may have possibly missed recording some APB-resistant OFF responses. Nevertheless, in the rabbit retina, we have obtained similar results recording from slices and from whole mount retina, suggesting that the functionality of the alternative pathway is independent of the preparation. Second, we dark-adapted the animals for a substantially longer time period than Soucy et al. (6 vs. 1 h), since in preliminary experiments, we observed that after short dark-adaptation periods the retina was unresponsive to low intensity stimulation. Therefore a difference in photoreceptor sensitivity between the two studies cannot be excluded. Finally, according to Soucy et al. (1998), the appearance of APB-resistant OFF
responses was dependent on stimulus contrast, being most prevalent at high contrast. To detect APB-resistant off responses at all intensities, we used the maximum possible contrast (100%), thus ruling out a difference between the stimulus employed to evoke responses in their experiments and in this study.

Although no more than 20% of rods were found to make contacts with the CB2, it was proposed that rod→rod gap junctions in the mouse retina may be the route by which all rod signals could be fed into CB2 cells and their corresponding GCs (Tsukamoto et al. 2001). It is well established, however, that GCs stratify at different strata in the inner plexiform layer, where each particular type receives input from only a few bipolar cell types (Boycott and Wässle 1999; DeVries 2000; Roska and Werblin 2001). Consequently, CB2 bipolar cells are expected to synapse onto some but not all GC types. Additionally, only an estimated 5% of rods were shown to possess flat contact synapses with glutamate receptors (Hack et al. 1999). Taking into account these considerations and consistent with our results, signaling through an APB-resistant pathway is expected to be transferred to only a subset of off and on-off GCs. Our data suggest that, in rodents, the percentage of off GCs might be considerably lower than in rabbit.

Soucy et al. (1998) also observed APB-resistant light responses in coneless mice, in which cones were genetically ablated. Genetic manipulation of particular cell types in the retina may result in widespread effects. For example, removal of cones from the retina results in degeneration of the rods (Ying et al. 2000). Moreover, in a retinal degeneration model, RB cells form ectopic contacts with cones in the absence of rods (Peng et al. 2000). Taken together, this may hint that, in the coneless mice, wiring changes could take place, such that a larger number of cone bipolar cell types receive rod input than in the normal mouse, thus explaining the results observed in the coneless mice. However, no difference in the APB sensitivity of off responses between the normal and coneless retina was observed, making an indirect effect due to cone ablation unlikely.

It remains to be elucidated in which other species the alternative pathways are present and whether they play an important role. In the developing retina of the ferret, APB-resistant off responses have been recorded under dark-adapted conditions from the majority of off and a small percentage of on-off GCs, although it is not known whether this pathway is also functional in the mature retina (Wang et al. 2001). In the cat retina, both on and off responses recorded under scotopic conditions from brisk sustained (X) and brisk transient (Y) cells were completely blocked by APB, and off responses were blocked by strychnine (Müller et al. 1988). These two GC classes comprise only about 50% of all GCs of the cat retina, and responses from other GC types have not been characterized so far. However, expression of a GluR1 glutamate receptor subunit has been observed in dendrites of off CB cells contacting rod spherules of the cat retina, suggesting the possibility that this pathway may also be present in the cat retina (Kolodziej et al. 2003). In the dark-adapted monkey all light-mediated behavior is abolished on APB application, whereas in mesopic and photopic conditions, only the on responses have been shown to be blocked by APB (Dolan and Schiller 1989). In addition, no contacts between rods and off cone bipolar cells have been reported in the primate retina (Boycott and Dowling 1969). However, it is possible that in the primate retina, where midget and parasol GCs make up ~90% of the GCs, an alternative rod pathway is only present in some of the remaining 10% of the GCs.

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