Neuron, Vol. 18, 637–650, April, 1997, Copyright ©1997 by Cell Press

The Light Response of Retinal Ganglion Cells Is Truncated by a Displaced Amacrine Circuit

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Summary

The vertebrate retina contains ganglion cells that appear to be specialized for detecting temporal changes. The characteristic response of these cells is a transient burst of action potentials when a stationary image is presented or removed, and often a strong discharge to moving images. These transient and motion-sensitive responses are thought to result from processing in the inner retina that involves amacrine cells, but the critical interactions have been difficult to reveal. Here, we used a cell-ablation technique to remove a subpopulation of amacrine cells from the mouse retina. Their ablation changed transient ganglion cell responses into prolonged discharges. This suggests that transient responses are generated, at least in part, by a truncation of sustained excitatory input to the ganglion cells and that the ablated amacrine cells are critical for this process.

Introduction

Retinal ganglion cells, the output neurons of the retina, can be sorted into different classes based on their responses to visual stimuli (Enroth-Cugell and Robson, 1966; Levick, 1975; Stone, 1983; Shapley and Perry, 1986). One of the main distinguishing features is the time course of the response: some ganglion cells respond to a step change in illumination with persistent firing, whereas others produce only a transient discharge (Rodieck and Stone, 1965; Werblin and Dowling, 1969; Werblin, 1970; Ikeda and Wright, 1972; Cleland et al., 1973). The cells in the latter group characteristically fire a brief burst of action potentials following a stimulus change and then shut down, suggesting that they may be specialized for detecting temporal changes. They generally respond strongly to stimuli moving across the receptive field. Many species show transient cell types with additional specialization, such as a selectivity for the direction and speed of the stimulus (Barlow et al., 1964).

The circuitry that generates these transient responses has not yet been established, but the critical processing is thought to occur in the inner plexiform layer of the retina (Werblin, 1977; Caldwell et al., 1978; Miller, 1979; Ariel and Daw, 1982; Marchiafava, 1983; Masland et al., 1984; Masland, 1988; Maguire et al., 1989). Cells distal to this layer carry only sustained signals. The responses of photoreceptors to a brief flash of light can last for seconds (Baylor et al., 1979). While the flash response of bipolar cells is shortened somewhat by the transfer function of the photoreceptor synapse (Bialek and Owen, 1990), it still lasts on the order of a second (Capovilla et al., 1987; Belgum and Copenhagen, 1988). As a result, a maintained step of light produces a sustained polarization in these outer retinal neurons. In contrast, the step response of transient ganglion cells lasts only tens of milliseconds (Wunk and Werblin, 1979; Belgum et al., 1983), even when elicited by sustained current injection into photoreceptors (Baylor and Fettiplace, 1977). While these results are best established in amphibian retinae, where intracellular recordings are readily available, similar differences in response dynamics also hold for mammalian retinae, as indicated by recordings from cat (Nelson and Kolb, 1983; Nelson et al., 1987) and rabbit retina (Dacheux and Miller, 1981; Amthor et al., 1989). Thus, the working hypothesis is that neural circuits in the inner retina convert the sustained bipolar signal to a transient ganglion cell response.

The operations performed in the inner retina have been difficult to unravel because of the complex array of cell types in this region. In particular, the amacrine cells are comprised of numerous subtypes, each with different morphological or biochemical properties (Perry and Walker, 1980; Masland, 1988; Wässle and Boycott, 1991) and each possibly serving different functions. Current pharmacological tools, such as neurotransmitter or ion-channel antagonists, are not specific enough to allow the functions of the subtypes to be examined separately, since many use the same neurotransmitters and receptors. In particular, immunohistochemical analyses in numerous species showed that many amacrine subtypes use γ-aminobutyric acid (GABA) as a primary transmitter (Mosinger et al., 1986; Masland, 1988; Pourcho and Owczarzak, 1989; Wässle and Chun, 1989). As a result, GABA agonists or antagonists act simultaneously at many sites in the inner plexiform layer.

An alternate way to examine circuit operations is to remove cells from the circuitry by ablation. Here, we used a novel ablation technique to remove a subset of amacrine cells from the mouse retina (Nirenberg and Cepko, 1993). The general method is the following: a set of cells is genetically engineered to express the gene for the enzyme β galactosidase (β gal). This can be achieved by generating a transgenic mouse that expresses β gal under the regulation of a cell type–specific promoter. The cells are then labeled with a fluorescent dye by treating them with a fluorogenic β-gal substrate. Once labeled, the cells can be killed by photodynamic damage. This approach provides a finer way of lesioning neural circuits: for example, the subtypes of amacrine cells often differ in their expression of neuropeptides or structural proteins. The promoters to the genes for these molecules can be used to target different subtypes separately. Alternatively, promoters identified by β-gal enhancer traps can serve to isolate cell type–specific genes (Allen et al., 1988; Gossler et al., 1989).

We have applied this ablation method to the circuitry of the inner retina by eliminating a subset of displaced amacrine cells and examining the effects on ganglion cell responses. The ablation of these amacrine cells altered the properties of the "transient ON" ganglion

cells: their light response became greatly prolonged. The results indicate that the transient ON response is shaped, at least in part, by a delayed suppression of a sustained excitatory input to the ganglion cell and that the ablated amacrine cells are involved in this circuit.

Results

A Subset of Displaced Amacrine Cells Was Targeted for Ablation

To target amacrine cells, we used a transgenic mouse line (provided by Malcolm Low and Richard Goodman) that expresses the enzyme β gal in a specific subpopulation of amacrine cells. This mouse line was generated using a construct with a putative promoter for the vasoactive intestinal peptide (VIP) gene linked to the gene for β gal. However, immunohistochemical analysis revealed that the amacrine cells that express β gal do not contain VIP (Figure 7A). Presumably, the transgene came under the control of genomic sequences near the integration site that can activate its expression in a cell-specific manner. Since the sequences that direct expression to this population of cells are not yet known, we designated these neurons as "V cells."

The V cells can be labeled with fluorescein using the β-gal substrate fluorescein-di-β-D-galactopyranoside (FDG). They are a population of amacrine cells that lie in the ganglion cell layer (displaced amacrine cells), as shown in Figure 1. They constitute ~25% of the displaced cells (Dräger and Olsen, 1981; Nirenberg and Cepko, 1993), with a density ranging from 400 cells/mm^2 in peripheral retina to 3000 cells/mm^2 in central areas. Confocal microscopy showed that most have 2–5 primary dendrites that extend toward the inner plexiform layer and begin to branch within ~25 μm of the soma (Figure 1B). The FDG label could not reveal the finer dendritic branches. Occasionally (5/38 cells), a labeled process extended for some distance in the plane of the cell bodies. To further verify that the V cells are in fact amacrine cells and not ganglion cells, we labeled the two populations with different dye tracers and tested for dye colocalization. Ganglion cells were stained by retrograde transport of the carbocyanine dye di-I from the superior colliculus, which receives afferents from most or all ganglion cells (Hofbauer and Dräger, 1985). We then isolated the retina and labeled the V cells with FDG. In fluorescence micrographs of live whole-mounted retinae restricted to regions with dense ganglion cell labeling, the two labels were found in distinct populations: 329 of 355 (93%) FDG-labeled cell bodies showed no overlap with di-I profiles (Figure 1C).

Ablation Is Effective and Specific

To ablate the V cells, FDG and a sensitizing agent, aminoethyl carbazole (AEC), were applied to the retina (Nirenberg and Cepko, 1993). The V cells were then photoablated by brief, intense illumination of the tissue. This method has been shown previously to kill >90% of targeted neurons with <2% nonspecific cell death. This was indicated by the selective uptake of the dye ethidium into targeted neurons shortly after ablation treatment and the ultimate disappearance of the neurons from the tissue without concomitant loss of neighboring cells (Nirenberg and Cepko, 1993).

Many amacrine cell types form gap junction connections to other cells that are permeable to small tracer molecules (Vaney, 1994; Mills and Massey, 1995). To further assess the selectivity of the ablation procedure, we tested whether fluorescein can spread through such putative junctions between the V cells and other neurons. We compared the set of cells stained by FDG with those stained by 5-bromo-4-chloro-3-β-D-galactoside (Xgal). Since Xgal forms a very focused precipitate, it likely does not pass through gap junctions and thus should mark exclusively cells expressing β gal. In several retinal whole mounts, we counted 132 fluorescent neurons, of which 129 had a corresponding Xgal stain (Figure 2). The exceptions were among the faintest fluorescent profiles and thus may have had low enzyme activity producing undetectable amounts of Xgal. Thus, it appears very unlikely that the fluorescein generated by the β-gal enzyme passes to other neurons.

