Research Article

Synchronous Bursts of Action Potentials in Ganglion Cells of the Developing Mammalian Retina

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The development of orderly connections in the mammalian visual system depends on action potentials in the optic nerve fibers, even before the retina receives visual input. In particular, it has been suggested that correlated firing of retinal ganglion cells in the same eye directs the segregation of their synaptic terminals into eye-specific layers within the lateral geniculate nucleus. Such correlations in electrical activity were found by simultaneous recording of the extracellular action potentials of up to 100 ganglion cells in the isolated retina of the newborn ferret and the fetal cat. These neurons fired spikes in nearly synchronous bursts lasting a few seconds and separated by 1 to 2 minutes of silence. Individual bursts consisted of a wave of excitation, several hundred micrometers wide, sweeping across the retina at about 100 micrometers per second. These concerted firing patterns have the appropriate spatial and temporal properties to guide the refinement of connections between the retina and the lateral geniculate nucleus.

NEURAL CONNECTIONS IN THE MAMMALIAN VISUAL SYSTEM are established in two phases; early diffuse connections are later refined, resulting in the precise adult pattern (*1, 2*). Initially, axons of the retinal ganglion cells grow into their target, the lateral geniculate nucleus (LGN), and extend branches and establish synaptic contacts with neurons throughout this structure (*3*). Later in development, cells in the LGN project axons to the primary visual cortex where their terminal arbors form diffuse synaptic connections throughout layer 4 (*4*). Thus, an initial pattern of connections is established, both in the LGN and in primary visual cortex, in which the regions driven by the two eyes overlap considerably. These early events do not require visual experience or electrical activity in the participating neurons (*5–7*). Subsequently, this coarse network is refined; the terminal arbors achieve their adult branching patterns by selective retraction and directed growth. These changes cause the afferent terminals driven by the two eyes to segregate into the eye-specific layers of the LGN and into the ocular dominance patches in layer 4 of the visual cortex. In the process, the initially rough topographic map between retina, LGN, and cortex is thought to acquire more precision (*8*).

The refinement of connections during the secondary phase of development depends on neural signals in the retina and can be grossly perturbed by abnormal electrical activity. For example, when action potentials in retinal ganglion cells are blocked by intracranial infusion of tetrodotoxin during early development, the formation of eye-specific layers in the LGN is prevented (*7, 9*). During the refinement of connections to visual cortex, injections of tetrodotoxin into the eye prevent the segregation of LGN axons into cortical ocular dominance columns (*6*). By eliminating electrical activity in the retina with tetrodotoxin and providing exogenous stimulation to the optic nerves, Stryker and Strickland (*10*) showed that correlated firing in fibers from the same eye was sufficient to produce ocular dominance columns in primary visual cortex. These observations can be understood if one postulates that synaptic terminals driven by one eye compete with those driven by the other eye for cells in the target region (*1, 2, 11*). It has been suggested that this competition is sustained by presynaptic electrical activity, with simultaneously active synapses onto a common target cell reinforcing each other at the expense of inactive synapses (*12*). Thus, target cells would eventually develop monocular input if fibers deriving from each eye fired synchronously, but out of synchrony with the fibers from the other eye.

So far, there has been no direct evidence for eye-specific correlations in activity during development of the visual system. Visual input itself might correlate the firing of neighboring retinal ganglion cells as soon as the retina is fully functional, and the animal opens its eyes. However, the terminals of retinal ganglion cells segregate into eye-specific layers before the photoreceptor cells are functional; this occurs in the cat between embryonic day 47 (E47) and E60 (*13*) and in the ferret between postnatal day 3 (P3) and P20 (*14*). Thus, the activity-dependent formation of eye-specific layers in the LGN requires (i) electrical signals that are generated endogenously within the retina, (ii) functional connections between the retinal ganglion cells and geniculate neurons, and (iii) a cellular mechanism that controls the activity-dependent modification of synapses in the LGN.

The synapses between optic nerve fibers and geniculate neurons are already functional during the period of rearrangement in the LGN (*15*). At the corresponding developmental stage, retinal ganglion cells of the fetal rat spontaneously generate action potentials (*16*), with individual neurons firing in rhythmic bursts. For such endogenous signals to be useful in guiding synaptic rearrangements, they must be correlated among neighboring ganglion cells. To search for such relations, we studied the simultaneous electrical

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activity of many ganglion cells in the retinae of newborn ferrets and fetal cats, using a multielectrode array (*17*).

