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In vivo evidence that retinal bipolar cells generate spikes modulated by light

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Retinal bipolar cells have been assumed to generate purely graded responses to light. To test this idea we imaged the presynaptic calcium transient in live zebrafish. We found that ON, OFF, transient and sustained bipolar cells are all capable of generating fast 'all-or-none' calcium transients modulated by visual stimulation.

In the retina, the visual signal begins as graded changes in membrane potential in photoreceptors, but the output is then delivered by ganglion cells in the form of spikes. Where does the conversion from analog to digital signaling begin? To reach the inner retina, the electrical signal travels through bipolar cells, and it has long been assumed that these are purely graded neurons[1]. Although calcium spikes have been recorded in one class of depolarizing bipolar cell in slices of goldfish retina, it is not clear whether this is an artifact of the preparation[2,3]. To investigate whether retinal bipolar cells can encode light with spikes, we imaged presynaptic calcium in live zebrafish expressing the reporter protein SyGCaMP2 (refs. 4,5 and **Supplementary Methods**).

SyGCaMP2 detected fast calcium transients in bipolar cell terminals, both spontaneously in the dark (**Fig. 1**) and modulated by light (**Fig. 2**). The amplitudes and kinetics of these calcium transients were markedly constant in many terminals (**Figs. 1c,d** and **Fig. 2c–h**). The time course of calcium influx was judged from the time derivative of the calcium transient and had a width at half-maximum of 50–80 ms (**Fig. 1e**). This pulse of calcium was immediately followed by an exponential decay with a time constant (τ) of 1.2 ± 0.6 s (24 terminals). A similar time constant of calcium decay has been measured in the synaptic terminal of bipolar cells using synthetic calcium dyes responding to brief (20 ms) depolarizations[6,7]. It therefore seems likely that fast presynaptic calcium transients observed *in vivo* are generated by stereotyped and transient depolarizations. These voltage signals are likely to be calcium spikes; in bipolar cells, these can be generated by the voltage-dependent calcium channels in the synaptic terminal that control neurotransmitter release[2,3,8–11].

Fast calcium transients were detected by thresholding the derivative of the SyGCaMP2 signal (**Supplementary Algorithm** and **Supplementary Fig. 1**). In a sample of 1,008 terminals in 9 different fish, 65% generated spikes over an observation period of 60 s. To test whether presynaptic spikes in bipolar cells might be used to encode visual information, we recorded SyGCaMP2 signals in response to a full-field stimulus consisting of a light step followed by modulation at 2.5 Hz. In the terminals in which we could not detect spikes

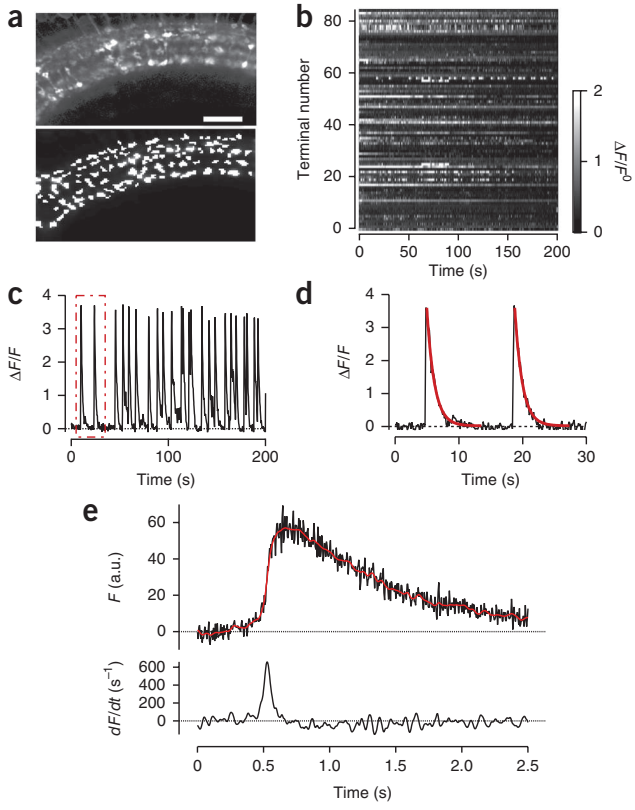

Figure 1 Imaging fast presynaptic calcium transients in bipolar cells *in vivo*. (**a**) Synaptic terminals of bipolar cells expressing SyGCaMP2 in a zebrafish (10 d post-fertilization). Regions of interest corresponding to terminals shown below. Scale bar represents 20 µm. (**b**) Raster plot showing spontaneous SyGCaMP2 signals in darkness. The sampling interval was 128 ms. (**c**) Spontaneous calcium transients in one terminal. Note the relatively fixed amplitude and time course. (**d**) Two calcium transients from **c** on an expanded timescale. An exponential fit to the first is shown in red (τ = 1.18 s) and superimposed on both spikes. From a sample of 1,008 terminals, 65% generate one or more calcium transients over a 60-s period. (**e**) A single presynaptic calcium transient sampled at 200 Hz (black) and smoothed by interpolation (red). a.u., arbitrary units. The lower trace is the derivative: the signal describing the rate of calcium influx had a width of 65 ms at half-maximum. All procedures were carried out according to the UK Animals (Scientific Procedures) Act 1986 and approved by the UK Home Office.