The Normal Mouse Retina Contains a Variety of Ganglion Cell Response Types

Since the functional properties of ganglion cells in the mouse have not yet been thoroughly characterized, we began by examining light responses in the normal retina. To assess the time course of the response, we used a simple stimulus of uniform illumination, whose intensity stepped up and down once every 2 s. The resulting ganglion cell spike trains were monitored by placing the retina on an array of extracellular microelectrodes in the bottom of a recording chamber.

The uniform flash stimulus revealed four major response types (Figure 3, Table 1): cells that responded with an increase in firing rate to the onset of the flash (ON cells), to the offset (OFF cells), to onset and offset (ON-OFF cells), and cells whose firing rate transiently decreased at the onset (SUPPRESSED cells). Slightly more than half the cells examined were ON cells. Among the ON and OFF groups, the responses ranged from a very transient burst of spikes following the light step (Figures 3A and 3C) to sustained firing that persisted for a second or more (Figures 3B and 3D). Transient responses predominated: in cat retina, this has been shown to result from a recording bias of extracellular electrodes in favor of the large cell bodies of α cells (Peichl and Wässle, 1979). All of these ganglion cell response types closely resemble those observed in other species (Stone, 1983), and the ON, OFF, and ON–OFF types have been reported previously in the mouse (Balkema et al., 1982; Stone and Pinto, 1993).

Ablation of V Cells Altered the Response Time Course of ON-Type Ganglion Cells

To test whether V cells play a role in shaping the response time course, we ablated them from the retina and assayed changes in ganglion cell responses to the flash stimulus. Since more than half of all ganglion cells recorded were ON-type cells, we focused our analysis of response dynamics on this group. OFF responses

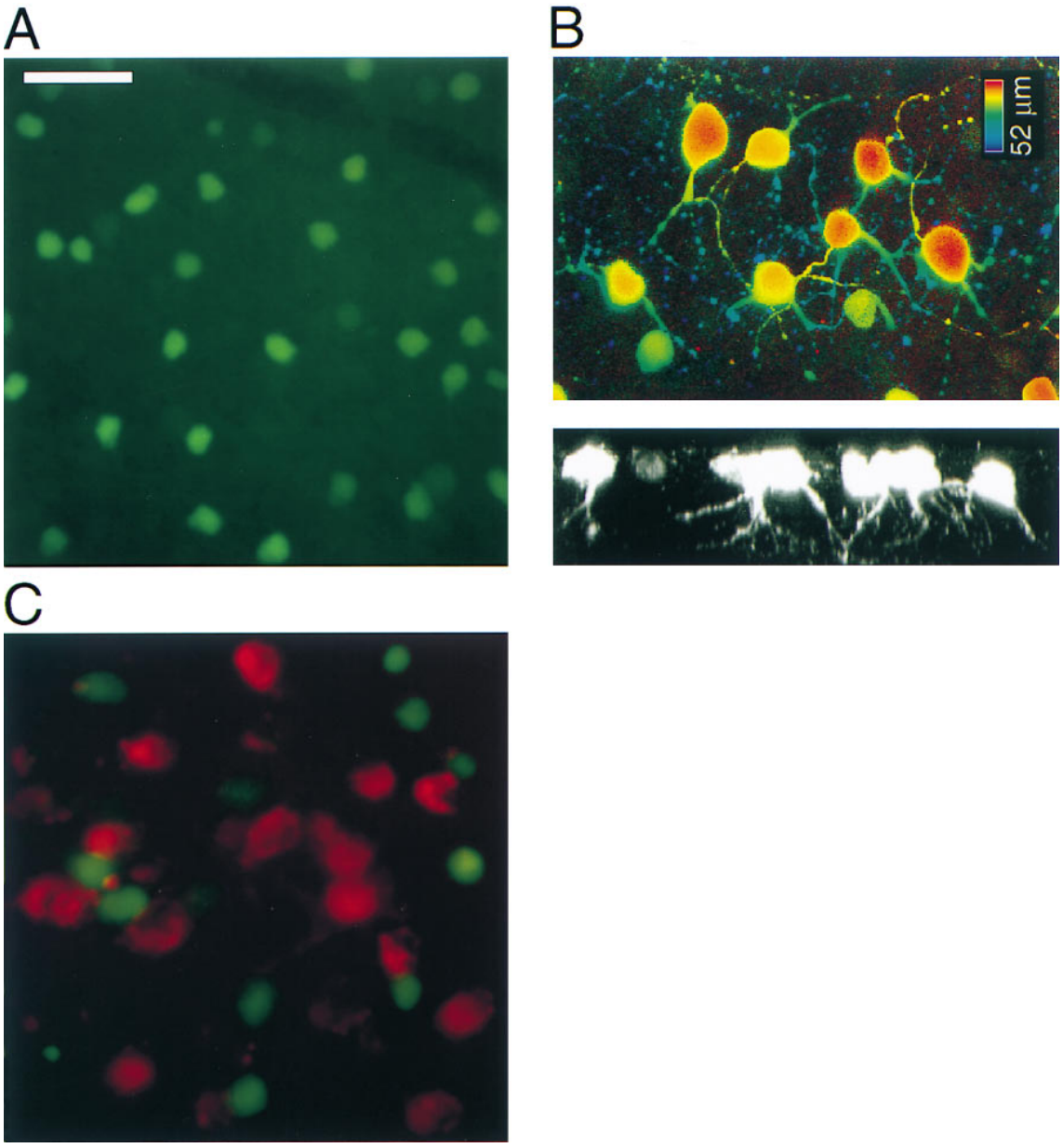


Figure 1. The V Cells Are a Subpopulation of Amacrine Cells That Lie Exclusively in the Ganglion Cell Layer

V cells labeled with FDG in live retina:

(A) Whole-mount, viewed ganglion cell–side up, imaged by standard fluorescence microscopy. Scale bar = 50 μm.

(B) Whole-mount, imaged by confocal microscopy (Zeiss LSM 410). The retina was optically sectioned (0.75 μm increments) from the fiber layer through the inner plexiform layer. Scale bar = 42 μm. Top: face view from the ganglion cell side. The depth axis is scaled in color, with red indicating the most superficial section (fiber layer) and blue indicating the deepest. Bottom: side view projection of a different field, with fiber layer at the top.

(C) Whole-mount in which ganglion cells are labeled with the carbocyanine dye di-I, imaged by standard fluorescence microscopy. Scale bar = 42 μm.

may be affected as well (Table 1) but due to their low incidence, they were not included in the present study.

In the first set of experiments, V cells were ablated in the eyes of anesthetized animals 2–4 days prior to isolating the retinae for electrophysiological recording. Ganglion cell responses from these retinae were then compared to responses from three control groups: nontransgenic mice (littermates) that received the identical treatment (FDG, AEC, and photoactivation of the dye), littermates that received FDG and AEC but no photoactivation, and untreated littermates (with and without the transgene).