Multielectrode recording. Retinas were isolated from adult or neonatal (P5 to P21) ferrets and two cats (E52 and P1; a cat at E52 is at the same developmental stage as a ferret at P10), deeply anesthetized with Nembutal (20 mg per kilogram of body weight). The ferret was chosen for most of these experiments, because the time course and pattern of development of its retinogeniculate pathway is essentially identical to that of the cat, with the advantage that the layers in the LGN of the ferret begin to form after birth (*14*). In the youngest of our experimental animals (P5), segregation of retinal ganglion cell axons has just begun, and it is largely complete by P21. Adult animals were dark-adapted for 30 min, and the retina was dissected in dim red light or under infrared illumination. All procedures were performed in Ringer medium (124 mM NaCl, 5 mM KCl, 1.15 mM KH_2PO_4, 1.15 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM D-glucose) or Ames medium (Sigma, buffered with 25 mM $NaHCO_3$ or 20 mM Hepes). Solutions buffered with bicarbonate were aerated with a mixture of 95 percent O_2 and 5 percent CO_2, others were aerated with O_2. For electrical recording, a piece of retina about 3 mm in diameter was placed with the ganglion cell layer facing down in a dish whose bottom contained a flat array of extracellular electrodes (*18*). This array consisted of 61 patches of platinum black, each about 10 μm in diameter, spaced regularly 70 μm apart over an area 0.5 mm in diameter (Fig. 1A). Each electrode was connected to a conducting lead that carried the electrical signal to the periphery of the dish. The retina was held in position with a fine nylon mesh so that the ganglion cell layer remained close to the bottom of the dish (Fig. 1B). The preparation was mounted on the stage of an inverted microscope, heated (33° to 37°C), and superfused with oxygenated medium. Under these conditions, the electrodes registered extracellular action potentials up to several hundred microvolts in amplitude (Fig. 2). For recording responses to light, the retina was stimulated with flashes of uniform intensity, which were projected onto the photoreceptor layer from above.

The voltage signals from the electrodes were amplified and filtered to give a bandwidth of 20 Hz to 2 kHz. Action potentials were detected and measured by 61 independent signal processors (Fig. 3A). Each signal processor detected a voltage spike when the signal crossed a preset threshold value. It then measured the spike's time of occurrence, its peak amplitude, and its width (at the threshold voltage), and transferred these data to a computer. The amplitude and width parameters describe the waveform of the action potential and were used to discriminate signals from different neurons recorded on the same electrode. When plotted in a two-dimensional histogram of peak amplitude and width, spikes recorded on an individual electrode fell into distinct clusters (Fig. 3B). Each of these clusters represents a stereotyped waveform of the action potential, and our analysis assumed that each cluster originated from a separate neuron. The spatial location of a neuron was taken to be the location of the respective recording electrode. When the action potentials of a single neuron were observed on two or more neighboring electrodes (Fig. 2) (*19*), we estimated its position as the average of the locations of these electrodes, weighted by the corresponding spike amplitudes. In the course of a strong burst, the action potential of a given cell sometimes varied in amplitude. Such an extended cluster in the amplitude-width plane could be recognized as deriving from a single cell because (i) all spikes in the cluster had the same estimated spatial location, (ii) their amplitude generally decreased monotonically with time throughout the burst, and (iii) spikes were separated by an absolute refractory period (the obligatory pause of a few milliseconds after a spike during which a neuron is unable to generate another action potential). On occasion, two neurons recorded on the same channel generated spikes of very similar waveform, and thus appeared as a single unit in the above analysis. Such a mixed spike train could be recognized by the absence of a clear refractory period. The results in Figs. 4, 6, and 7 are derived from spike trains of single neurons, as determined by this criterion, whereas Fig. 5 includes spikes from some clusters in peak-width space that may have contributions from more than one cell. We also recorded some action potentials from passing fibers. These were clearly identified by their brief triphasic waveform and their sequential occurrence along a string of electrodes at a velocity characteristic of axonal conduction in the retina, about 0.5 m/s. These spikes were not analyzed, since the location of the cell body was unknown.

