Mechanisms Underlying Developmental Changes in the Firing Patterns of ON and OFF Retinal Ganglion Cells during Refinement of their Central Projections

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Patterned neuronal activity is implicated in the refinement of connectivity during development. Calcium-imaging studies of the immature ferret visual system demonstrated previously that functionally separate ON and OFF retinal ganglion cells (RGCs) develop distinct temporal patterns of spontaneous activity as their axonal projections undergo refinement. OFF RGCs become spontaneously more active compared with ON cells, resulting in a decrease in synchronous activity between these cell types. This change in ON and OFF activity patterns is suitable for driving the activity-dependent refinement of their axonal projections. Here, we used whole-cell and perforated-patch recording techniques to elucidate the mechanisms that underlie the developmental alteration in the ON and OFF RGC activity patterns. First, we show that before the refinement period, ON and OFF RGCs have similar spike patterns; however, during the period of segregation, OFF RGCs demonstrate

significantly higher spike rates relative to ON cells. With increasing age, OFF cells require less depolarization to reach their action potential threshold and fire more spikes in response to current injection compared with ON cells. In addition, spontaneous postsynaptic currents and potentials are greater in magnitude in OFF cells than ON cells. In contrast, before axonal refinement, there are no differences in the intrinsic excitability or synaptic drive onto ON and OFF cells. Together, our results show that developmental changes in ON and OFF RGC excitability and in the strength of their synaptic drives act together to reshape the spike patterns of these cells in a manner appropriate for the refinement of their connectivity.

Key words: developing retina; activity-dependent segregation; ferret visual system; spike patterns; action potential threshold; spontaneous activity; ON-center ganglion cells; OFF-center ganglion cells

In many parts of the developing CNS, the early patterns of connectivity are refined by processes that rely on action potential activity (Goodman and Shatz, 1993; Katz and Shatz, 1996). Temporal cues relayed by the activity patterns of presynaptic cells are thought to be key to the refinement process (for review, see Bi and Poo, 2001; van Ooyen, 2001). In the ferret visual system, the axonal projections of functionally distinct ON-center and OFF-center retinal ganglion cells (RGCs) innervate distinct sublaminae within their central target, the dorsal lateral geniculate nucleus (dLGN) (Stryker and Zahs, 1983). ON and OFF sublamination occurs before eye opening but requires neuronal activity (Hahm et al., 1991; Cramer and Sur, 1997) that is generated spontaneously by the retina (Wong, 1999).

We observed previously that ON and OFF RGCs alter their activity patterns during development (Wong and Oakley, 1996). Before the period when ON and OFF retinal projections segregate, ON and OFF RGCs periodically undergo synaptically driven rhythmic bursting activity that is synchronized between neighboring cells (Feller et al., 1996; Wong and Oakley, 1996; Wong, 1999). Calcium-imaging studies showed that during the period of ON–OFF segregation, periodic elevations in intracellular calcium levels occurred much more frequently in OFF cells

compared with ON cells. This difference in mean activity levels, together with a decrease in synchronous activity between the two cell types, can drive the ON and OFF segregation process under a Hebbian model of synaptic competition (Lee and Wong, 1999). Because patterned activity in the RGCs is potentially important for the refinement of retinogeniculate circuitry (Goodman and Shatz, 1993; Crair, 1999; Wong, 1999), we sought here to determine the mechanisms that underlie the developmental change in the activity patterns of the ON and OFF RGCs.

First, we demonstrate that the difference in calcium activity patterns between maturing ON and OFF RGCs reflects differences in spiking activity. This is important, because temporal information relevant for the segregation process is likely to be encoded in the spike patterns. We then considered whether OFF cells may fire action potentials more readily compared with ON cells for a given excitatory input, which would result in a relatively greater mean firing rate in OFF cells. Next, because the circuitry of the inner retina matures during the ON–OFF segregation period (Sernagor et al., 2001), we examined whether differences in synaptic drive contribute to a higher firing rate in OFF cells compared with ON cells. It is possible that OFF RGCs become more active compared with ON RGCs at the older ages because OFF RGCs receive a greater net excitatory drive. This could be attributable to OFF cells receiving a relatively stronger excitatory drive and/or weaker inhibitory drive. To investigate these possibilities, we compared the physiological properties and synaptic drives of morphologically classified ON and OFF cells across two age groups, before and during the period of ON–OFF axonal

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segregation, using whole-cell and perforated-patch recording techniques.

MATERIALS AND METHODS

Tissue preparation

Ferrets aged between postnatal day 7 (P7) and P24 were killed with 5% halothane followed by decapitation. The eyes were enucleated and the retinas were separated from the pigment epithelium in cold oxygenated Ames medium (Sigma, St. Louis, MO) that was buffered with 20 mM HEPES and titrated to a pH of 7.4 with 5 M NaOH. Each retina was spread flat onto a glass slide, divided into two to four pieces, and mounted ganglion cell side up on black filter paper (HABP045; Millipore, Bedford, MA). The peripheral region (outer third) of the retina was positioned over a hole in the filter paper, through which cells could be viewed and selected for recording.