In control retinae (Figure 4A left), most ON responses were very transient. In contrast, ON responses from V cell–depleted retinae (Figure 4A right) were greatly prolonged. For each ON ganglion cell, we estimated the

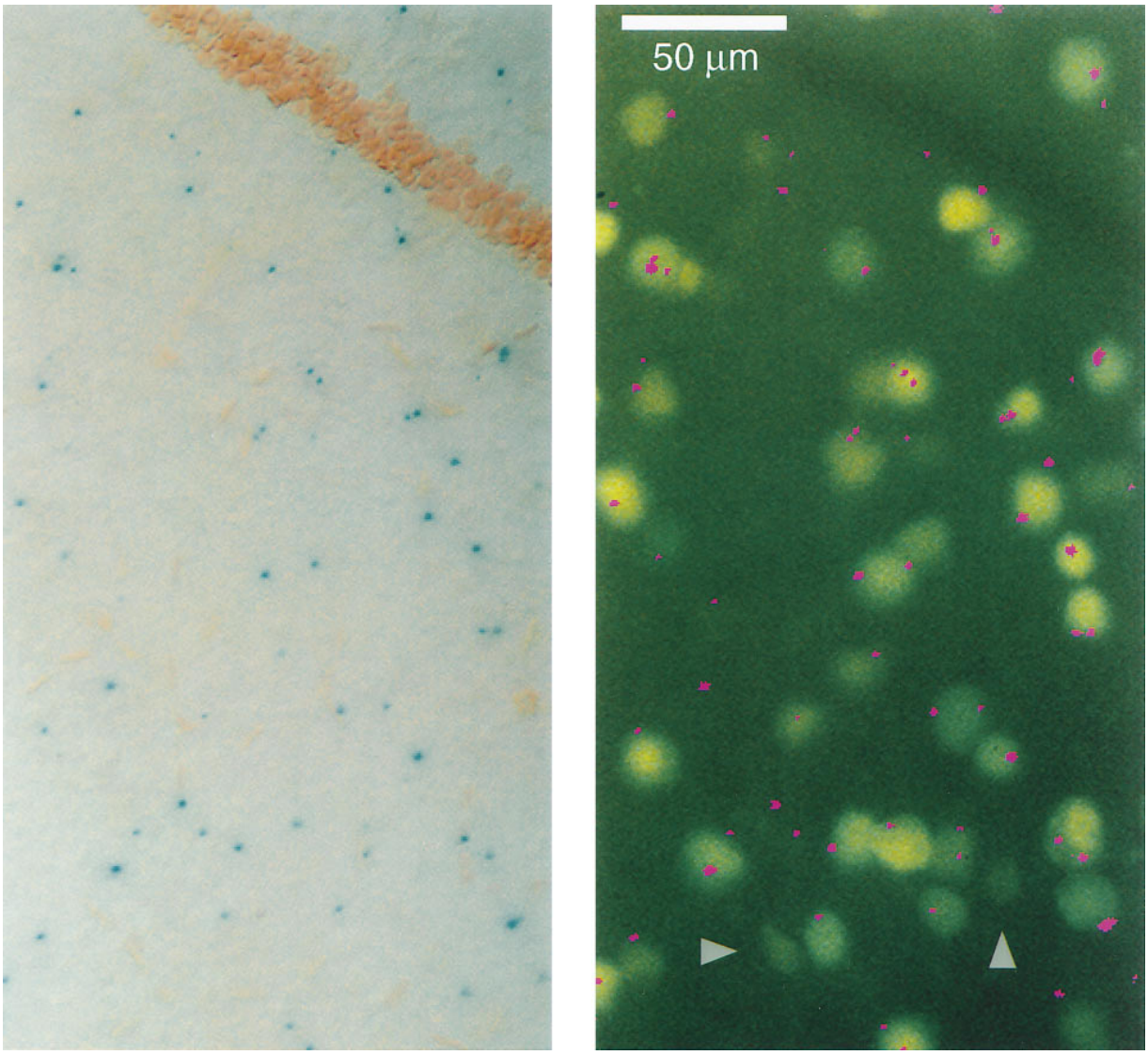


Figure 2. Fluorescein Does Not Spread from V Cells to Other Neurons

Correspondence of labeling with FDG and Xgal in the same retina. Left: fixed retinal whole-mount stained with Xgal. Right: the same region labeled with FDG prior to fixation. The pink overlay is a transfer of the Xgal spots from the left panel. Almost all fluorescent neurons also produced Xgal stain. Arrows indicate the only two exceptions. A small fraction of Xgal-stained cells (4/60) did not label with FDG (see Experimental Procedures).

duration of the light response as the time during which its firing rate was elevated from the baseline by more than 1/*e* of its peak value (Figure 4B). By this measure, the median response duration in control retinae was 120 ms (105 cells; 15 retinae), compared to 220 ms (32 cells; 5 retinae) in V cell–depleted retinae. Histograms of the response duration (Figure 4C) showed that it had the same distribution within each of the three control groups, whereas the distribution in V cell–depleted retinae was shifted significantly toward long durations ($P < 0.01$; chi-squared test). This was caused by a shift from transient to prolonged responses, rather than simply a loss of transient cells, since the fraction of ganglion cells with ON responses was the same in the three control groups and the V cell–depleted retinae ($P > 0.05$; ANOVA; Table 1). In addition, the baseline firing rate of ON cells was elevated in V cell–depleted retinae: 2.40 ± 0.43 Hz (mean ± SEM) compared to 0.31 ± 0.06 Hz in control retinae ($P < 0.05$; Student's *t*-test). However, the peak firing rate was not affected by the ablation: 23 ± 3 Hz compared to 21 ± 2 Hz in control retinae ($P > 0.1$; Student's *t*-test).

In the second set of experiments, the ablation was performed in vitro during the recording session. This allowed the responses of individual ganglion cells to be followed before and after the perturbation (Figure 5A). The results were similar to those obtained in the first set of experiments. V-cell ablation produced a marked increase in the response duration of all ON ganglion cells by a factor of 2.9 (median ratio for 13 cells in four experimental retinae). By contrast, sham photoablation treatment did not increase the response duration; in

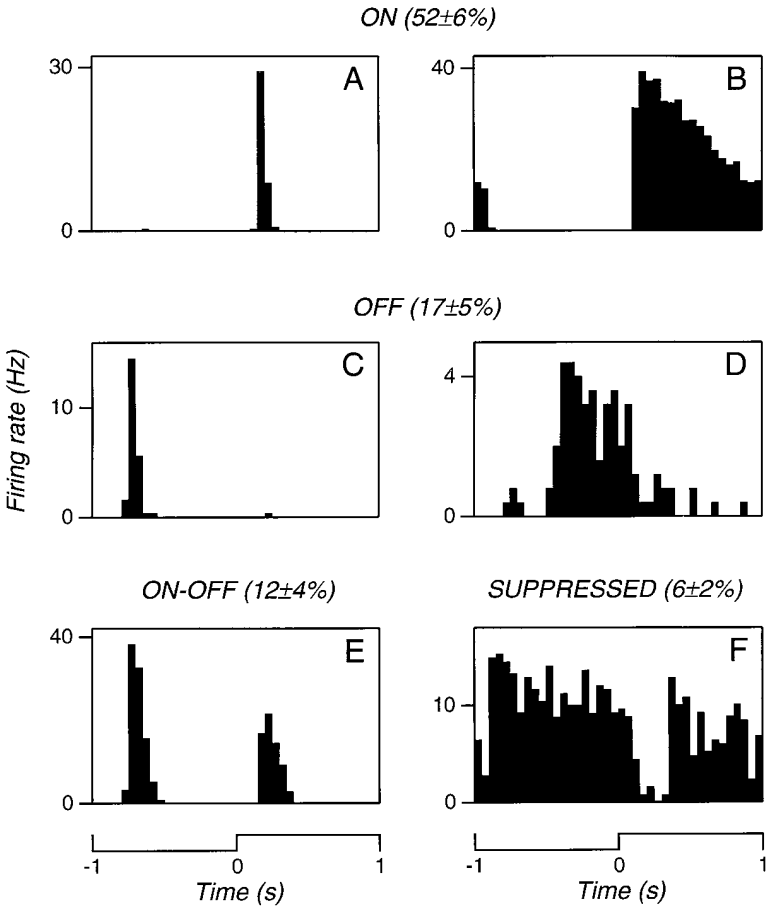


Figure 3. Responses of Ganglion Cells in the Normal Mouse Retina to a 0.5 Hz Uniform Flash

Each plot shows the average firing rate of a ganglion cell throughout the stimulus period, estimated from a PSTH over at least 50 repeats. The percentage of observed cells with a similar response is indicated at the top (mean ± SEM from seven retinae). ON and OFF responses ranged from very transient ([A] and [C]) to sustained ([B] and [D]); see also Figure 4C. The bottom trace shows the time course of the light intensity.

