SUPPORTING ONLINE MATERIAL

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

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1. Methods and Materials

2. Algorithm for detecting spikes in SyGCaMP2 signals

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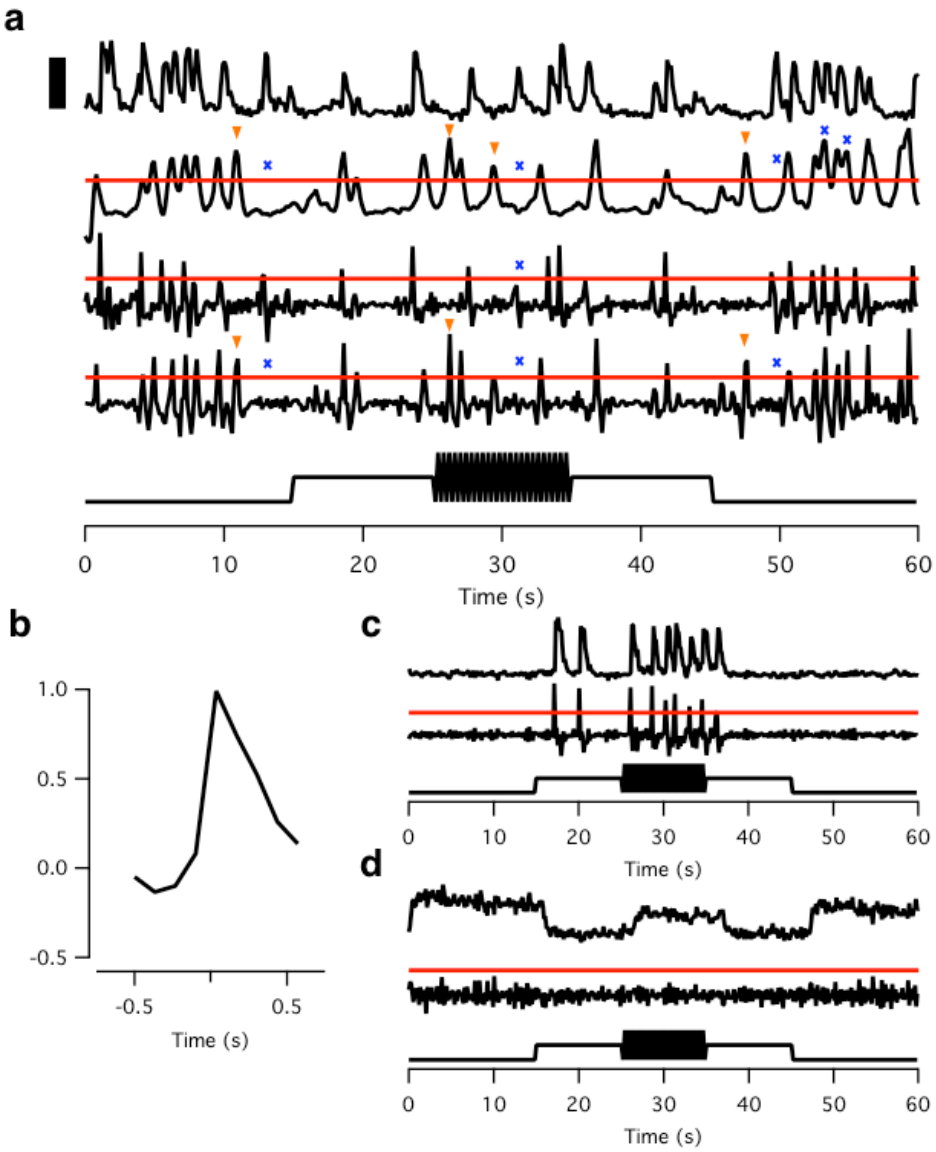

Supplementary Figure 1. Algorithm for detecting spikes in SyGCaMP2 signals

Graphical representation of the three different spike detection methods used to extract spikes from the SyGCaMP2 recordings *in vivo*. **a**. Top trace: example raw SyGCaMP2 signal. Spurious events are marked by the orange triangles and missed events by the blue crosses. The stimulus time-course is shown at the bottom of the panel. The light intensity was modulated as a square wave from 25 to 35 s (100% contrast, 2.5 Hz; maximum intensity = 1.7 nW/mm^2). Second trace: matched filtering of the raw trace with the spike template shown in **b**. The red line represents the detection threshold of 3 times the standard deviation of the whole trace. Third trace from top: first derivate of the raw trace with the same thresholding criterion. Fourth trace: matched filtering of the first derivate of the raw trace with the first derivate of the template. Threshold of three times the standard deviation (red line). Scale bar: $\Delta F/F = 1$. **b**. Spike template obtained by averaging 50 manually selected spikes over 10 different traces. **c**. First derivate detection algorithm at work with a series of spikes. **d**. First derivative detection algorithm at work with a graded response. No spikes are detected in this case.