Whole-cell and perforated-patch recordings

For all recordings, the extracellular solution was Ames medium (Ames and Nesbitt, 1981). The retinas were maintained in oxygenated media at room temperature until they were transferred to a recording chamber. Perforated-patch and whole-cell recordings were performed at 32°C (Wong and Oakley, 1996; Wong et al., 2000a).

Perforated-patch recordings were performed using the cation-selective ionophore gramicidin D (Sigma) to gain electrical access to the cell while maintaining the endogenous intracellular anionic composition. This configuration also minimizes the washout of intracellular contents over time, and thus permits recording of sodium action potentials over a much longer time, compared with what is possible with whole-cell recordings. The pipette solution for gramicidin perforated-patch recordings consisted of 119 mM KCl and gramicidin D (92.3 μ g/ml) dissolved in methanol.

Whole-cell voltage-clamp recordings were performed to measure excitatory and inhibitory synaptic currents. In the whole-cell configuration, we could isolate the excitatory synaptic inputs at a holding potential of -55 mV, the reversal potential for chloride. Inhibitory synaptic currents mediated predominantly by chloride channels were isolated at a holding potential of 0 mV, the reversal potential for the excitatory currents (Wong et al., 2000a). For these whole-cell voltage-clamp recordings, the pipette solution contained (in mM): 133 cesium gluconate, 10 TEA-chloride, 0.4 MgCl_2 , 10 NaCl, and 7 Na-HEPES. Cesium and TEA were included to block voltage-gated potassium channels in the recorded cell and to facilitate the recording of synaptic inputs at positive holding potentials.

Whole-cell, current-clamp recordings were performed to measure spontaneous EPSPs. For these recordings, the composition of the recording (pipette) solution was similar to the endogenous ion concentrations. It consisted of (in mM): 90 K-gluconate, 30 KCl, 5 EGTA, and 10 Na-HEPES. For these experiments, lidocaine *N*-ethyl bromide (QX-314) (pipette concentration of 5 mM) was also included in this pipette solution to block sodium-dependent action potentials while recording the membrane potential. The QX-314 current-clamp recordings enabled us to measure the amplitudes of the membrane depolarizations underlying the spike activity.

Drugs were applied using a gravity flow superfusion system. Unless otherwise indicated, all drugs were obtained from Sigma. The standard superfusate that was used to block excitatory and inhibitory inputs consisted of (in μ M): 100 D-AP-5 (Precision Biochemicals, Vancouver, British Columbia, Canada), 20 1,2,3,4-tetrahydro-6-nitro-2,3-dioxobenzof[*q*]quinoxaline-7-sulfonamide (NBQX), 100 dihydro- β -erythroidin hydrobromide (DH β E), 150 picrotoxin, and 1 strychnine.

Ganglion cells were viewed using a water immersion objective [63 $\times$, numerical aperture (N.A.) 0.9 Zeiss, Thornwood, NY] and a Nomarski optics filter on a Zeiss Axioskop fixed-stage microscope. To gain access to the cell bodies of ganglion cells, glial endfeet above a selected cell were cleared with a patch pipette. The debris was removed by suction through a patch pipette with a broken tip. All recordings were obtained using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Electrodes were pulled from borosilicate glass (TW150F-4; World Precision Instruments, Sarasota, FL) using a Flaming/Brown P-87 puller (Sutter Instruments, Novato, CA) and had resistances of 5–10 M Ω . Membrane potentials were corrected for junction potentials (-5.3 mV for gramicidin solutions, -14.5 mV for whole-cell voltage-clamp solutions, and -12.0 mV for whole-cell current-clamp solutions). Series resistance was not compensated. Data were filtered at 2 kHz with the eighth pole Bessel

low-pass filter on the amplifier and digitized and stored on a Pentium computer using a Labmaster data acquisition board (Scientific Solutions, Solon, OH). Spontaneous events were digitized and recorded on a digital audio tape recorder (PS75; Dagan, Minneapolis, MN). The sampling rate was 11 kHz. Patchit software (White Perch Software, Belmont, MA) was used to generate voltage and current commands.

Analysis

Voltage recordings. We defined long duration, sustained EPSPs as “sustained EPSPs.” For voltage recordings in the presence of QX-314, sustained EPSPs were identified by searching for local maxima using MiniAnalysis (Synaptosoft, Leonia, NJ). An event was defined as a sustained EPSP if its amplitude was at least 8 mV positive to the baseline membrane potential and its area was >160 mV $\cdot$ sec. The baseline potential was obtained by averaging a 1 sec segment before the local maxima. When the algorithm occasionally selected two candidate sustained EPSPs that overlapped, the longest duration event was classified as the sustained EPSP. The duration of a sustained EPSP was defined as follows. Its onset was the time at which the voltage reached 0.5% of the peak amplitude and its offset was the time at which voltage returned to 1% of the peak amplitude. Sustained EPSPs had durations of much longer than 1 sec. Shorter duration EPSPs (called “transient EPSPs”) occurred between the sustained EPSPs. An event occurring between sustained EPSPs was defined as a transient EPSP if its amplitude was ≥ 4 mV positive to the baseline membrane potential. Transient EPSPs had durations of <0.5 sec.