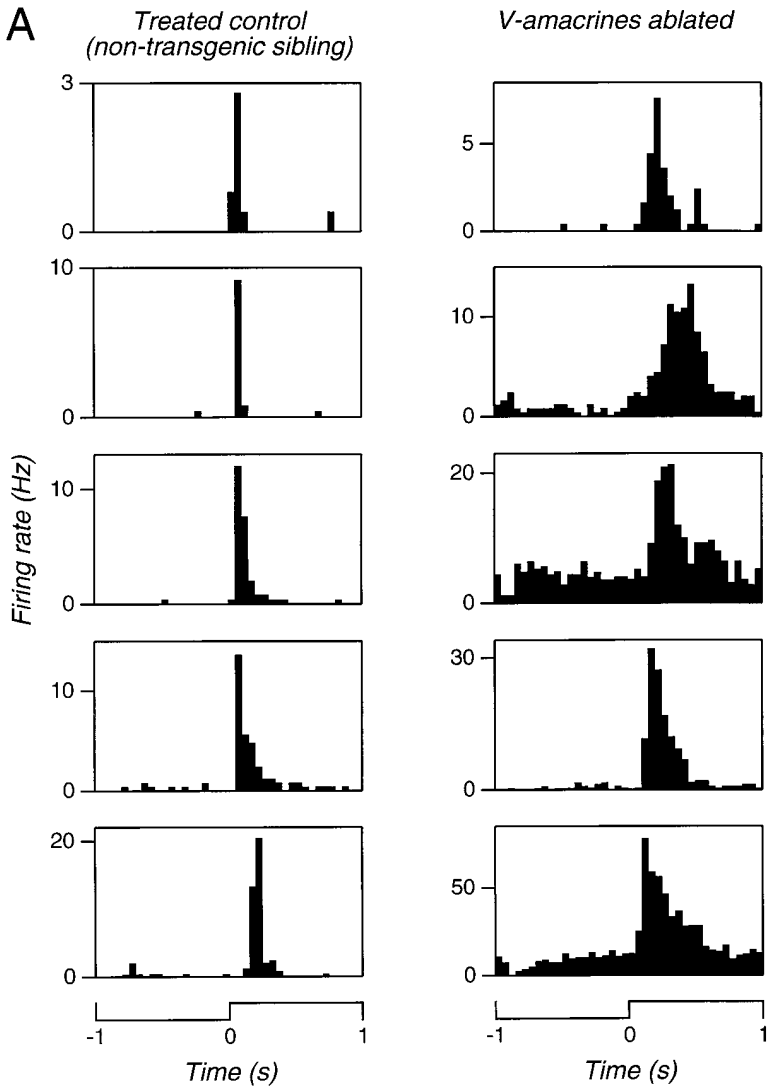
fact, it produced slightly shorter responses, by a factor of 0.9 (15 cells in three control retinae). This probably resulted from changes in photoreceptor kinetics due to light adaptation during the intense illumination (Jakiela et al., 1976). Histograms of the effect on response duration confirmed a clear difference between experimental and control retinae (Figure 5B; $P < 0.01$; Student's *t*-test comparing ratios from experimental retinae with ratios from controls). For about half of the recorded ON ganglion cells, V-cell ablation also produced an increase in the response latency (Figure 5C), measured as the time between light onset and the point where the firing rate above baseline crossed 1/*e* of its peak value (see Figure 4B). This effect was weaker than that on the response duration, with a median latency increase of 1.6-fold. For individual cells, the size of the latency change did not correlate with the change in response duration.

V Cells Shape the Response Duration through the Receptive Field Center

The results presented above suggest that in the normal retina, V cells play a key role in sharpening the step response of ganglion cells. One possibility is that they achieve this by mediating the ON cell's inhibitory surround (Werblin and Copenhagen, 1974). Under diffuse illumination, the surround signal might provide a delayed suppression of the center response (Enroth-Cugell and Lennie, 1975), and reduction of the surround by amacrine ablation could result in a prolonged response to the light step. To evaluate this proposal, we compared the ganglion cell response to uniform illumination with the response to a small spot covering only the receptive field center. The optimal center stimulus for each cell was found by varying the size and position of the spot to produce the maximal peak firing rate

Table 1. Proportion of Ganglion Cell Response Types in Control and V-Amacrine-Depleted Retinae (Mean ± SE)

Response type	Untreated 122 cell/7 retinae	Transgenic sib FDG, AEC, no photoactivation 56 cells/4 retinae	Nontransgenic sib FDG, AEC, photoactivation 44 cells/4 retinae	V-amacrine cells ablated 62 cells/5 retinae
On	0.52 ± 0.06	0.47 ± 0.06	0.54 ± 0.10	0.54 ± 0.08
Off	0.17 ± 0.05	0.12 ± 0.03	0.12 ± 0.08	0.03 ± 0.04
On-Off	0.12 ±0.04	0.10 ± 0.03	0.09 ± 0.03	0
Suppressed	0.06 ± 0.02	0.08 ± 0.01	0.04 ± 0.04	0.04 ± 0.03
Other	0.13 ± 0.04	0.23 ± 0.03	0.21 ± 0.08	0.39 ± 0.07



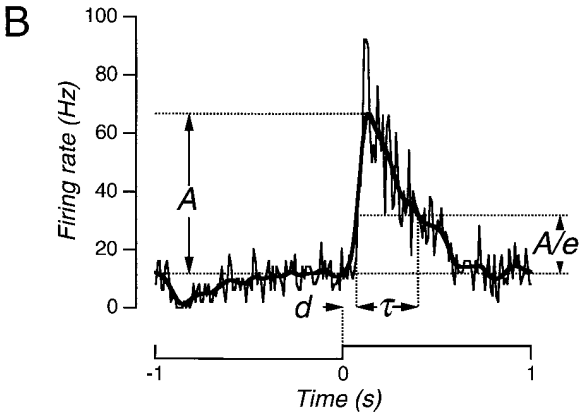


Figure 4. ON Ganglion Cell Responses Are Prolonged in V Cell–Depleted Retinae

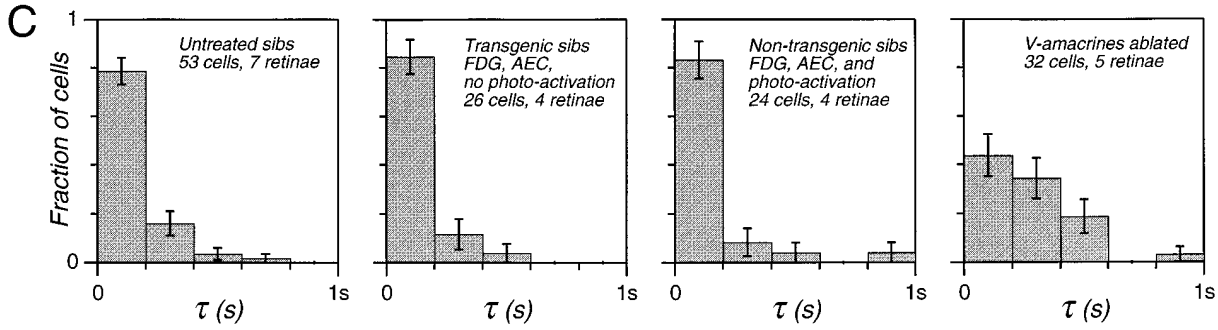
(A) Responses to a 0.5 Hz uniform flash, presented as in Figure 3. Right: responses of all ON cells recorded from a V amacrine–depleted retina. Left: responses of all ON cells recorded from a nontransgenic control retina that received the identical treatment (FDG, AEC, and photoactivation). Though this limited sample suggests an effect of ablation on the peak firing rate, this was not observed systematically (see text).

(B) Measurement of response duration. Each PSTH (see Figure 3) was accumulated with 10 ms bins (thin line), then smoothed by convolution with a Gaussian (SD = 30 ms; thick line). Baseline activity was taken as the mean firing rate during the 500 ms period before the stimulus transition. A response threshold was set at 1/*e* of the peak firing rate above baseline, *A*. Response latency, *d*, was measured as the delay from light onset to when the firing rate crossed threshold. Response duration, τ, was measured as the time interval during which the firing rate exceeded threshold.

(C) Histograms of response durations for all ON cells from control retinae and V amacrine–depleted retinae. Note that the smoothing Gaussian placed a lower bound on response durations of 85 ms.

(*Figure 4 continued on next page*)

during the ON discharge. Reducing the illumination from a uniform field to the optimal center stimulus produced an increase in the peak firing rate by a factor of 1.52 ± 0.26 (mean ± SEM), a clear indication of suppressive surround interactions. However, eliminating the surround stimulus had little or no effect on the time course of the ON response for six of seven cells tested (Figure 6). Even including the outlier cell, the response duration changed by only a factor of 1.09 ± 0.08, which is substantially smaller than that observed with V-cell ablation (Figure 4C). Since stimulation of the receptive field surround does not significantly affect the response duration



of these ganglion cells, it appears that V cells must shape the function of the center pathway.