It is essential to determine which retinal neurons generate these action potentials. Spikes recorded from axonal fibers clearly originate in retinal ganglion cells. To determine whether amacrine cells might also contribute to the electrode array recordings, we used single, glass capillary electrodes to record from individual neurons in the ganglion cell layer before filling them with a fluorescent dye (2.5 percent carboxyfluorescein). In all cases (four cells in cat E50-P5, six cells in ferret P10-P23), neurons that generated action potentials were identified as retinal ganglion cells by their dendritic morphology and the presence of an axon. This result suggests that most of the signals that we analyzed were derived from retinal ganglion cells.

Synchronized firing in the retina. Spontaneous action potentials

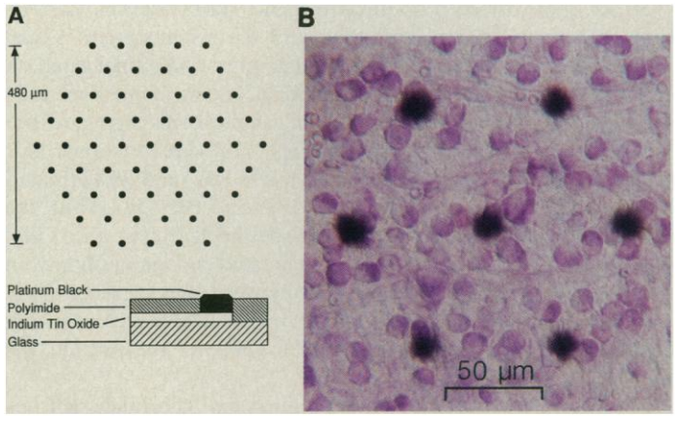

Fig. 1. (**A**) (Top) Plan diagram of the electrode array. Each dot represents a platinum black recording site. The connecting leads are not shown. (Bottom) Section through the electrode array, not to scale. Conducting traces of indium tin oxide carry the signal from the electrodes to connections at the periphery of the dish. A layer of polyimide serves as insulator. (**B**) The ganglion cell layer of a P18 ferret retina stained with toluidine blue and placed on the electrode array. The electrodes appear as black spots just out of the focal plane. Their conducting leads are transparent and thus not visible here.

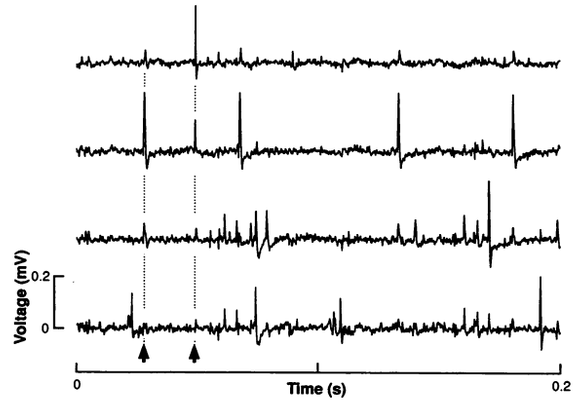

Fig. 2. Voltage signals recorded from the retina of a P21 ferret on four neighboring sites of the electrode array. Negative voltage is plotted upward. Arrows indicate action potentials that appear on several electrodes.