Current recordings. To identify and quantify sustained EPSCs and sustained IPSCs, we performed the following analysis in MatLab (MathWorks, Natick, MA). Candidate events were selected based on their threshold amplitude above (IPSCs) or below (EPSCs) the baseline membrane current. The onset and termination of each event were defined as the times when the current deviated from and returned to baseline. Short-duration currents (called transient EPSCs and “transient IPSCs”), which occurred during the period between the sustained EPSCs and sustained IPSCs, respectively, were defined by MiniAnalysis as those events with amplitudes greater than four times the root mean square of baseline noise.

Morphological identification of ON and OFF RGCs

We focused our study on β RGCs that we had assessed previously by calcium imaging (Wong and Oakley, 1996). After recording, cells were classified morphologically as either ON or OFF β RGCs by intracellular filling with Lucifer yellow. The patch pipettes contained 0.01–0.02% Lucifer yellow. The dye diffused into the cell during whole-cell recording or after breaking through the cell membrane after perforated-patch recording.

In the mature retina, physiologically classified OFF RGCs have dendritic arbors that stratify exclusively in sublamina a, whereas ON RGCs stratify in sublamina b of the inner plexiform layer (IPL). Typically, sublamina b comprises the inner three-fifths of the IPL and sublamina a comprises the outer two-fifths of the IPL (Nelson et al., 1978). Stratified β RGCs are present in the ferret retina for both age groups included in our study (Wong and Oakley, 1996; Wang et al., 2001). Figure 1a is an example of a pair of P21 ferret β cells that were dye-filled with sulforhodamine 101 (1%; Molecular Probes, Eugene, OR); their dendritic arbors were clearly separated, ramifying in either sublamina a (OFF) or b (ON). The well-established relationship between dendritic morphology and physiology holds true for the developing retina. In the ferret, when light responses can be first measured at P21, RGCs with dendrites stratifying in sublamina b (ON) increase their firing rates at light onset, whereas those cells stratifying in sublamina a (OFF) increased their firing rates at light offset (Wang et al., 2001). The relationship between dendritic stratification and ON or OFF responses also extends to RGCs which, during development, have yet to confine their dendrites to one sublamina (Bodnarenko and Chalupa, 1993; Bodnarenko et al., 1995; Lohmann and Wong, 2001). Unstratified RGCs have both ON- and OFF-center responses (Wang et al., 2001). It should be noted that before the third postnatal week in ferrets, photoreceptors are not yet mature, and thus our classification of ON and OFF cells for the early age group (P7–P10) is based entirely on morphological criteria (Wong and Oakley, 1996; Bodnarenko et al., 1999).

To determine the stratification level of the recorded and dye-filled cells, we measured the depth of the dendritic arbor relative to the inner and outer borders of the IPL. These borders were determined under transmitted light illumination and Nomarski optics that allowed us to

Figure 1. Dendritic stratification patterns of morphologically classified ON and OFF β RGCs. *a*, Top, Three-dimensional reconstruction of the arbors of morphologically defined ON and OFF β cells of a P21 ferret (see Materials and Methods). The stack of images was obtained at 0.25 μ m step sizes from the base of the cell body using a two photon microscope (Zeiss 40 $\times$ oil, N.A. 1.3) (1024M; Bio-Rad, Richmond, CA). Each image was acquired with 800 nm of excitation. Bottom, A 90° rotation of the image stack demonstrating the dendritic arbors of the ON and OFF RGCs ramifying in distinct sublaminae of the IPL. Sublaminae *a* (s_a) and *b* (s_b) are indicated. *b*, *c*, Epifluorescence image of Lucifer yellow labeling of a P21 β cell after whole-cell recording. Focusing from the base of the cell body (0 μ m) at the IPL/ganglion cell layer border, the dendritic arbor of this cell was observed to terminate 6 μ m into the IPL. We would classify this cell as an ON cell because its dendrites stratified within sublamina *b*. Scale bars, 10 μ m.

assess the z-depth of the ganglion cell layer/IPL border and the inner nuclear layer/IPL border. Both the ganglion cell and inner nuclear layers are easily distinguished under Nomarski optics because they comprise only cell bodies. Simultaneous viewing of the Nomarski image and the fluorescent processes allowed us to define the location of the dendritic terminals relative to the IPL borders. Z-depth was obtained in micrometers from the z-adjustment controls (1 μ m steps) on the microscope stage (Fig. 1*b,c*). Typically, in mid-peripheral retinas, the IPL was 20–22 μ m thick by the end of the first postnatal week and 23–25 μ m in the adult.