Immunohistochemical Analysis Suggests That Most or All V Cells Are Inhibitory

The above experiments cannot determine whether the V cells participate directly in the ON pathway or act indirectly as modulators. To begin to address this question, we examined V cells for markers associated with the classical amacrine cell neurotransmitters GABA and acetylcholine (Figures 7B and 7C). Preliminary immunohistochemical analysis suggests that most V cells are inhibitory: when treated with antibodies raised against the GABA transporter GAT-1 (Brecha and Weigmann, 1994), 85% of V cells (64/75) examined in thin vertical sections showed immunoreactivity. Furthermore, the V cells are likely not cholinergic: of 169 V cells examined, only 3 reacted with antibodies against choline acetyltransferase (ChAT). The same preparation revealed a regularly spaced population of neurons positive for ChAT in the ganglion cell layer, presumably the starburst amacrines (Masland, 1988). Thus, V cells do not overlap with the starburst amacrine cell type.

Discussion

It has long been postulated that the transient ganglion cell responses are shaped by the circuitry of the inner retina, but the specific mechanisms are not yet known. Two general models have been proposed (Figure 8). In the first, the transient response is generated by a circuit mechanism: a sustained excitatory signal from the outer retina is truncated by a delayed inhibitory signal from the inner retina. An amacrine cell is presumed to mediate this process by antagonizing either the output of the bipolar cell or the input to the ganglion cell. This model postulates an inhibitory amacrine cell (Werblin, 1972; Werblin and Copenhagen, 1974; Caldwell and Daw, 1978; Belgum et al., 1984; Barnes and Werblin, 1986; McReynolds and Lukasiewicz, 1989; Maguire et al., 1989). The neural machinery needed for such an inhibitory loop is present in the retina: ultrastructural studies have revealed reciprocal synapses between bipolar and amacrine cells (Witkovsky and Dowling, 1969), and GABA-mediated inhibitory responses have been elicited from bipolar cell terminals (Tachibana and Kaneko, 1988).

In the second model, the transient ganglion cell response is generated by a cellular mechanism: a sustained excitatory signal from the outer retina is converted to a transient response by the membrane properties of an intermediary cell between the bipolar and ganglion cell or by the ganglion cell itself (Werblin, 1977; Victor and Shapley, 1979; Masland et al., 1984; Sakai and Naka, 1987). In this scenario, a cell rather than a circuit transforms the response; this cell would have to be an excitatory neuron. This model is supported by observations that some amacrine cell types have active membrane conductances that could transform a sustained depolarizing input into a fast, transient signal (Barnes and Werblin, 1986).

Our results are not consistent with this "transient excitation" model. It cannot account for the finding that V-cell ablation transforms ON ganglion cell responses from transient to sustained. Clearly, the V cells are not the critical intermediary cells since their ablation did not produce a loss of ON responses (Figure 8) but rather a change in ON-cell response dynamics. If other amacrines were serving as the intermediary cells, then V-cell ablation should not have changed the time course of the ganglion cell response, unless the V cells influenced the membrane properties of these intermediary cells.

By contrast, the effects of V-cell ablation are readily understood within a circuit mechanism for transient responses. Specifically, the V cell might lie along a delayed inhibitory pathway that interferes with a sustained excitatory signal conveyed to ganglion cells by bipolar cells. In this case, ablation of the V cell would cut the inhibitory pathway and thus allow a prolonged influence of the direct excitation (Figure 8), as observed. Consistent with this model, the peak firing rate of ganglion cells during the ON response was not affected by V-cell ablation: this peak value is determined by the strength of the direct excitatory input in the short interval before inhibition takes effect and should not depend on the presence of the inhibitory pathway. Furthermore, we observed a slight increase in the baseline firing rate of transient ON cells following V-cell ablation. This can be understood within the "delayed inhibition" model, if V cells also mediate some tonic inhibition in this pathway. For a fraction of ON cells, V-cell ablation also increased the response latency. This effect did not correlate with the change in response duration, suggesting that V cells may modulate these two response properties through independent mechanisms.

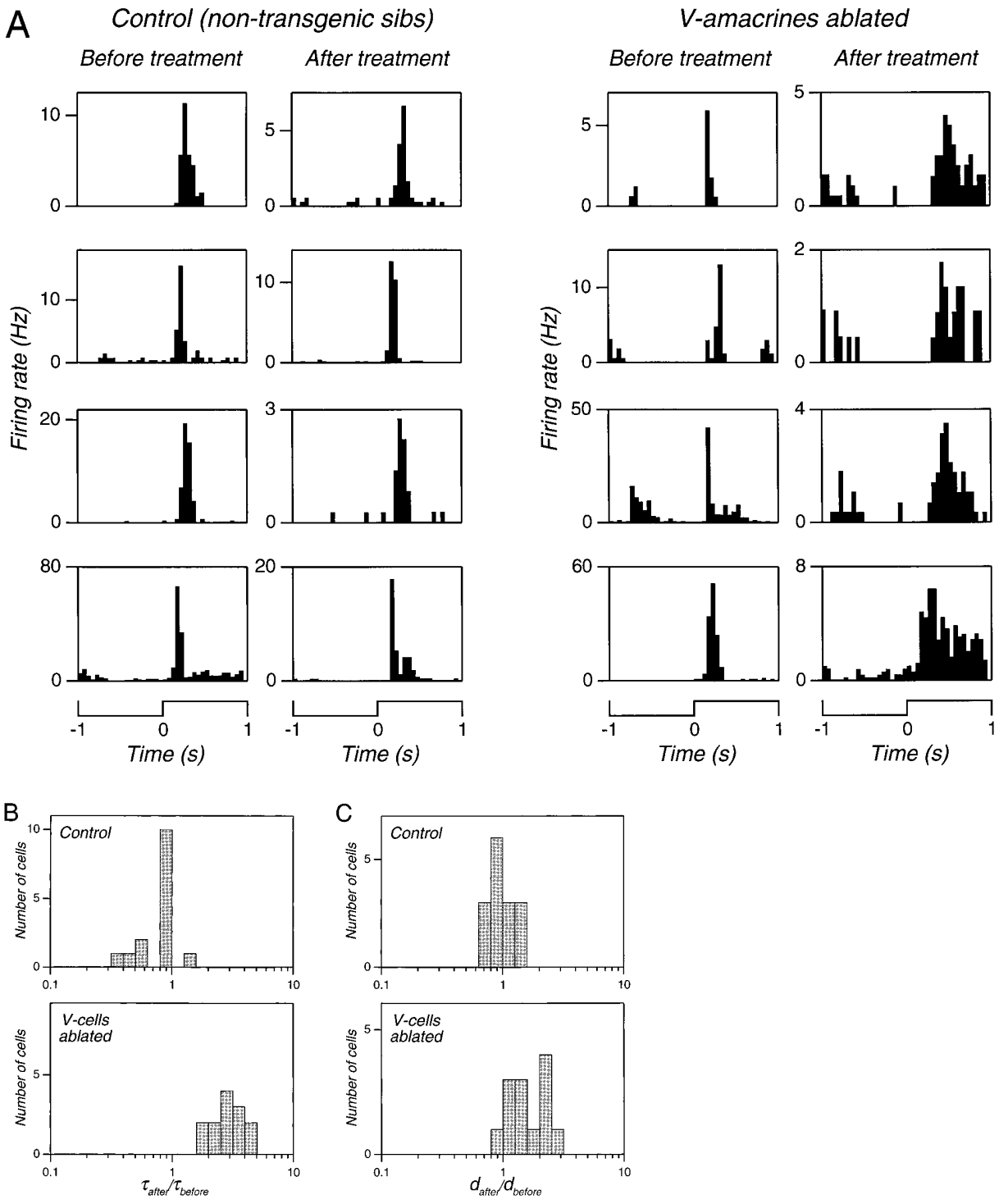


Figure 5. Acute Ablation of V Cells Extends the Transient ON Ganglion Cell Response

(A) ON ganglion cell responses to a 0.5 Hz uniform flash. Each pair of adjacent plots shows the response of the same ganglion cell before and 45–60 min after in vitro ablation treatment. Left: responses from nontransgenic control retinae. These retinae were treated identically to experimental retinae (receiving FDG, AEC, and photoexcitation) but did not suffer ablation since they did not carry the β-gal transgene. Right: responses from transgenic retinae. Each PSTH was accumulated over at least 50 repeats.