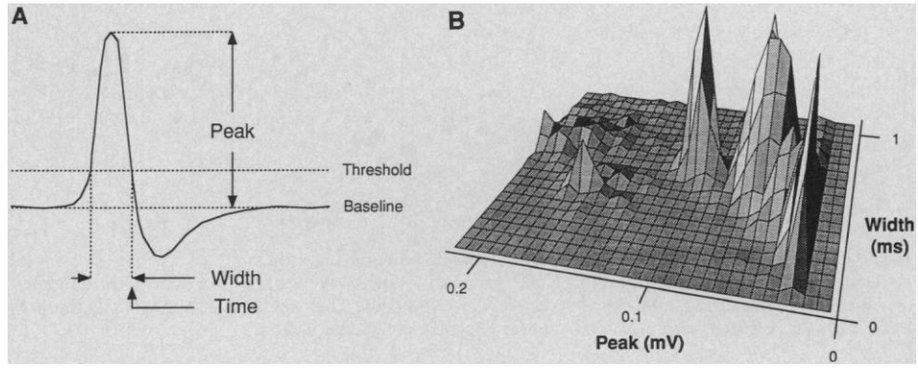

Fig. 3. (**A**) Signal processing during data acquisition. Only events in which the signal voltage crosses a preset threshold are recorded. After such a threshold crossing, the signal processor measures peak amplitude, width, and time of arrival of the pulse. (**B**) A two-dimensional histogram of peak amplitude and width for 2350 voltage spikes recorded on an individual electrode during the experiment of Fig. 2. The number of spikes in each amplitude and width bin is plotted upward. In this instance, one can distinguish contributions from at least four neurons. The sharp peak near the origin of the histogram is due to electrical noise.

were observed in all of the retinas studied. In retinas from fetal and neonatal animals, but not in those from adult ferrets, this spike activity occurred in rhythmic bursts that were strikingly synchronized across all of the cells monitored by the electrode array (Fig. 4A). The retina was silent except for brief intervals lasting a few seconds during which many neurons became active simultaneously. These bursts of action potentials were spaced 1 to 2 min apart. The retina generated this activity spontaneously, in the absence of mature photoreceptors, and without external stimuli.

When examined on an expanded time scale, these bursts were not strictly simultaneous for all cells (Fig. 4B). Different neurons began firing at times that were staggered over several seconds. To determine whether the timing of a burst might be systematically related to the location of the cell, we mapped the activity on the electrode array. In Fig. 5, we show the firing rate for all the recorded neurons in the form of successive 0.5-s snapshots over a 4-s period covering the burst shown in Fig. 4B. Each cell is drawn at its estimated location as a dot whose area is proportional to the cell's firing rate. During this particular recording period, cells near the bottom edge of the electrode array began to fire first. Then cells in the middle of the array joined in, and these were followed by neurons near the top edge. The overall pattern of firing during the burst resembled a wave of neural activity sweeping across the patch of retina. Most of the bursts in Fig. 4A had a wavelike appearance, but at least six different directions of propagation were observed in this 20-min interval. The retina maintained this pattern of activity for 3 hours.

We have observed such rhythmic correlated bursting in the retinas from each of nine ferrets between the ages of P5 and P21 and in two cat retinas at E52 and P1. The duration of individual bursts of action potentials usually ranged from 2 to 4 s, although bursts of up to 8 s were observed. The quiet periods lasted from 40 to 100 s. In seven experiments, where the number of cells recorded was large enough to distinguish the wavelike nature of the bursts, the wave front propagated at a velocity of 80 to 140 μm/s. Within our present sample, we found no clear dependence of these parameters on the age of the animal. However, the retina of an adult ferret generated no such pattern of synchronized firing. In the dark, neurons in the ganglion cell layer of the adult retina fired at steady rates ranging from 1 to 20 Hz. In addition, these cells showed "on" and "off" responses when the retina was stimulated with diffuse flashes of light.

Further studies were aimed at understanding the nature of these bursts of activity in the developing retina, and how they propagate across the network. All spiking activity ceased when 1 μM tetrodotoxin was added to the bath, indicating that the action potentials were generated by voltage-sensitive Na^+ channels rather than Ca^{2+} channels. Omission of $CaCl_2$ from the bathing solution (*20*) drastically altered the pattern of activity, both the duration of each burst and the length of the period between bursts being shortened by about a factor of 10. Nevertheless, firing remained nearly synchronous across many cells (Fig. 6). While Ca^{2+} clearly plays a role in modulating the synchronized activity, this observation suggests that the signal propagating excitation across the retina does not rely exclusively on Ca^{2+}-mediated synaptic transmitter release. To determine whether the active cells might communicate by Ca^{2+}-independent synapses (*21*) or gap junctions, we searched for temporal correlations between their firing patterns on the millisecond time scale. The correlation function between the spike trains of two nearby neurons in the retina of a P5 ferret (Fig. 7A) shows a smooth peak, a few seconds wide, centered near zero delay time. This implies that the two cells tended to fire within a few seconds of each other, as expected, since they participated in the same synchronous bursts. The correlation function reveals no interesting features near zero delay time. For example, we would expect to see sharp peaks close to the origin if action potentials from one neuron synaptically excited the other neuron and thus increased its probability of firing. Because none of the two-cell correlations that we computed from these experiments showed any such peaks on a millisecond time scale, direct synaptic interactions between these neurons must be very weak or rare. We conclude that the signal that synchronizes bursting among many cells probably does not spread via fast synapses.

