SUPPLEMENTARY METHODS

Photostimulation thresholds as a function of ChR2-GFP expression level

Brain slice experiments were performed to determine the relationship between action potential thresholds and ChR2-GFP expression. The slice preparation and recording conditions were as described in Petreanu *et al* [1]. Photostimuli were delivered using an LED. The firing threshold was determined in terms of photostimulation intensity. Expression levels were estimated by measuring ChR2-GFP fluorescence ('Brightness') using a cooled CCD (Retiga 2000, QImaging, Canada). Lower photostimulus intensities were required to fire brighter neurons (Supplementary Fig. 2).

Characterization of the photostimuli

Absolute intensity was calibrated using a calibrated power meter (Fieldmaster, with a LM-2 VIS, Coherent, CA). The spatial distribution of the LED light at the surface of the brain was mapped using a CCD beam profiler (WinCamD, Dataray, CA). The LED was positioned 500 μm from the CCD chip, corresponding to the distance between the LED and brain surface in the *in vivo* experiments (Supplementary Fig. 5a, b). The maximal intensity (corresponding to 200mA driving current) averaged over a 2 mm diameter circle was ~ 8 mW/mm^2. For the experiments of Figure 1 the recorded neurons were within 500 μm of the center of the LED, corresponding to an average max intensity of I_{max} = 11.6 mW/mm^2.

We further estimated the spatial distribution of light intensity in the brain. A mouse brain was rapidly dissected (without perfusion), mounted in agar, and cut along a coronal plane through the barrel cortex. A skull plate containing a 2 mm diameter craniotomy was repositioned over the barrel cortex, with the craniotomy centered on the cut surface of the brain (Supplementary Fig. 5a). The LED was positioned as in the behavioral experiments, 500 μm from the surface of the brain (dashed blue line). The upper half of the LED was covered with a razorblade to prevent direct illumination of the cut surface of the brain (Supplementary Fig. 5d). The distribution of blue light on the cut surface was imaged using a cooled CCD camera (QICAM, QImaging, Canada) on a dissecting microscope (MVX10, Olympus). The intensity distribution was measured 250 μm below the pia (dashed red line, Supplementary Fig. 5a, f). Because the light reaching the camera is scattered on its way out of the tissue, the width of the measured light intensity distribution (Supplementary Fig. 5c) is likely an overestimate of the actual width of the distribution in the brain.

Analysis of axonal excitability

We used brain slice measurements to asses the axonal excitability during photostimulation. Some ChR2-GFP-positive neurons were found beyond the imaging window (Fig. 3c) and although the somata and dendrites of these neurons receive very little light (Supplementary Fig. 5c), it is possible that their axons project under the imaging window to the barrel cortex. If these axons were excited by our light stimuli [1] this could lead to errors in our estimates of the number of neurons driving behavior (Fig. 3d). Similarly, these neurons could have been excited by scattered light. Here we show

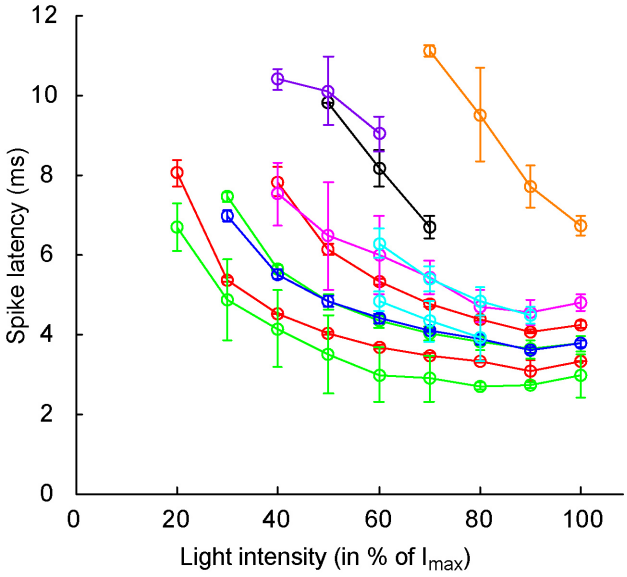
that under our experimental conditions, predominantly neurons that were directly illuminated by a diffuse source were excited. Photostimulation of long-range axons and photostimulation by scattered light were negligible beyond ~ 250 μm.