(B) Histogram of the change in response duration, expressed as the ratio of the value after treatment to the value before treatment.

(C) Histogram of the change in response latency.

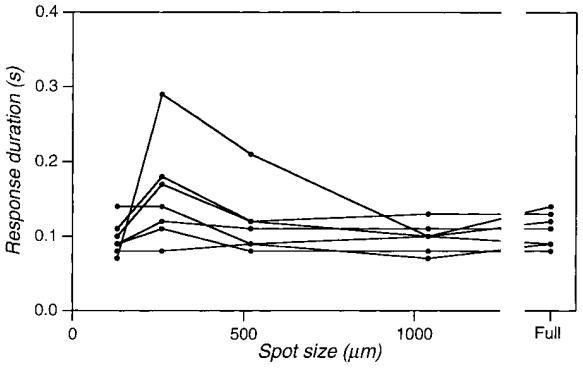


Figure 6. The Time Course of the Transient ON Cell Response Is Not Shaped by Input from the Receptive Field Surround

Response durations for seven ON cells as a function of spot size. Each cell was presented with flashing spots of increasing radius, ranging from a small spot inside the receptive field center to uniform illumination. For each spot size, the firing rate was averaged over 10 repeats of the stimulus, and the response duration was measured as described in Figure 4B.

If the V cells participate in such an inhibitory feedback circuit, do they act locally—on the receptive field center of an individual ganglion cell—or through a wide-field mechanism, by which distant stimuli can modulate local responses (Werblin and Copenhagen, 1974; Fischer et al., 1975)? Two observations suggest a local circuit. Experiments with small flashing spots showed that the duration of the transient ON response is not altered by inputs from a broad inhibitory surround (Figure 6). Furthermore, the V cells are not a sparse amacrine cell population, which would have indicated a wide-field modulatory role. Instead, the number of V cells is similar to the number of transient ON ganglion cells strongly affected by the ablation: about half of all ON cells underwent a significant change in response duration (Figure 4C). Since about half of the cells in our recordings were ON cells, this means ~25% of the ganglion cells examined were affected. If the mouse retina contains 70,000 total ganglion cells (Dräger and Olsen, 1981), the number of affected ganglion cells is 17,500, compared to ~15,000 V cells (Nirenberg and Cepko, 1993). If our recordings were biased in favor of the ON–transient ganglion cells, the ratio of V cells to affected ganglion cells would be even greater. In this context, it should be noted that mechanisms other than those involving V cells may contribute to the shaping of transient responses since some ON cells retained their transient character even after the ablation treatment (Figure 4C).

A second question is whether the V cells provide the inhibition, or whether they act indirectly to alter the strength of an inhibitory input. To determine whether a direct inhibitory function is at least possible, we tested the cells for molecules involved in GABA-ergic and cholinergic transmission: most V cells were immunoreactive to the GABA transporter GAT-1, but virtually no cells showed evidence for ChAT. Thus, the majority of V cells could, in principle, provide the negative feedback signals directly.

We therefore tested whether a pharmacological blockade of GABA-ergic transmission can produce the effects seen on V-cell ablation. The retina was bathed in picrotoxin, which blocks inhibitory transmission at GABA-A and most GABA-C receptors (though not on rat bipolar cells; see Feigenspan et al., 1993). Ganglion cell responses were recorded to the stimulus used in the V-cell ablation experiments (Figure 9). Picrotoxin had numerous effects on the ON ganglion cells: (1) for most cells, the peak firing rate and the average firing rate were significantly elevated. (2) Most cells (17/23) still produced a transient burst of spikes after light onset, but this was followed by a pause of 50–100 ms, and then a second, more extended response phase. (3) For many cells (10/23), a transient OFF response appeared in picrotoxin, whose peak firing rate was often greater than that in the ON response. Therefore, its emergence cannot be explained by a lowering of the firing threshold in ganglion cells but probably involves presynaptic changes. None of the effects of picrotoxin has the simple appearance of extending the response duration, as observed following V-cell ablation.

Studies in other species confirm that a block of GABA-ergic transmission has very broad effects on retinal function (Caldwell and Daw, 1978; Caldwell et al., 1978; Bolz et al., 1985), presumably because the transmitter is used at many sites within the circuit. In rabbit, picrotoxin affects the response of transient ON cells with an increase in peak firing rate, appearance of maintained activity, introduction of an OFF response, and sometimes extension of the response time course (Caldwell and Daw, 1978; Caldwell et al., 1978). In contradiction to this, a recent cat study concluded that picrotoxin made retinal responses more transient (Frumkes and Nelson, 1995; Frumkes et al., 1995). Clearly, the pleiotropic action of these transmitter blockers produces a variety of new response components, whose effects may well mask the action of GABA at any particular synapse. Consequently, it has been difficult to obtain direct evidence for the "delayed inhibition" model by these pharmacological methods. By comparison, the perturbation caused by ablation of the V cells is far more restricted, as it eliminates only a fraction of the amacrine cells and leaves the outer retina untouched. As a result, the observed effects on ganglion cell function are more specific and distinguish more cleanly between alternative models.

Our immunocytochemical studies also showed that the V cells are not cholinergic; thus, they do not overlap with another well-characterized class of displaced amacrine cells: the ON-starburst cells. Do the V cells constitute a single cell type? The intensity of the FDG label varied somewhat among V cells (Figure 1A), and only 85% of them stained with GAT-1 antibody, which allows for more than one neuronal class within the population. Thus, it is possible that only a subset of the ablated cells are responsible for shaping the transient ON response. Such questions could be resolved by examining the physiology of individual cells through intracellular recording and by analyzing their morphology and synaptic connections. The β-gal marker will be useful for these studies: by loading the retina with low concentrations of FDG, the β gal–expressing cells can be visualized in the living preparation (Figure 1) for dye-injection, high resolution microscopy, or intracellular recording.