In our recorded population, most cells had dendritic arbors confined to one sublamina; a smaller percentage had dendrites in both sublaminae (18% at P7–P10, $n = 38$; 1% at P18–P24, $n = 84$). Only cells that stratified completely in the ON or OFF sublamina were analyzed in detail, because we wanted to directly compare the activity patterns of ON and OFF cells across ages. A total of 19 ON cells and 12 OFF cells were recorded and analyzed for P7–P10, and 50 ON cells and 33 OFF cells were recorded and analyzed for P18–P24.

Statistics

All summaries are means $\pm$ 1 SEM. Comparisons of the percentage of events above action potential threshold were made with a z test in SigmaStat (SPSS, Chicago, IL). All other statistical comparisons were made with Mann–Whitney rank–sum tests in SigmaStat. Statistical significance was $p < 0.05$.

RESULTS

Patterns of spontaneous spike activity in developing ON and OFF RGCs

Our voltage recordings demonstrate that temporal patterns of spontaneous action potentials in ON and OFF RGCs alter with development. Figure 2*a* illustrates examples of the spike patterns of an ON and an OFF RGC from the early age group (P7–P10), recorded in perforated-patch mode. Both cell types generated periodic trains of action potentials (Fig. 2*a*). The trains of action potentials rode on sustained EPSPs.

Figure 2. Spike patterns of developing ON and OFF RGCs. Perforated-patch recordings showing patterns of spontaneous spiking in ON and OFF RGCs at the younger (*a*) and older (*b*, *c*) ages are shown. The bottom traces in *b* and *c* are filtered versions of the raw data, revealing underlying sustained EPSPs of relatively long duration (asterisks in *b*) (see Materials and Methods). *c*, An example of a sustained EPSP from each of the two P21 cells in *b* shown at an expanded time scale. Note that there is little or no spiking before and after a sustained EPSP in the ON cell, but there is considerable spiking during these periods in the OFF cell.

The spike patterns of ON and OFF RGCs became distinct during the period when their axonal terminals segregate in the dLGN (P18–P24) (Fig. 2*b*). ON cells continued to rhythmically exhibit sustained EPSPs, with few or no action potentials between these events (Fig. 2*b,c*). In contrast, although OFF cells also periodically demonstrated sustained EPSPs and trains of action potentials, spiking occurred very frequently between the sustained events (Fig. 2*b,c*). The intervals between the sustained EPSPs for ON and OFF cells were not significantly different (Table 1).

We applied a mathematical filter (moving average function with a sampling window of 0.5 sec) to the raw voltage traces recorded from P18–P24 cells to define the duration of the sustained EPSPs without interference from the action potentials. Figure 2*b,c* shows that the filtering closely follows the shape and duration of the underlying sustained EPSPs. This enabled us to compare the frequency of spiking for ON and OFF cells during

Table 1. Summary of time intervals between sustained events measured under different recording conditions for ON and OFF cells

	P7–P10		P18–P24	
	ON	OFF	ON	OFF
Sustained EPSPs (filtered)			66.2 ± 11.8 (14)	40.1 ± 9.1 (7)
Sustained EPSPs (in QX-314)			59.4 ± 7.3 (11)	69.4 ± 9.5 (9)
Sustained IPSCs	87.7 ± 14.2 (7)	79.2 ± 10.2 (4)	54.4 ± 6.0 (5)	61.6 ± 7.8 (8)
Sustained EPSCs	85.0 ± 10.7 (7)	90.6 ± 3.6 (4)	55.7 ± 4.3 (5)	68.7 ± 10.3 (8)

Numbers are means ± SEM in seconds. The numbers in parentheses indicate the number of cells. For all conditions, the intervals between events in ON cells were not significantly different compared with intervals between events in OFF cells ($p > 0.05$).

the sustained EPSPs and during the periods between these events. For sustained EPSPs that elicited action potentials, the average firing rate during the sustained EPSPs for ON cells was 1.70 ± 0.25 Hz, compared with 4.12 ± 0.64 Hz for OFF cells. During the intervals between the sustained EPSPs, ON cells seldom spiked (average firing rate of 0.01 ± 0.01 Hz), in contrast to OFF cells (0.52 ± 0.44 Hz). Overall, the mean spike rates were higher for OFF (1.17 ± 0.44 Hz) compared with ON (0.22 ± 0.03 Hz) cells. All differences in firing rates between ON and OFF cells were significant ($p < 0.02$ for 16 ON cells and 6 OFF cells).

Action potential thresholds

We considered whether OFF cells could fire spikes more often compared with ON cells, because the OFF cells require less depolarization to reach action potential threshold. To address this possibility, we recorded the firing patterns of ON and OFF RGCs in response to a series of injected current steps. We compared the responses of ON and OFF cells from the two age groups, before (P7–P10) and during (P18–P24) ON–OFF segregation in the dLGN. Recordings were performed in perforated-patch mode in a cocktail of neurotransmitter receptor antagonists (in μM : 100 D-AP-5, 20 NBQX, 100 DH β E, 150 picrotoxin, and 1 strychnine) to block the inputs that contribute to spontaneous activity in the RGCs (Wong et al., 2000a).