We used whole-cell or cell-attached recordings to detect photostimulation-evoked action potentials in the presence of CPP (5 μM) and NBQX (10 μM). We first performed laser scanning photostimulation (LSPS) using a small laser spot (10-15 micrometers, see Petreanu *et al* [1] for details) to confirm that the recorded cell had an intact axon in L5 and in neighboring columns (Supplementary Fig. 7a). We then mimicked the diffuse illumination of our *in vivo* assays using a large light spot produced by the fluorescence illuminator of our microscope (diameter 100-200 μm; GFP filter set; Chroma, blue circles, Supplementary Fig. 7a) covering the dendritic arbor or parts of the axonal arbor with blue light for 2 ms. Using the aperture stop of the microscope we adjusted the intensity of the photostimulus on the somadendritic compartment close to the firing threshold of the recorded cell. By removing calibrated neutral density filters we measured how much additional intensity was required to cause action potentials when illuminating L2/3 axons either in L5 (450 micrometers below the recorded cell) or in L2/3 (250 micrometers lateral and medial to the recorded cell, Supplementary Fig. 7a). Excitation of axons in L5 required 10-fold higher intensities (range 2-60; n = 10), despite the fact that L2/3 -> L5 is a massive axonal projection. 22-fold higher intensities were required in neighboring columns in L2/3 (range 4-60; n = 5, Supplementary Fig. 7b). These experiments demonstrate that axons are much less excitable than dendrites when using spatially extended photostimuli, such as diode illumination used in vivo. These

experiments also indicate that excitation by scattered blue light is limited to distances smaller than ~ 250 micrometers. Neurons are therefore photostimulated primarily in a well-defined excitation volume, which does not extend much beyond the spatial distribution of incident intensity on the brain (see also Supplementary Fig. 5).

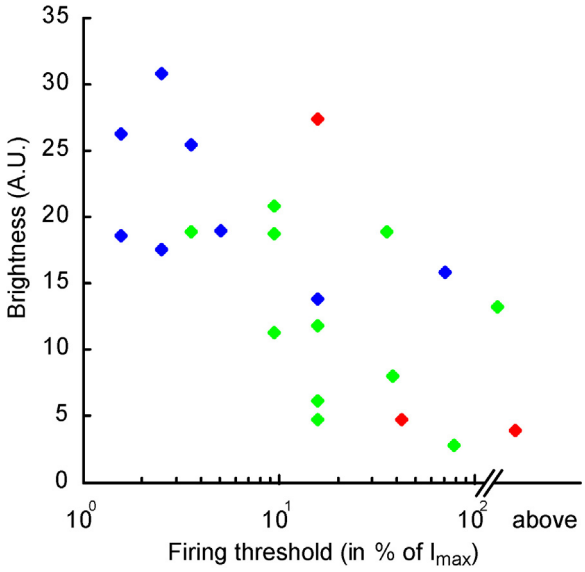
1. Petreanu, L., Huber, D., Sobczyk, A. & Svoboda, K. Channelrhodopsin-2-assisted circuit mapping of long-range callosal projections. Nat Neurosci 10, 663-8 (2007).

Supplementary Figure 1



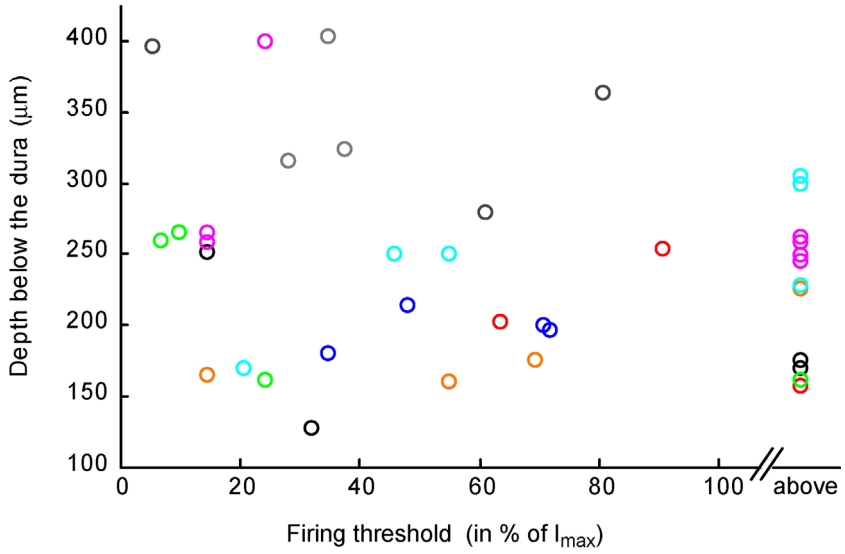
Supplementary Figure 1: Spike latencies as a function of light intensity. Same neurons and color code as in Figure 1g. Error bars: standard deviation.