Examination of these correlations on a longer time scale revealed a marked dependence on the intercellular distance. The shape of the curve in Fig. 7A, from a pair of cells about 40 μm apart, was typical of short intercellular distances. By contrast, the correlation function computed for two cells located approximately 370 μm apart was quite different (Fig. 7B). This pair of cells tended to fire out of synchrony because of the finite speed with which bursts of activity

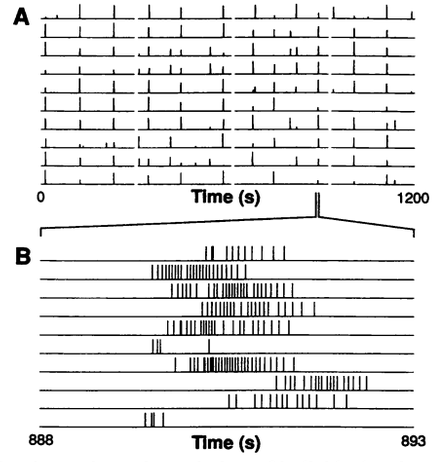

Fig. 4. (**A**) Firing rate of ten neurons recorded in the retina of a P5 ferret, plotted over a 20-min period. For each cell, spikes were binned in 1-s intervals, and the resulting histogram was normalized to the largest bin. The corresponding maximal firing rates were, from top to bottom 25, 19, 26, 19, 17, 13, 24, 21, 19, and 16 Hz. Recording was interrupted briefly every 300 s; this is indicated by the broken baseline. (**B**) Portion of the record in (A) from 888 s to 893 s, expanded along the time axis to show timing of individual action potentials.

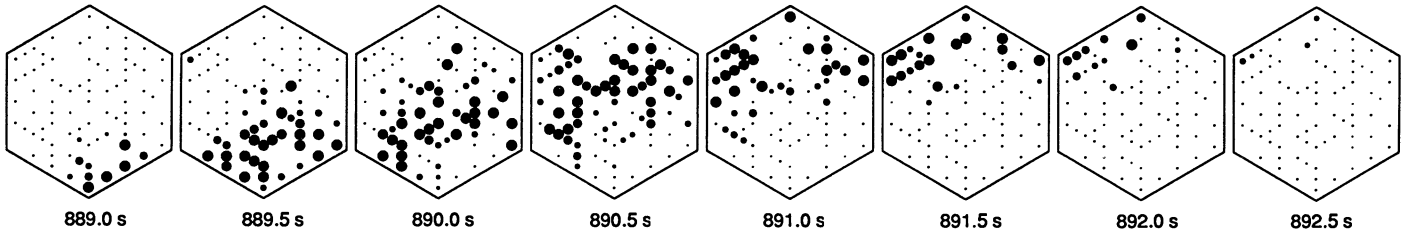

Fig. 5. Time course of spike activity over the electrode array during a burst covering the time interval from 889 s to 893 s of Fig. 4. Successive frames show the averaged firing rate over successive 0.5-s intervals. Each of 82 neurons is represented with a small dot at its approximate spatial location over the electrode array. The dot area for an electrically active cell is increased proportionally to the averaged firing rate of the neuron during the respective 0.5-s interval.

spread across the retina. The correlation function shows several peaks, corresponding to different delays between bursts from the two neurons, as the waves of excitation propagated in different directions during the period of observation.