We first compared the responses of ON and OFF RGCs to current injections at P18–P24. Resting potentials for ON and OFF cells in Ames medium were -76.6 ± 1.6 mV ($n = 24$ cells) and -72.0 ± 3.0 mV ($n = 12$ cells), respectively, which were not significantly different. For a fixed-amplitude current injection, OFF cells fired action potentials more readily (Fig. 3a). Figure 3b quantifies the firing rate during the duration of each current step for the recorded population. For a large range of current amplitudes (10–50 pA), ON cells fired significantly fewer spikes than OFF cells. Next, we calculated the magnitude of depolarization required for ON and OFF RGCs to reach their spike thresholds. The action potential threshold was defined as the potential at which the voltage maximally accelerated on the first evoked action potential (Fig. 3c). ON RGCs required 25 ± 3 mV, whereas OFF RGCs required only 15 ± 2 mV to reach their respective action potential thresholds from rest (Fig. 3d). Thus, OFF RGCs rest closer to their action potential thresholds compared with ON RGCs, and relatively larger depolarizations are required to evoke spiking in ON cells compared with OFF cells.

In contrast, at P7–P10, the amount of depolarization required to reach action potential threshold was similar for ON and OFF RGCs (Fig. 3d). Interestingly, with age the magnitude of depolarization required to reach spike threshold decreases in OFF cells but not in ON cells (Fig. 3d).

We also asked whether the difference in ON and OFF spike

thresholds occurs at rest, when synaptic transmission is present. By recording and applying current steps in between the sustained EPSPs, we found that the magnitude of depolarization required to reach spike threshold still differed significantly between ON and OFF cells in the presence of tonic synaptic transmission (data not shown). Thus, a difference in spike threshold between ON and OFF cells is likely to contribute to their disparate spike rates.

Spontaneous postsynaptic currents

To compare the properties of the inputs onto developing ON and OFF RGCs, we obtained whole-cell voltage-clamp records of their spontaneous synaptic currents. To observe EPSCs, RGCs were voltage clamped at -55 mV, the chloride reversal potential set by the pipette and external solutions (see Materials and Methods). IPSCs were measured at a holding voltage of 0 mV, the reversal potential determined previously for glutamatergic- and cholinergic-mediated excitatory currents (Wong et al., 2000a).

At P7–P10, we found that long-duration, periodically occurring EPSCs (sustained EPSCs) and IPSCs (sustained IPSCs) occurred in both ON and OFF RGCs (Fig. 4a). The sustained postsynaptic currents (PSCs) resemble the compound EPSCs described previously in immature ferret ganglion cells (Feller et al., 1996) and rabbit ganglion cells (Zhou, 1998; Zhou and Zhao, 2000). Both the sustained EPSCs and sustained IPSCs occurred at a frequency similar to that observed for sustained EPSPs recorded in current-clamp mode (Table 1). The average charge transfer (area under the current traces) of the sustained PSCs was not significantly different for ON and OFF RGCs (Fig. 5).

In the older age group (P18–P24), periodic sustained EPSCs and sustained IPSCs were also observed in ON and OFF RGCs (Fig. 4b). The average charge transfer for both types of sustained events was greater in OFF compared with ON cells. This suggests that for a set driving force, both excitatory and inhibitory conductance changes were larger in OFF cells compared with ON cells (Fig. 5). However, the sustained EPSCs and IPSCs occurred at similar frequencies for ON and OFF cells (Table 1). In addition to the sustained EPSCs and IPSCs, we also noted that in this older age group there were frequently occurring smaller amplitude and short-duration EPSCs and IPSCs in the ON and OFF RGCs (Fig. 4c). These transient EPSCs occurred at 1.1 ± 0.6 Hz ($n = 4$ cells) in ON cells and at 6.4 ± 0.8 Hz ($n = 6$ cells) for OFF cells. The transient EPSC frequency was significantly different between the ON and OFF cells ($p = 0.01$). Transient IPSCs occurred at 1.8 ± 0.8 Hz for ON cells ($n = 5$ cells) and 7.6 ± 2.6 Hz for OFF cells ($n = 6$). Although the transient IPSCs tended to occur at higher frequencies in the OFF cells, this was not significantly different from that of ON cells ($p = 0.08$).

Figure 3. Action potential thresholds of ON and OFF RGCs. Perforated-patch recordings from RGCs are shown. Voltage responses were recorded in a cocktail of receptor antagonists to block synaptic input. *a*, Depolarizing current steps, 800 msec in duration, were injected into the P18–P24 cells at the times indicated by the elevated horizontal lines below the traces. *b*, Mean firing rate $\pm$ SEM for the population of P18–P24 RGCs over the range of amplitudes of injected current. For each current amplitude, asterisks indicate a significant difference ($p < 0.05$) in the responses of ON compared with OFF RGCs. *c*, Example showing the voltage at which a spike is generated (the action potential threshold). *d*, The average $\pm$ SEM of the magnitude of depolarization [action potential threshold (Threshold) minus resting potential (V_{rest})] required for ON (white bars) and OFF (black bars) cells to reach their respective action potential thresholds. The depolarization required to reach action potential threshold was significantly greater for ON compared with OFF RGCs for the P18–P24 age group ($p = 0.02$) but not for the P7–P10 group ($p = 0.42$). There was a significant decrease in the magnitude of depolarization required to reach threshold with increasing age in OFF RGCs ($p = 0.005$) but not in ON RGCs ($p = 0.66$). Recordings were from 10 ON cells and 6 OFF cells from P7–P10 and 8 ON cells and 7 OFF cells from P18–P24. Asterisks indicate statistically significant differences.