Supplementary Figure 2



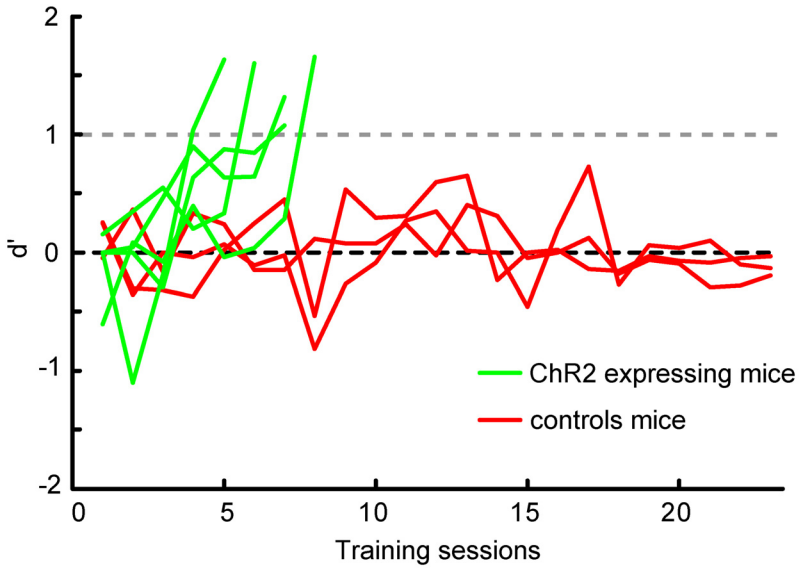
Supplementary Figure 2: Relationship between brightness of ChR2-GFP fluorescence (measured with a cooled CCD camera) and the firing threshold. Different colors indicate separate animals.