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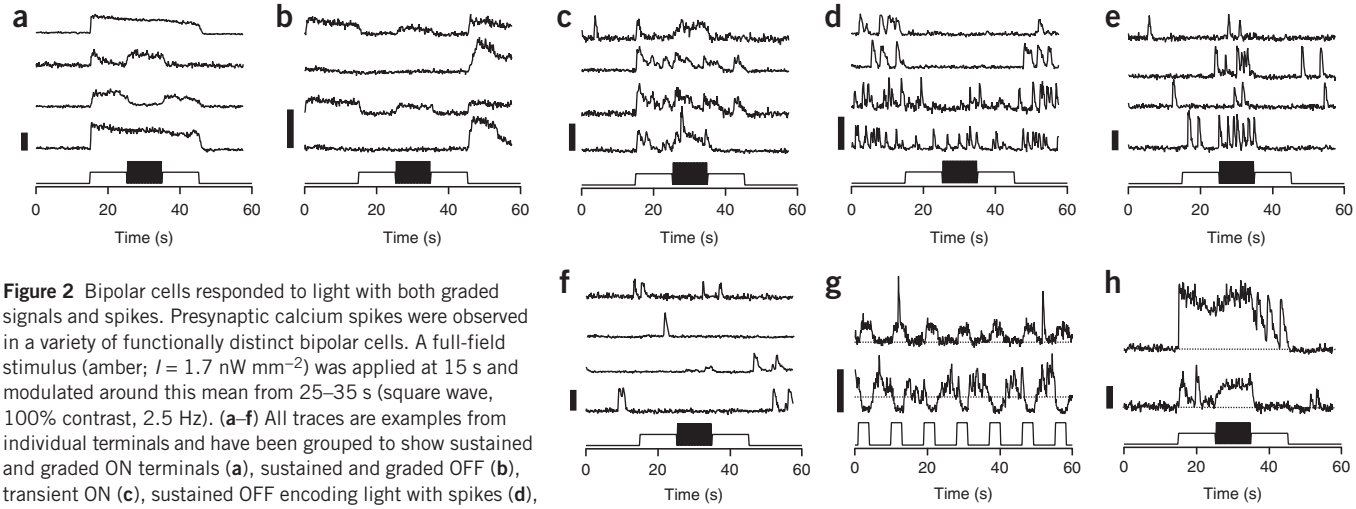

Figure 2 Bipolar cells responded to light with both graded signals and spikes. Presynaptic calcium spikes were observed in a variety of functionally distinct bipolar cells. A full-field stimulus (amber; $I = 1.7$ nW mm^{-2}) was applied at 15 s and modulated around this mean from 25–35 s (square wave, 100% contrast, 2.5 Hz). (**a**–**f**) All traces are examples from individual terminals and have been grouped to show sustained and graded ON terminals (**a**), sustained and graded OFF (**b**), transient ON (**c**), sustained OFF encoding light with spikes (**d**), transient ON terminals generating calcium spikes in response to temporal contrast (**e**), terminals generating calcium spikes at low rates, but without clear modulation by the stimulus (**f**). Scale bars represent $\Delta F/F = 2$. (**g**) Sustained ON and OFF terminals also generating spikes (upper and lower traces, respectively). (**h**) Upper trace, sustained ON terminal generating a slow response to contrast and then spikes. Lower trace, transient ON cell that spikes at light onset, but then generates a slow sustained response to contrast (maximum intensity = 1.7 nW mm^{-2}). Scale bars in **g** and **h** represent $\Delta F/F = 1$.

(35%), slow sustained changes in calcium were observed with variable kinetics and polarities, reflecting ON and OFF cells responding to the stimulus in different ways (**Fig. 2a**,**b**). The terminals that generated fast spike-like calcium transients (65%) were modulated in a variety of ways. We observed both transient and sustained ON terminals (**Fig. 2c**), sustained OFF (**Fig. 2d**) and transient ON terminals responding especially strongly to temporal contrast at 2.5 Hz (**Fig. 2e**). In other terminals, spikes were not clearly modulated by this stimulus, although we cannot rule out the possibility that other stimuli might have been effective (**Fig. 2f**). Thus, ON and OFF bipolar cells with different kinetics have the ability to generate spikes modulated by light.

It was often possible to detect small and slow changes in baseline calcium coincident with changes in spike frequency (for example, **Fig. 2c**,**d**). In some ON cells, spikes could occur in darkness when resting calcium was low, reflecting a hyperpolarized state, and in light when mean calcium levels were higher, reflecting depolarization (**Fig. 2g**). In some OFF cells, spiking occurred continuously in the dark but switched off completely in the light, coincident with a fall in baseline calcium (**Fig. 2g**). Notably, the same terminal could sometimes switch between generating sustained signals and generating spikes (**Fig. 2h**), reminiscent of the bistable membrane potential observed in isolated bipolar cells[8,10]. These results indicate that bipolar cells in the retina of zebrafish respond to visual stimulation with a combination of graded and spiking signals.

The functional design of the retina is strongly conserved across vertebrates, so it is worth investigating whether bipolar cells in other species are also capable of encoding a visual stimulus with spikes as well as graded voltage signals.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

Experiments were designed by E.D., F.E. and L.L. and performed by E.D., F.E. and L.L. Analysis was carried out by E.D., F.E. and L.L. The manuscript was written by F.E., T.B. and L.L.

COMPETING FINANCIAL INTERESTS

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