Figure 4. Spontaneous currents in developing ON and OFF RGCs. Voltage-clamp recordings in whole-cell configuration of ON and OFF RGCs from the younger (*a*) and older (*b*) age groups are shown. Cells were voltage clamped at 0 mV (top trace of each pair, EPSCs) or at -55 mV (bottom trace of each pair, IPSCs). The scale bars in *b* apply to both holding potentials and to all cells shown in *a* and *b*. In both age groups and for ON and OFF cells, periodic, sustained EPSCs and IPSCs were observed. *c*, Transient EPSCs and IPSCs in older RGCs (indicated by asterisks).

Spontaneous postsynaptic potentials

We subsequently asked whether OFF cells may spike more frequently compared with ON cells because they receive a stronger net excitatory drive. Although the amplitude of the PSCs differed between ON and OFF cells at P18–P24, the current recordings alone do not reveal how the underlying excitatory and inhibitory conductances act together to affect the membrane potential. In part, this is because the spontaneous excitatory and inhibitory events cannot be easily observed and measured simultaneously during voltage-clamp recording. The perforated-patch, current-clamp experiments provided a reasonable estimate of the relative amplitudes of the EPSPs of ON and OFF cells (Fig. 2*b*), but this required low-pass filtering of high-frequency components to remove the spikes from the raw traces. To directly measure the net depolarization resulting from the excitatory and inhibitory drives, we performed whole-cell voltage recordings in the presence of QX-314, which diffuses into the recorded cell and blocks its sodium action potentials.

Both ON and OFF RGCs showed sustained, periodic EPSPs resembling the depolarization pattern underlying the high-frequency spiking shown in Figure 2 (compare Fig. 6 with Fig. 2*b*). The average duration of the sustained EPSPs was significantly longer for the ON cells than for the OFF cells ($p = 0.015$). For the ON cells the mean duration was 15.3 ± 1.1 sec (105 EPSPs from 11 cells), whereas for the OFF cells the mean duration was 11.6 ± 0.5 sec (92 EPSPs from 9 cells). The sustained EPSPs of ON and OFF RGCs, observed in the presence of QX-314, occurred at similar

Figure 5. Quantification of sustained EPSCs and IPSCs of ON and OFF RGCs. Bars indicate the average $\pm$ SEM of the absolute value of the average charge transferred per sustained EPSC or sustained IPSC. At P7–P10, there were no significant differences in charge transfer per sustained EPSC or sustained IPSC between ON and OFF cells ($p = 0.53$ for IPSCs and EPSCs; 7 ON and 4 OFF RGCs). However, at P18–P24, OFF cells had larger charge transfer per sustained EPSC or sustained IPSC compared with ON cells ($p = 0.003$ for sustained IPSCs; $p = 0.002$ for sustained EPSCs; 5 ON and 8 OFF RGCs). Asterisks indicate statistically significant differences.

frequencies (Table 1). In both ON and OFF cells, transient EPSPs were also detected between the sustained EPSPs. The durations of the transient EPSPs were not different between the ON and OFF cells ($p > 0.99$). For ON cells the duration of the transient EPSPs was 201 ± 12 msec (917 EPSPs from 11 cells); for the OFF cells the duration was 197 ± 6 msec (1814 EPSPs from 9 cells).

To quantify and compare the EPSP properties of the ON and OFF cells, we plotted the areas under the EPSPs against their peak amplitudes (Fig. 7*a*). Two clusters were readily identified in these plots by area, corroborating the presence of two major types of EPSPs. The peak amplitudes of both the sustained and transient EPSPs were on average greater in OFF cells compared with ON cells (Fig. 7*b,c*) despite overlap in their respective distributions. To assess whether the peak amplitudes of the EPSPs were sufficient to cause spiking, we compared their amplitudes with the average depolarization required to reach action potential threshold in ON and OFF cells (Fig. 7*a*, vertical lines). Events with amplitudes that are greater than the depolarization required to reach action potential threshold (represented by symbols to the right of the appropriate bars in Fig. 7*a*) are likely to elicit spikes. Figure 7*a* shows that whereas 33.3% of the sustained EPSPs in ON cells can lead to spiking, only 0.8% of the transient EPSPs exceeded the spike threshold for these cells. Thus, for ON cells, spiking may be limited because many of the EPSPs do not bring the cell above spike threshold. For OFF cells, the majority (92.5%) of the sustained EPSPs would be expected to lead to spiking. In contrast to ON cells, a larger percentage (5.3%) of the transient EPSPs in OFF cells are sufficient to cause spiking.