Supplementary Figure 3



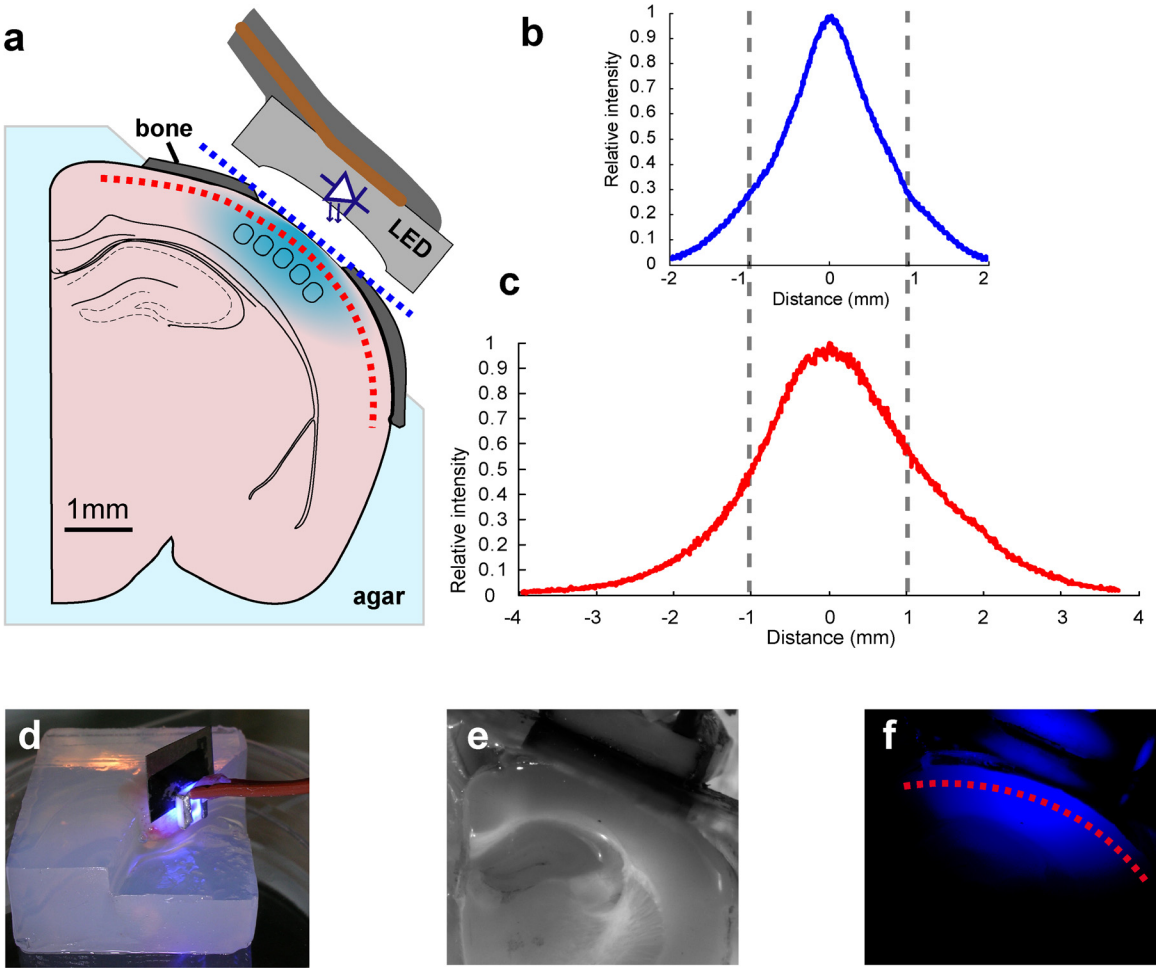
Supplementary Figure 3: Firing threshold as a function of the depth of the recorded neurons. Same neurons and color code as in Figure 1g.

Supplementary Figure 4



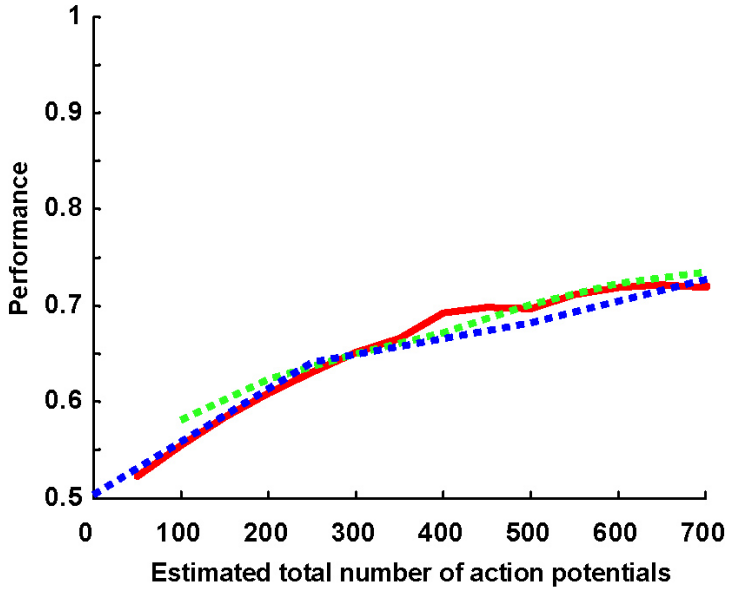
Supplementary Figure 4: Learning curves for the initial stimulus/reward association. The response is expressed as d' (d'=z(hit rate)-z(false alarm rate)). The data is presented in terms of d' because our protocol included a bias correction, resulting in unequal numbers of stimulated and non-stimulated trials (see Methods). Red and green lines show the performance of the individual mice. Black dashed line: chance level, grey dashed line: criterion performance (d'=1, ~65-70 % correct) to change to next behavioral protocol.

