Supplementary Data 1

Axial resolution in brain slices

To determine the properties of the uncaging volume in brain slices we scanned the ultraviolet (UV) uncaging beam over fluorescent beads placed beneath the surface of cerebellar brain slices at different depths. The uncaging volume was visualized in brain slices by scanning the uncaging beam over fluorescent beads to generate scanning images. Single beads of diameter 1 μm or 4.7 μm (Fluoresbrite Bright Blue carboxylate microspheres, Polysciences, Inc., Warrington, PA) were attached by suction to a micropipette tip and advanced to various depths within brain slices.

Brain tissue scatters UV light. As expected, with increasing depth the spot grew dimmer (**Supplementary Fig. 1a**). The peak fluorescence decreased exponentially with a length constant of 22 μm, and the total estimated focal excitation decreased with a length constant of 32 μm. The lateral and axial FWHM of the uncaging spot grew slightly in the first 30 microns of the slice, reaching values of 3 μm (lateral) and 17 μm (axial) at a depth of 25 microns (**Supplementary Fig. 1b,d**). Thus scattering reduced the total amount of light reaching the uncaging volume, and in the first 20 microns of the slice the focal properties of the volume itself were approximately preserved.

To quantify axial localization of biological responses, we uncaged glutamate over dendrites and moved the uncaging volume axially above and below the uncaging locations. At the slice surface, as the uncaging location was moved axially from a dendritic branchlet, responses to MNI- glutamate became smaller (**Supplementary Fig. 1c**), with a full-width half-maximal distance of 22-23 μm. At a depth of 34-40 microns below the surface, full-width half-maximal distances were 34-53 μm. These distances

may reflect in part responses to scattered and unfocused light since the detector itself, *i.e.* the dendrite, is spatially distributed[1]. To test this we used double-caged glutamate (bis-CNB-glutamate), in which glutamate is released only after two uncaging events. This approach limits glutamate production to the focal volume, a chemical two-photon effect[1]. Using 140-200 μM bis-CNB-glutamate, at a depth of 20-35 microns the full-width half-maxima improved to 26-33 μm (**Supplementary Fig. 1d**). This resolution was comparable to responses at the slice surface and to the axial resolution of fluorescent bead images.

1. Pettit, D. L., Wang, S. S.-H., Gee, K. R. & Augustine, G. J. Chemical two-photon uncaging: a novel approach to mapping glutamate receptors. *Neuron* **19**, 465-471 (1997).