A role for retinal activity in visual development. By recording simultaneously from many cells, we have shown that neurons within the ganglion cell layer of the developing ferret and cat retina display locally synchronized spontaneous activity long before photoreceptors mature. Several lines of evidence suggest that the synchronized bursts of action potentials represent normal physiological activity, and are not merely caused by our recording methods. (i) The patterns of rhythmic activity occurred in every preparation of fetal or neonatal retina. Under identical conditions, the adult retina showed a maintained firing rate characteristic of mammalian retinal ganglion cells monitored in vivo (*22*) as well as light responses. (ii) These bursts of action potentials persisted for many hours, and the firing rates of retinal ganglion cells during the bursts are well within the physiological range recorded from ganglion cells in vivo. Furthermore, this electrical activity was dependent on physiological environmental conditions; we generally observed no or very irregular firing when the temperature was below 33°C or above 39°C, or when the oxygenation was inadequate. (iii) Similar neuronal activity has been observed in ganglion cells of other developing retinas. Masland (*23*) recorded bursts of action potentials from the developing rabbit retina in an eyecup preparation. These patterns had a periodicity and a burst duration very similar to those that we observed. Galli and Maffei (*16*) reported in vivo measurements of spontaneous activity in retinal ganglion cells of the fetal rat. Individual neurons fired action potentials in brief bursts lasting a few seconds separated by silent intervals of tens of seconds. Occasional paired recordings showed that nearby cells were correlated in their discharge patterns (*24*). (iv) Periodic bursting activity has also been seen in vivo in the superior colliculus (*25*) and the lateral geniculate (*26*) of immature rabbits. These signals are thought to be of retinal origin because eye removal or pressure blockade can abolish them. Taken together, these observations indicate that the bursting activity that we have seen in isolated retinas also occurs in vivo.

Our observations provide a few clues regarding the mechanism that generates and sustains the traveling waves of activity. In the ferret retina at P5 to P10 and the cat retina at E52, the ganglion and amacrine cells are likely to be synaptically connected, while the bipolar cells are still immature and probably lack functional connections to ganglion and amacrine cells (*27*). Thus, the observed firing patterns are probably generated entirely within the ganglion and amacrine cell layers of the retina. Since a given region of the retina sustains waves traveling in various directions, the direction of propagation is not determined by any oriented connectivity in the underlying neuronal network. In a simple scheme, one might treat the retina as a uniform excitable medium (*28*) in which ganglion cells interact locally in a mutually excitatory manner through non-directional connections. By this positive feedback, any small focus of activity could rapidly grow in size and intensity. If, in addition, the network becomes refractory after a certain period of firing, the excitation will spread outward into the quiescent region as a radial wave. In the refractory region behind the wave, the threshold for excitation might then decrease gradually until the network became sensitive to another local disturbance leading to the next radial wave. Triggering events might occur anywhere within the network without the need for a dedicated pacemaker cell, since we observe the wave of excitation traveling in many different directions. Furthermore, this scheme could explain the uniform duration of the quiet intervals, which would be determined by the period of refractoriness rather than the interval between random triggering events.

The wave of activity crosses the distance between two cell bodies in about 0.2 s. This time sets limits on the nature of the postulated

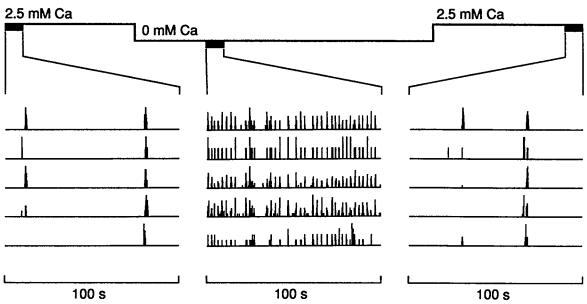

Fig. 6. Effects of lowering extracellular Ca^{2+} ion concentration. The firing rate of five neurons from the retina of a P5 ferret (same experiment as Fig. 5) is plotted over three 100-s periods: before, during, and after perfusion with medium of low Ca^{2+} concentration. For each cell, spikes were binned in 0.25-s intervals and the histogram normalized to the maximal value observed during the respective 100-s period.