Supplementary Figure 5



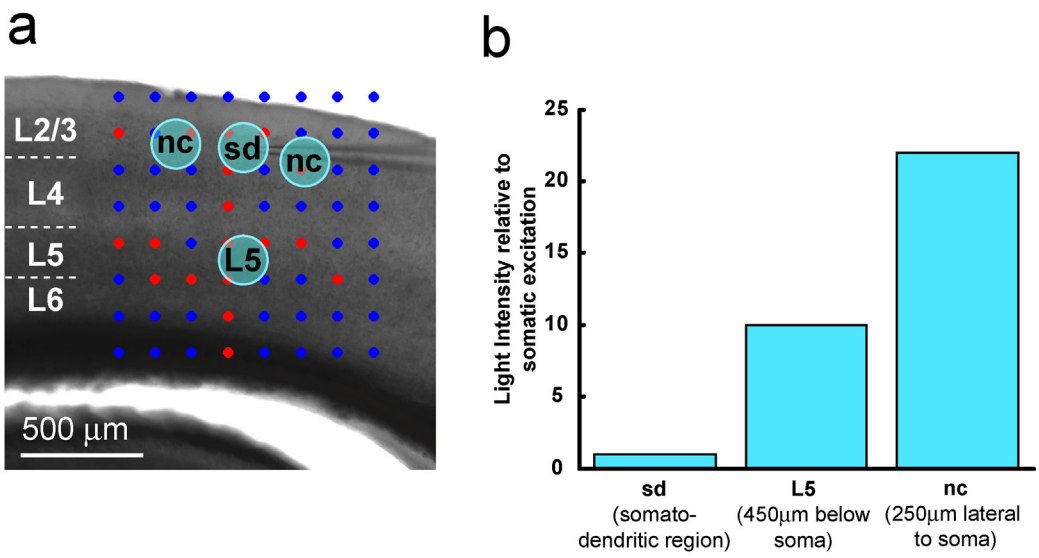
Supplementary Figure 5: Measurement of the spatial distribution of light intensity in the brain. **(a)** Schematic of the arrangement of brain tissue in an agar block, skull plate with craniotomy and measurement planes in relationship with the LED. The incident intensity distribution was measured along the dashed blue line (b). The intensity distribution inside the brain was measured along the dashed red line (c). **(b)** Normalized light intensity distribution at the brain surface, 500 µm from the LED (dotted blue line in a). The dashed grey line indicates the edges of the craniotomy. **(c)** Normalized light intensity distribution in the brain 250 µm below the surface. **(d-f)** Actual images of the experimental setup and light distribution.

Supplementary Figure 6



Supplementary Figure 6: Performance as a function of the total number of action potentials. The thin red line corresponds to the performance in response to a single photostimulus (same data as in Fig. 3d). The dashed green (two photostimuli per trial) and blue (five photostimuli per trial) are the performance curves of Fig. 3d scaled by the number of photostimuli. Assuming that all photostimuli in a train reliably trigger action potentials, the horizontal axis represents the total number of action potentials directly evoked by light.

Supplementary Figure 7



Supplementary Figure 7: Analysis of axonal excitability in brain slices. **(a)** Brightfield image of a brain slice with the recording electrode (translucent blue circle, sd, cell-attached). The red (action potential) and blue (no action potential) spots indicate the laser positions (beam diameter, 10-15µm) and evoked responses during laser scanning photostimulation. Note that axons were excited in L5 and in neighboring columns (nc). The translucent blue circles indicate the positions of the large photostimulus spots produced by the fluorescence burner of the microscope on the soma and dendritic arbor (sd), axons in layer 5 (L5, 450 µm below the soma), and in neighboring columns (nc, 250 µm medial and lateral from the soma) **(b)** Light intensity of a diffuse illumination spot necessary to excite the recorded neurons by illuminating their axons in layer 5 (L5) or neighboring columns (nc), normalized to excitation intensity at the soma (sd).

Supplementary movie 1: Two-photon laser scanning microscopy image stack of layer 2/3 neurons expressing ChR2-GFP and RFP.

Supplementary movie 2: A mouse performing a detection task during photostimulation (see also Fig. 2). The mouse was expressing ChR2-GFP in layer 2/3 neurons.