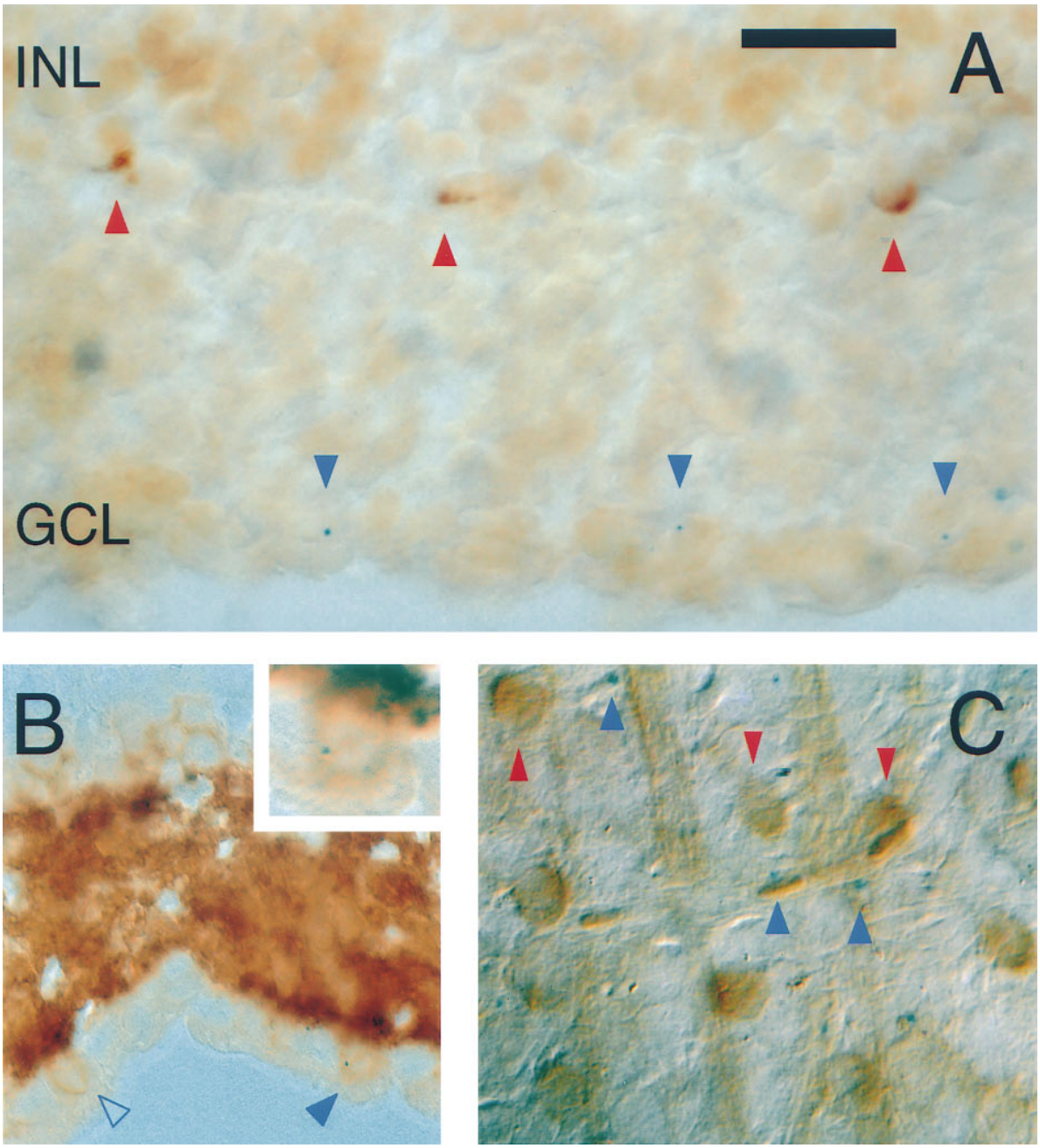


Figure 7. The V Cells Do Not Express VIP or ChAT but Are Likely GABAergic

(A) Vertical 15 μm section of the retina, double-labeled with VIP-antibody (horse radish peroxidase reaction product, HRP) and Xgal (blue precipitate). Red arrows: VIP-positive cells in the inner nuclear layer show no Xgal stain. Blue arrows: Xgal in V cells in the ganglion cell layer, which do not show VIP stain. Scale bar = 20 μm.

(B) Vertical 6 μm section, double labeled with GAT-1 antibody and Xgal. Filled arrow: a cell labeled with both markers (inset). Open arrow: a cell labeled only with GAT-1 antibody. Scale bar = 25 μm.

(C) Whole-mount retina double labeled with ChAT antibody and Xgal. Red arrows: examples of ChAT-positive cells without Xgal stain. Blue arrows: Xgal-stained cells without ChAT stain. Note that blood cells also show HRP reaction product; they are distinguished from ChAT-positive cells by their size, shape, and proximity to blood vessels. Scale bar = 34 μm.

In conclusion, we have shown that a subpopulation of displaced amacrine cells plays a critical role in shaping ganglion cell response dynamics. Their ablation substantially increases the response duration of transient ON-type ganglion cells, probably by abolishing a negative feedback signal. V cells may also modulate the latency of ganglion cell responses, and further analysis using more complex stimuli will allow these effects to

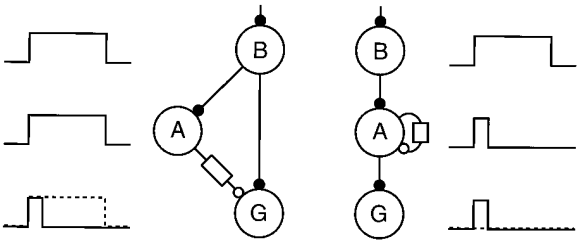


Figure 8. Two Candidate Mechanisms for the Generation of Transient Ganglion Cell Responses

Simplified circuits summarizing the "delayed inhibition" (left) and "transient excitation" (right) schemes. Bipolar (B), amacrine (A), and ganglion (G) cells interact by excitatory (closed circles) and inhibitory (open circles) connections. Boxes represent temporal delay in a pathway. Using a parallel formalism, the transient transfer function of the amacrine cell in the "transient excitation" scheme is symbolized by a delayed inhibitory feedback loop: this is likely implemented by the gating properties of membrane conductances, and no chemical autapses are implied by this diagram. Traces represent an idealized time course of each neuron's membrane potential during a step of light (solid lines) and the response predicted after direct or indirect inactivation of neuron A (broken lines).

be examined more thoroughly. In the future, other cell types in the mouse retina can also be ablated—by targeting cells based on genes that they express, such as enzymes, neuropeptides, or structural proteins, or by utilizing "enhancer traps." Eventually, this may allow a systematic dissection of retinal circuit mechanisms.

Experimental Procedures

Ablation of Amacrine Cells In Vivo

The ablation method has been described previously in detail (Nirenberg and Cepko, 1993). Briefly, the procedure was as follows: the eye of the anesthetized mouse was injected with 0.5–1.0 μl of 65 mg/ml FDG, 10 μg/ml AEC in 100% dimethyl sulfoxide (DMSO), and the solution was allowed to penetrate the tissue for 5 min. Assuming a vitreal volume of 5–10 μl, the solution was diluted 10- to 20-fold in the eye. The fluorescein-labeled cells were then visualized by placing the mouse on the microscope stage so that the microscope objective could be focused through the optics of the eye onto the retina. Corneal refraction was reduced by placing a glass coverslip on the animal's cornea. The retina was then illuminated for 8 min using a fluorescence microscope (Zeiss Axiophot; 200 W mercury short arc lamp; fluorescein filter set: exciter filter, 450–490 nm; dichroic filter, 510 nm; barrier filter, 520–560 nm). FDG uptake into the retina was sometimes incomplete and patchy. Thus, it was necessary to verify for each experiment that the V cells were ablated. After each recording session, the tissue was fixed and stained with Xgal to test for the presence of β gal–expressing cells (Cepko, 1989); only retinal areas where >90% of β gal–expressing cells were eliminated, compared to control regions, were analyzed.

Labeling Ganglion Cells with Di-I and V Cells with FDG in Live Tissue

Ganglion cells were labeled by injecting 10 μl of 20 mg/ml Di-I in DMSO into the superior colliculus of the anesthetized mouse and allowing 3–4 days for transport to the retina. The retina was then removed from the animal and placed ganglion cell–side up in a small volume of Ringer's just sufficient to cover the tissue. 50–100 μl of 2.6 mg/ml FDG in 2.5% DMSO–97.5% Ringer's solution was then dropped on the retinal surface and allowed to penetrate the tissue for 5–10 min. The tissue was then washed with Ringer's solution three times and inspected under fluorescence optics using an FITC–Texas red filter (Olympus).

FDG and Xgal Labeling in the Same Tissue

Several pieces of retina (~2 mm × 4 mm) were labeled with FDG as described above. Each piece was then placed in a tunnel slide to hold the tissue flat. The retinal piece was then photographed to produce an image of all of the fluorescent cells. Afterward, the piece was fixed in the same flat configuration by flowing 0.5% glutaraldehyde in phosphate-buffered saline (PBS) into the slide. The tissue was then washed several times and stained for Xgal as described previously (Cepko, 1989). The retina was mounted, coverslipped, and photographed again, and this image was compared to the FDG photograph.

Stimulation and Recording of Ganglion Cell Responses

Spike trains were recorded extracellularly from the ganglion cell layer using a multielectrode array as described previously (Meister et al., 1994). The retina was isolated into oxygenated Ringer's solution (110 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1.6 mM $MgCl_2$, 10 mM D-glucose, buffered with 22 mM $NaHCO_3$, and 5% CO_2–95% O_2 [pH 7.4]) under dim red illumination, then dark adapted for 30 min. A piece of retina 2–3 mm on a side was cut from the superior periphery and placed ganglion cell–side down on an array of 61 electrodes spaced 60 μm apart. The isolated retina, maintained at 37°C, remained stable for 4–6 hr. Most if not all of the spikes recorded extracellularly probably derive from ganglion cells rather than displaced amacrine cells. In recording from the ganglion cell layer of the cat, which has a higher proportion of displaced amacrine cells than the mouse, one very rarely encounters neurons that cannot be driven retrogradely from the optic tract (H. Wässle, personal communication). Furthermore, ablation of V cells, a population of displaced amacrine cells, did not alter the number of cells in our recordings, indicating that one does not regularly record from these displaced amacrine cells.

A computer monitor (Apple RGB Display) delivered uniform illumination of the retina with white light, flashing on and off at 0.5 Hz, at a mean intensity of 5 mW/m² and temporal contrast (on−off)/(on+off) of 0.99. For experiments involving in vitro ablation, these uniform flashes were delivered by a green (peak emission at 565 nm) light emitting diode (LED) with a mean intensity of 200 mW/m². To quantitate the effect of the monitor and the green LED on each of the three mouse photoreceptors, we provide the equivalent photon flux of a monochromatic light source at the wavelength of peak absorption (λ_{max}) (Table 2).

