Supplementary Materials and Methods

Image analysis. For image analysis with *Bouton Project* (**Figs. 2-4**) regions of interest (ROIs) were chosen using a high-resolution 1x1 image as a guide. CFP and YFP pixel intensities were averaged in each frame over the ROIs and results were exported to *Microcal Origin 6.0*. FRET was calculated as YFP/CFP. ΔFRET was calculated by subtracting the mean rFRET from the maximal FRET response. Because background levels did not follow the spatial gradient observed for synaptic regions and because the reporter also localized to the membrane at lower levels, postsynaptic data was not background subtracted. Slight differences in the membrane topography gave different local background levels which greatly affected the magnitude of background subtracted ΔFRET. In spite of this, image sequences that were background subtracted also showed a ΔFRET gradient. Rather than introduce biases dependent on the choice of background area and because background did not seem to affect our main results, we chose to calculate ΔFRET from the raw data. Image sequences obscured by muscle contraction were discarded, as were data obtained from postsynapses where the CFP signal-to-background ratio was less than 1.3.

For extended experiments where two frames were acquired before and after stimulation (**Figs. 5-7**), images were analyzed in *Matlab 7.0* (Mathworks). To align the YFP and CFP channels, the YFP frame was horizontally scanned over the CFP to the point of highest correlation, followed by a similar vertical scan. The YFP image stack was then divided by the aligned CFP stack to yield the FRET stack. For image display ΔFRET matrices were convolved with a low pass Gaussian filter (size = 4 µm x 4 µm, σ = 0.8 µm).

To obtain ΔFRET values for each postsynapse the images were partitioned into ROIs. An image segmentation algorithm was implemented to detect clusters of pixels of high fluorescence[1]. First, contiguous islands (24-720 μm) of pixels brighter than all adjacent pixels were detected in the YFP image. Clusters were then partitioned into ROIs containing individual postsynapses. To do this, intensity peaks within each cluster were detected by re-running the algorithm with higher constraints (3.2-9.6 μm). To expand the ROI from the fluorescence peak to the rest of the postsynapse area the remaining pixels within the cluster were listed in descending order of brightness. The first pixel in the list adjacent to an intensity peak was added to the peak as part of the new ROI. The list was repeatedly scanned until all pixels belonged to a ROI. To correct for muscle drift during imaging, each ROI was moved to the brightest region in the next frame within 3 pixels in any direction.

The area of a postsynapse was defined as the number of pixels within the ROI. For axonal branch studies, the distance-to-end of a postsynapse was the distance from the postsynapse's center to the center of the terminal postsynapse on the same branch. Axon branches and their ends were manually marked.

The calculation of fractional fluorescence changes after presynaptic imaging was done as before[2]. Co-expression of mDsRed enabled visualization of the axonal arbors expressing SpH. Background was subtracted locally from the intensity of individual boutons. ΔFRET/FRET (YC2.3) was calculated after local subtraction of the background in both channels individually. Data was analyzed off-line by custom software programmed in IDL (RSI).

References

1. Ponomarev, A.L. & Davis, R.L. An adjustable-threshold algorithm for the identification of objects in three-dimensional images. *Bioinformatics* **19**, 1431-5 (2003).
2. Reiff, D.F. et al. In vivo performance of genetically encoded indicators of neural activity in flies. *J. Neurosci.* **25**, 4766-78 (2005).