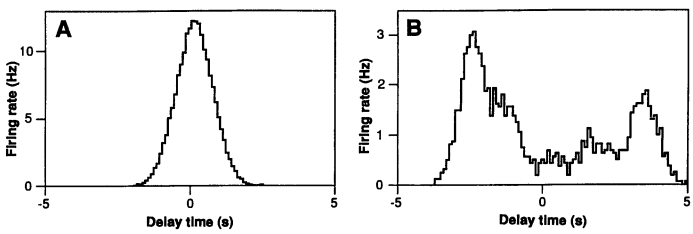

Fig. 7. (**A**) Correlation between the spike trains of two neurons from the retina of a P5 ferret (same experiment as Fig. 5), located about 40 μm apart. The spike rate of one cell is plotted as a function of time relative to the firing of an action potential by the other cell. This spike rate was averaged over the 20-minute period shown in Fig. 5, including 12 major bursts. (**B**) Correlation between the spike trains of two different neurons in the same retina as (A), located about 370 μm apart.

excitatory connections. In particular, they are unlikely to be formed by electrical gap junctions or by fast chemical synapses with integration times of the order of milliseconds. In fact, the signal propagates slowly enough to involve diffusion of an excitatory substance through the extracellular space: in 0.2 s, a small molecule can diffuse about 10 μm, which is comparable to the distance between the cell bodies of neighboring ganglion cells. The excitatory substance could be a neurotransmitter or potassium ions released during an action potential.

Whatever mechanism produces it, the spontaneous activity in the retina contains cues that might be used to instruct synaptic refinement during development of the LGN. Retinal ganglion cells lying close to each other appear to be correlated in their activity; they generate action potentials in synchronized bursts and thus are most likely to fire within a few seconds of each other. The activity of cells in one eye is not expected to correlate with that of cells in the other eye because the two retinas do not communicate or receive a common efferent signal during development. Because the bursts are short compared to the quiet periods that separate them, two cells from opposite eyes will rarely fire together. More precisely, for short times (of the order of the burst length, 2 to 4 s) the correlation function between the spike trains of two cells in the same retina is higher than that of two cells from opposite eyes by the ratio of quiet interval to burst length, roughly a factor of 20. Thus, target neurons within the LGN could reliably identify axonal terminals that tend to fire within the same 2 to 4 s as deriving from the same eye. The time course of the activity patterns places constraints on the mechanism that modifies synaptic connections in response to correlated firing; this process cannot require correlation times longer than a few seconds, the observed duration of a burst. In fact, temporal correlation between the firing of two terminals should be ineffective at times greater than the burst duration, in order to optimize the distinction between the two eyes. The stimulation experiments of Stryker and Strickland (*10*) suggest that a process satisfying these criteria can drive ocular segregation in cat primary visual cortex during postnatal life.

A further cue of potential importance in development is provided by the spatial properties of the observed firing patterns. When excitation spreads across the retina in waves, nearby ganglion cells (Fig. 7A) are more closely synchronized in their firing than distant cells (Fig. 7B). Under a mechanism of correlation-based synaptic competition, the axons of nearby retinal ganglion cells would be more likely to form synapses onto the same target cell in the LGN. This would further refine the topographic map between retina and LGN. Little is known about the detailed development of this ordered projection, but we would predict that any process driven by the observed waves of retinal activity could only refine the retinotopic map to a grain comparable to the width of the wave, about 200 to 400 μm as measured on the retina.

Although electrical activity in afferent fibers plays an important role in the development of the LGN, other forces must also be at work. For example, the hypothesized activity-driven competition between synapses on a common target cell can explain the appearance of monocular neurons in the target tissue, but not its large-scale organization into regions of cells with the same ocular dominance. In models of cortical development, the structure of ocular dominance stripes has been explained by excitatory interactions among cortical neurons on the scale of the width of a stripe (*2*). However, unlike the visual cortex, the adult LGN shows the same sequence of ocular dominance layers in every animal. This stereotypic organization might be the result of a systematic bias toward one or the other eye before the onset of activity-driven synaptic rearrangement. In fact, axons from the contralateral eye innervate the LGN about 3 days before those from the ipsilateral eye (*13*), leading to a dominance of the contralateral eye in the layer furthest from the optic tract that is maintained throughout development.

Our observations of concerted neural activity in the immature retina provide a mechanistic basis for the notion that electrical signals guide the synaptic development of the mammalian visual system long before the onset of vision. They place quantitative constraints on the nature of the cellular process that couples the action potentials in the afferent fibers to synaptic change in the target region. At this stage in development, the retina sends instructive rather than sensory signals to the brain. Their message is contained entirely in the relative timing of spikes on different optic nerve axons. Thus the immature retina encodes information in massively parallel form, distributed over many nerve fibers. It still remains to be seen how these signals control the chain of events leading to synaptic specificity in the visual system.

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