DISCUSSION

Developmental changes in the activity patterns of RGCs

Our results show that ON and OFF RGCs develop different rates of spontaneous action potential activity during the period when their axonal projections segregate in the dLGN. We have also

Figure 6. Spontaneous EPSPs in maturing ON and OFF RGCs. Voltage changes were recorded in whole-cell configuration with QX-314 in the pipette to block action potentials. *a*, Examples of spontaneous EPSPs in an ON and an OFF RGC. The durations of the sustained EPSPs are indicated by the horizontal bars (see Materials and Methods). *b*, Examples of sustained EPSPs from the cells in *a* shown in an expanded time scale. Asterisks indicate the transient EPSPs between the sustained EPSPs.

shown that RGCs are capable of generating higher spike rates with maturation, in agreement with previous studies (Skaliora et al., 1993; Rothe et al., 1999). With increasing age, OFF β RGCs spike more frequently compared with ON β RGCs. This age-related change in the relative spike rates of ON and OFF RGCs is also observed for α cells, another major RGC class, as shown by calcium-imaging studies (for review, see Wong, 1997) and extracellular recordings from cell pairs (Lee and Wong, 1999). Thus, the divergence in spike patterns observed in β RGCs is not unique to this cell class but rather is a developmental feature characteristic of ON and OFF subtypes of RGCs.

Although ON and OFF β RGCs develop different spike patterns with maturation, we did observe similarities in their activity patterns even in the older age group. At P18–P24, both ON and OFF β cells exhibit sustained spontaneous EPSPs. Two observations suggest that the periodically occurring sustained EPSPs represent synchronized activity among neighboring cells. First, the time intervals between sustained EPSPs are similar for ON and OFF RGCs across age groups (Table 1). These intervals, ~ 1 – 2 min, are similar to the intervals between spontaneous retinal waves that are known to coordinate the activity of neighboring RGCs (for review, see Wong, 1999). Second, simultaneous calcium imaging and voltage recordings show that sustained EPSPs occur when many cells in the field of view exhibit an elevation in intracellular calcium levels (K. Myhr and R. Wong, unpublished data). Thus, it is likely that the sustained EPSPs of ON and OFF RGCs at P18–P24 are driven by the lateral network underlying retinal waves (Wong, 1999). Transient EPSPs are also observed in P18–P24 ON and OFF β RGCs. These EPSPs may instead reflect the emergence of the photoreceptor–bipolar pathway that develops after the second postnatal week (Miller et al., 1999; Wang et al., 2001). Indeed, OFF RGCs may demonstrate a higher frequency of spiking compared with ON RGCs because OFF bipolar cells are relatively more depolarized in the dark (Werblin and Dowling, 1969).

Figure 7. Quantitative comparison of EPSPs in maturing ON and OFF RGCs. *a*, For each EPSP that was >4 pA above baseline, its area versus its amplitude is plotted for the recorded populations of ON cells (11 cells, open symbols) and OFF cells (9 cells, filled symbols). The EPSPs forming the upper cluster (squares) consist of sustained EPSPs; the lower cluster (diamonds) consists of transient EPSPs that occur between the sustained EPSPs. The vertical lines mark the average and SEM of the amplitude of depolarization required to reach the respective action potential thresholds (AP Thresh) of ON (dotted vertical line) and OFF (thick solid vertical line) RGCs (Fig. 4). *b*, *c*, Cumulative amplitude plots for sustained EPSPs (*b*) and transient EPSPs (*c*) for ON cells (dotted lines) and OFF cells (solid lines). Both types of EPSPs are significantly smaller in amplitude in ON cells compared with OFF cells ($p = 0.019$ for sustained EPSPs; $p < 0.001$ for transient EPSPs).

Mechanisms underlying the differences in ON and OFF spike patterns

We observed a fundamental difference in the intrinsic ability of ON and OFF cells to fire action potentials during ON–OFF segregation in the dLGN. OFF cells rest closer to their action potential threshold compared with ON cells and respond with a higher firing rate for a fixed-amplitude current input. What could underlie these differences in the excitability of ON and OFF cells? One possibility is that with maturation OFF cells express a greater density of sodium channels than ON cells. Although many studies have shown that RGC sodium channel expression increases with development (Skaliora et al., 1993; Schmid and Guenther, 1996; Rothe et al., 1999), comparisons between ON and OFF cells have not been undertaken. It is also possible that ON and OFF cells differ in the kinetics of their sodium channel inactivation rather than in their channel density. Wang et al. (1997) showed that the speed of recovery from inactivation of the sodium current in rat RGCs increased with age and with increasing excitability of these cells. Although such kinetics can vary with RGC class in the adult retina (Kaneda and Kaneko, 1991), possible differences between ON and OFF RGCs have yet to be examined systematically.