Table 2. Photon Flux at λ_{max} (in Photons/μm²/s) Corresponding to 1 W/m²

Light source	Rod λ_{max} = 500 nm	S-Cone λ_{max} = 370 nm	M-Cone λ_{max} = 511 nm
White monitor	$1.06 \cdot 10^6$	$7.72 \cdot 10^4$	$1.08 \cdot 10^6$
Green LED	$6.15 \cdot 10^5$	$8.74 \cdot 10^{-1}$	$1.00 \cdot 10^6$

In picrotoxin experiments, the stimulus was delivered by the monitor. Ganglion cell responses were recorded in normal Ringer's solution, after 15–30 min of superfusion with Ringer's solution containing 100 μM picrotoxin, and following washout with normal Ringer's solution for >20 min. Some experiments used lower doses of picrotoxin (10–40 μM) with very similar results.

Analysis and Classification of Ganglion Cell Responses

For each cell, a peristimulus time histogram (PSTH) was accumulated over 50 stimulus repeats with 50 ms bins. ON and OFF cells were distinguished by comparing their mean firing rates during the on portion (r_{on}) and the off portion (r_{off}) of the periodic light stimulus. For OFF cells, r_{on}/r_{off} was <0.25; for ON cells, r_{on}/r_{off} was >4. Cells were classified as ON–OFF if they showed a transient increase in firing rate at light onset and offset, with $0.25 < r_{on}/r_{off} < 4$. SUPPRESSED cells showed a single transient decrease in firing rate after light onset. Cells classified as OTHER were not driven by the uniform flash stimulus or produced rare response patterns.

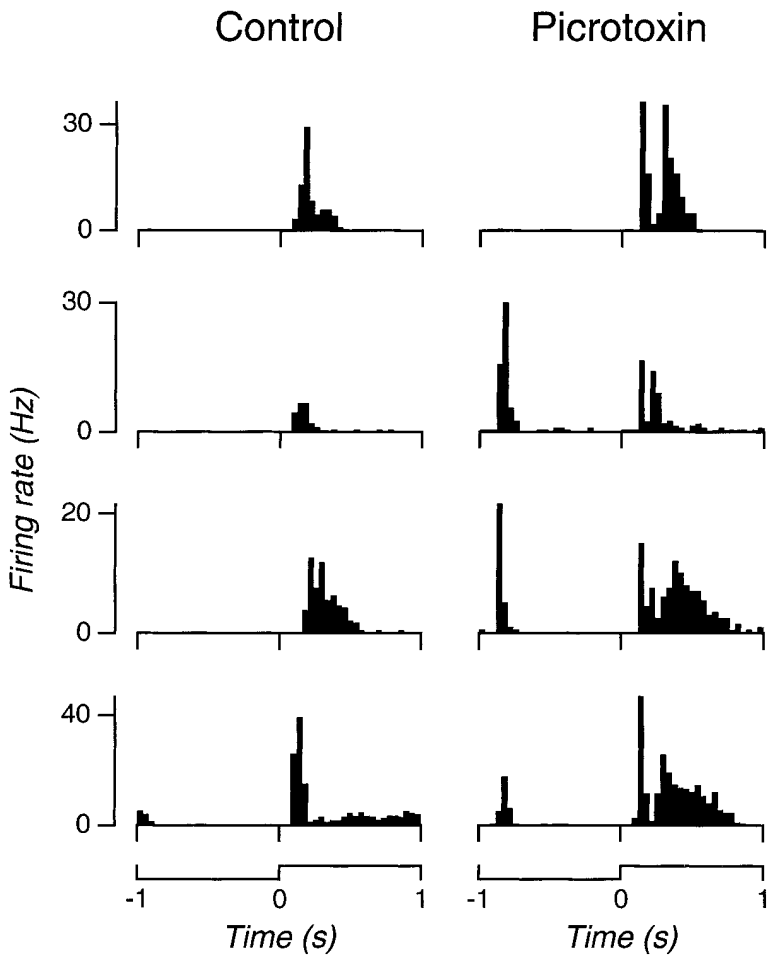


Figure 9. Effects of Picrotoxin on Responses of ON Ganglion Cells

ON ganglion cell responses to a 0.5 Hz uniform flash, presented as in Figure 3. Each pair of adjacent plots shows the response of the same ganglion cell before and during application of 100 μM picrotoxin. Each PSTH was accumulated over at least 50 repeats.

Ablation of Amacrine Cells during the Recording Session

For in vitro ablation of amacrine cells during the recording session, the tissue was bathed for 8 min in 20 μl Ringer's solution containing 1.5 mg/ml FDG, 35 μg/ml AEC, and 2.5% DMSO, then transferred to the electrode array and dark adapted for 30 min. Ganglion cell responses were recorded under flashes from the LED. To trigger photoablation, the retina was illuminated for 3.5 min using the fluorescein excitation beam of an Olympus IMT2 microscope, focused on the ganglion cell layer with a 10×, 0.3 NA objective. The retina was then dark adapted again for 30 min to allow photosensitivity to return, and responses to the same stimulus were measured at 15–30 min intervals for 1–4 hr. Success of the ablation was confirmed by the brown photoconversion product of AEC. Controls were nontransgenic retinae treated identically. The intense LED stimulus was used to compensate for the loss of photoreceptor pigment during photoablation. Note, however, that the absolute firing rates of many cells decreased immediately following the ablation procedure (Figure 5A) in experimental retinae and controls, presumably as a result of pigment bleaching and light adaptation.

Comparing Ganglion Cell Responses to Uniform and Center-Only Illumination

Using the computer monitor, a circular flashing spot was presented on a uniform background. The spot flashed on (10 mW/m^2) and off (0 mW/m^2) at 0.5 Hz, with the background held at the same mean intensity (5 mW/m^2). For each cell examined, the receptive field center was located by varying the location and the size of the flashing spot to produce the maximal ON discharge, as determined by ear with an audio monitor. The cell was then presented with spots of increasing radius, beginning with one limited to the receptive field center and increasing in size by a factor of two until the spot covered the entire piece of retina. Note that this last condition is identical to that used in all tests of response duration involving V-cell ablation. PSTHs were accumulated over 10 flashes at each spot size.

Immunohistochemistry

Retinae were fixed in 4% paraformaldehyde, stained with Xgal overnight (Cepko, 1989), then washed three times in 10 mM PBS. The tissue was then cryoprotected in 30% sucrose–70% PBS. For whole-mount staining, the retinae were transferred to glass coverslips and frozen and thawed twice on dry ice. They were then incubated overnight at 4°C in blocking serum (Vectastain ABC kit, Vector Labs) with 0.3% Triton-X-100, followed by incubation with primary antibody for 48 hr at dilutions of 1:750 for the GAT-1 antibody (Chemicon), 1:500 for the ChAT antibody (Chemicon), and 1:40 for the VIP antibody (gift of Seymour Reichlin and Elio Raviola). This was followed by three several-hour PBS washes, treatment with a Vectastain biotinylated secondary antibody, three washes, treatment with the Vectastain avidin–biotin horseradish peroxidase complex (ABC), three washes, and reaction with 3,3′-diaminobenzidine (DAB) and H_2O_2 (Vector Labs). For cryostat sections, retinae were cut vertically in sections 7–10 μm thick. Sections were then fixed to gelatin-coated slides with 4% paraformaldehyde for 5 min and washed several times. The antibody staining was performed as described above, except that the incubations with the blocking serum, the antibodies, and the ABC complex were 1 hr each, and the washes were 10 min each. Negative control retinae received the same treatment, excluding the primary antibody.

Acknowledgments

We thank Malcolm Low and Richard Goodman for the generous gift of their transgenic mouse line and Seymour Reichlin and Heinz Wässle for the use of antibodies. We are also grateful to John Assad and Ralph Nelson for helpful discussions and to David Warland, Elio Raviola, Shigang He, Dave Smith, and Peter Latham for their generous technical assistance and advice. We thank Denis Baylor, Michael Berry, John Dowling, Richard Masland, Pam Reinagel, and Stelios Smirnakis for comments on the manuscript. This work was supported by an NRSA to S. N. and grants from the Whitaker Foundation and the Pew Scholars Program to M. M.

Received October 21, 1996; revised March 4, 1997.

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