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1. Methods and Materials

Transgenic Zebrafish

All procedures were carried out according to the UK Animals (Scientific Procedures) Act 1986 and approved by the UK Home Office. Transgenic zebrafish expressing SyGCaMP2.0 under the Ribeye-A promoter[1], were kept on a 14:10 h light:dark cycle and bred naturally. Larvae were grown in 200 μM 1-phenyl-2-thiourea (Sigma) from 28 h post-fertilisation to inhibit melanin formation[2].

Multiphoton imaging *in vivo*

Whole zebrafish larvae (9-11 dpf) were immobilised in 2.5% low melting point agarose (Biogene) on a glass coverslip. Fish were mounted with one eye pointing up towards the objective and the retina imaged using a custom-built multiphoton microscope equipped with a mode-locked Chameleon titanium–sapphire laser tuned to 915 nm (Coherent) and an Olympus LUMPlanFI 40x water immersion objective (N.A. 0.8). Emitted fluorescence was captured through both the objective and a sub-stage oil condenser, filtered through a HQ 520/60m-2P GFP emission filter (Chroma Technology) and detected by photomultiplier tubes (Hamamatsu). Scanning and image acquisition were controlled under ScanImage v.3.6 software[3]. Full field light stimuli were delivered using amber LEDs (Luxeon) projected through the objective or delivered by a light guide. Stimuli were controlled using Igor Pro v. 4.01 (WaveMetrics) and were time-locked to image acquisition. Intensity of light stimulation was I = 1.7 nW/mm^2. Image sequences were acquired at 7.83 Hz (1 ms per line, and 128 x 128 pixels per frame) or at 1 KHz by performing line scans at 1 ms per line.

Image processing

Movies of SyGCaMP2 signals were first pre-processed by alignment and Kalman filtering in ImageJ (National Institutes of Health) and then analyzed using a custom-written suite of macros named SARFIA[4], running in Igor Pro v. 6.10. ROIs defining synaptic terminals were defined by applying a Laplacian transform to the averaged image and then thresholding, as described in detail. SyGCaMP2 signals were quantified as the average fluorescence per pixel after subtraction of the background and normalized as variations of intensity over the resting fluorescence (ΔF/F).

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2. Algorithm for detecting spikes in SyGCaMP2 signals

Three different algorithms for detecting spikes in SyGCaMP2 traces were compared, and examples of their performance are shown in Supplementary Figure 1a. The top trace shows the raw SyGCaMP2 signal from an individual terminal. The first method we tested was match filtering the original trace followed by application of a threshold, with all upwards threshold crossings counted as a spike (second trace from the top). The template was obtained by averaging 50 isolated "spike-like" events from 10 different terminals, and is shown in Supplementary Fig. 1b. Using a threshold of three times the standard deviation of the whole trace, most events judged to be spikes by eye were counted. However, this method introduced two artefacts: spurious events (orange triangles) and missed events (blue crosses). Missed events occurred when the spike rose more slowly than average or when a second spike occurred before the first had recovered to baseline. We therefore tested a more straightforward approach: applying a threshold to the time derivative of the original trace (third trace from top). This algorithm performed well, only failing to detect one event that might be judged by eye to be a spike in this sample. The third algorithm we tested was a hybrid of the first two: the derivative of the spike template was used as a matched filter on the derivative of the raw SyGCaMP2 trace (bottom trace in Supplementary Figure 1a). This method improved detection of spikes running into each other as compared to the simple matched filter, but missed more events. These considerations led us to detect spikes by simply applying a threshold to the time derivative of the raw SyGCaMP2 signal. A threshold set to three times the standard deviation reliably differentiated calcium spikes from sustained signals that rose more slowly, as can be seen by comparing Supplementary Figures 1c and d.

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