Differences in potassium channel expression could also contribute to differences in spike rates and patterns (Hille, 1992). In the more mature ferret retina (P30–P45), relatively sustained firing can be converted to burst-like activity when calcium-activated potassium conductances sensitive to apamin are pharmacologically blocked (Wang et al., 1998). Interestingly, in that study, a subset of α and β RGCs was found to be insensitive to apamin. The morphological identity of this subset is as yet unknown, but it may exclusively comprise ON or OFF RGCs. It may also be possible that immature ON and OFF β RGCs have similar densities and types of non-calcium-dependent voltage-gated potassium channels that change with development (Skaliora et al., 1995; Rothe et al., 1999). Future experiments determining the developmental profiles of sodium and potassium channel expression and kinetics in identified ON and OFF cells would be highly informative with regard to their contribution to the distinct firing patterns of these cell types.

In addition to differences in intrinsic excitability, we found that the synaptic drives onto P18–P24 ON and OFF RGCs are also different. In this age group, recordings in the presence of QX-314 showed that the amplitudes of spontaneous sustained EPSPs are on average larger for OFF cells compared with ON cells. The relatively larger sustained EPSPs in OFF cells may result from a

stronger excitatory drive. Consistent with this, our current recordings indicated that sustained EPSCs were significantly larger in OFF cells compared with ON cells. These currents were shown previously to be glutamate-mediated for the older age group, suggesting a contribution from bipolar cell inputs (Wong et al., 2000a).

In the older age group, sustained IPSCs were also significantly greater in amplitude in OFF cells compared with ON cells: The Cl^- conductance changes of P18–P24 OFF cells were larger than those of ON cells. In conducting our experiments in whole-cell mode, we abolished any endogenous differences in $[\text{Cl}^-]_i$ in the P18–P24 ON and OFF cells. Such a difference in endogenous $[\text{Cl}^-]_i$ between ON and OFF cells may exist, as has been shown for neighboring neurons in the developing spinal cord (Rohrbough and Spitzer, 1996). An uncertainty in endogenous chloride driving forces such as this would make it difficult to determine in which direction the chloride-mediated inputs contributed to the voltage responses. More likely, however, the larger IPSCs in OFF cells may simply reflect larger GABA-mediated synaptic inputs. We could not determine the timing of excitatory and inhibitory inputs, which interact to give rise to the sustained EPSPs. However, because the sustained EPSPs were larger in OFF cells compared with ON cells, the net excitatory drive must be greater in OFF cells.

Together our observations suggest that the intrinsic excitability and synaptic drive onto ON and OFF cells alter with age and act together to shape the firing patterns of these cells. An intriguing question to pursue is how these mechanisms are developmentally regulated to produce spike patterns of ON and OFF RGCs that are appropriate for the activity-dependent refinement of their axonal projections.

Developmental implications of diverging ON and OFF RGC activity

The dendrites of RGCs also undergo reorganization and stratify into one of the two major sublaminae within the IPL during the period of ON–OFF axonal segregation (Bodnarenko et al., 1999; Lohmann and Wong, 2001). The dendritic stratification process may also depend on spontaneous activity. Before vision, blockade of glutamatergic transmission in the developing retina perturbs the dendritic stratification patterns of ON and OFF RGCs (Bodnarenko and Chalupa, 1993; Bodnarenko et al., 1995). Moreover, neurotransmission regulates dendritic remodeling in both ON and OFF RGCs during the period of synaptogenesis and dendritic stratification (Wong and Wong, 2000; Wong et al.,

2000b). Unstratified RGCs in the older age group receive both ON and OFF bipolar inputs (Wang et al., 2001). Thus, dendritic remodeling in RGCs and the subsequent loss of one type of bipolar input may involve competition between ON and OFF bipolar cells and possibly a process driven by differences in their spontaneous and light-evoked activity patterns. In the present study, we did not compare the spike patterns of unstratified RGCs with those of well-stratified RGCs because we encountered only one unstratified cell at P18–P24. It would be interesting in the future to determine the spontaneous spike patterns of these unstratified RGCs to elucidate the physiological properties of the “transiently present” bipolar inputs and to determine how these inputs are eliminated as the ON and OFF pathways segregate within the retina.

Although it is clear that with age ON and OFF RGCs are contacted by synaptic inputs with different properties, these RGC subtypes also appear to be intrinsically different. We demonstrated recently that the dendrites of ON and OFF RGCs form dendrodendritic contacts only between cells of the same subtype, thus implying that these two populations of cells possess distinct cell-to-cell recognition cues (Lohmann and Wong, 2001). Our physiological recordings here lend additional support to the existence of molecular differences between ON and OFF RGCs; with development, we found that ON and OFF RGCs exhibit different intrinsic membrane properties.

In summary, our results demonstrate how the spike patterns of ON and OFF RGCs are developmentally regulated to convey information relevant for the activity-dependent refinement of their connections with dLGN neurons at each stage of development. The challenge remains for us to ascertain how in the visual system and in other neural networks patterned spike activity and molecular differences act in concert to set up precise connectivity patterns with maturation.

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