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Supplemental Information

Two-Photon Optogenetic Mapping of Excitatory Synaptic Connectivity and Strength

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Supplemental Information

Transparent Methods

Animal handling and experimentation were done according to the US National Institutes of Health and Columbia Institutional Animal Care and Use Committee guidelines. Animals of both sexes were used and were housed and maintained in a temperature-controlled environment on a 12-h light-dark cycle, with *ad libitum* food and water, in a Columbia University Animal Facility.

C57BL/6 mice aged postnatal day (P)0 to P1 were injected with AAV-DJ-CaMKIIa-C1V1(E162T)-TS-P2A-eYFP-WPRE or AAV8(Y733F)-CaMKIIa-C1V1(E162T)-TS-P2A-eYFP-WPRE at concentrations of $1.8 \cdot 10^{13}$– $5.5 \cdot 10^{13}$ GC·mL^{-1} (Neuroscience Gene Vector and Viral Core, Stanford, CA). Pups were anesthetized using hypothermia. They were placed on aluminum foil on a cooling block placed on crushed ice. Viral injections were made using a 10 µL PCR glass pipette (Drummond Scientific Company, PA) pulled to a sharp micropipette and attached to a custom made manipulator. After penetration of the pup's skin and skull, 400 nL of virus containing solution were expelled into left visual cortex using repetitive 30 ms pulses of 7-8 psi applied with a Picospritzer II (Parker Hannifin, NJ). After retracting the pipette, animals were rewarmed on a circulating warm water blanket. Before returning to the home cage, pups were placed on the cage/dam bedding to facilitate acceptance by the dam due to familiar smell.

On day 18-34 after virus injection, coronal sections of the neocortex of the injected animals were prepared using a Leica VT1200S vibratome. Following deep anesthesia via inhalation of isoflurane, the animal was decapitated, and the brain quickly removed. Slices of 300 µm thickness were prepared in ice-cold slicing solution containing (in mM): 93 N-Methyl-D-glucamine, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES , 25 glucose , 5 Na-ascorbate , 3 Na-pyruvate , 10 $MgSO_4$, 0.5 $CaCl_2$, pH adjusted with HCl to 7.3, bubbled with 95% O_2 and 5% CO_2 (modified after Ting et al., 2014). After a short recovery period (4-8 min) in 35-37 °C warm slicing solution, slices were kept at room temperature in artificial cerebral spinal fluid (ACSF) until transferred into a recording chamber. ACSF contained (in mM): 126 NaCl, 26 $NaHCO_3$, 1.145 NaH_2PO_4, 10 glucose, 3 KCl, 1 $MgSO_4$ and 2 $CaCl_2$, 0.1 Na-pyruvate, 0.8 ascorbic acid, bubbled with 95% O_2 and 5% CO_2, Osmolarity ~300 mOsm.

Experiments were performed with a custom-made two-photon laser scanning microscope based on a modified Olympus BX50WI microscope equipped with a water immersion 40x/0.8 NA objective (Olympus). Patch pipettes were pulled from borosilicate glass (1.5 mm O.D, 0.86 mm I.D, Sutter Instruments Co., CA) using a DMZ Universal Electrode Puller (Zeitz-Instruments, Martinsried, Germany) with a resistance of 4-5 MΩ when filled with internal

solution containing (in mM): 130 K-gluconate, 5 NaCl, 2 $MgSO_4$, 10 HEPES, 5 EGTA, 4 MgATP, 0.4 Na_2GTP, 7 Na_2-phospocreatine, 2 pyruvic acid, 0.007 Alexa 594 hydrazide, pH adjusted to 7.3, ~280-290 mOsm). Whole cell patch-clamp recordings were established using a Multiclamp 700B amplifier (Molecular Devices, Union City, CA). For mapping experiments cells were clamped to -70 mV. Experiments were performed at room temperature. Access resistance was left uncompensated. Voltage and current signals were acquired with a sampling rate of 10k Hz. Current was offline filtered with a cutoff frequency of 1 kHz.

All experiments were performed using a Ti:sapphire laser as the light source (Coherent Chameleon Ultra II, 140-fs pulses, 80-MHz repetition rate). Laser power was modulated by a Pockels cell (350-160, Conoptics, Danbury, CT). Images were acquired using Fluoview 2.1.22 with 800 nm excitation to visualize neurons filled with fluorescent dye via whole cell recordings, or 940 nm excitation to visualize C1V1-EYFP-expressing neurons. Appropriate emission filters were used to separate the red (Alexa 594) and green (EYFP) signal. Optical signals were amplified through photomultiplier tubes (H7422-P40 Hamamatsu) connected to a signal preamplifier (Model 5113, Signal Recovery AMETEK Advanced Measurement Technology, PA). To normalize fluorescence intensity across experiments, we added 5 µL of 6.0 µm–diameter polystyrene microspheres of 0.3% relative intensity fluorescence with excitation/emission wavelength of 505/515 nm (InSpeck Green Calibration Kit, Thermo Fisher Scientific) on top of each slice at the beginning of the experiment. Fluorescence of the microspheres was collected simultaneously when imaging EYFP expression at 940 nm. Mean fluorescence intensity of EYFP-expressing neurons was calculated using ImageJ (National Institutes of Health, MD) and normalized by the mean fluorescence intensity of microspheres from the same image stack. To calculate mean fluorescence intensity, we made an individual sum projection of the image stack. We manually draw a region of interest around the neuron or microsphere and calculated mean fluorescence intensity and subtracted background fluorescence before normalizing the data.

To excite C1V1, the laser was tuned to 1040 nm. For mapping experiments, cell bodies identified by EYFP expression were selected manually using custom-written Matlab code. A random path in which the neurons were stimulated one after the other was generated. A “non-neighbor” algorithm was implemented, which prevented a neuron from being chosen as the next one to be stimulated if another neuron had been stimulated within 15 µm in the last 10 seconds. If no neuron fulfilled this criterion, the minimal time of 300 ms between stimulations was increased to satisfy the criterion. The path file was imported into custom-written software (Nikolenko et al., 2003) used to move the laser beam to the chosen points in the field of view. To stimulate a neuron, a pattern of point stimulations (53 points, lasting about 54 ms total) was carried out, starting at the border of the cell, moving in a spiral inwards with an external

diameter of 9 µm (Fig. 1A). The same pattern was used for calibration experiments. For calibration of lateral resolution, the stimulation pattern was first placed on the center of the soma. Subsequently, stimulations were performed in a radius of 10, 20, and 30 µm around the soma, with 8 stimulations per circle as is shown in Figure 2A. Interstimulation time was 500 ms, and 2 s between repeats of the complete calibration run. For axial calibration of resolution the stimulation placed at the center of the soma and was repeated three times with a 2 s interstimulation time, then the laser focus was moved 3 µm up or down. Finally, in Figure 2I and J, we performed a calibration of lateral resolution in 3 dimensions. In these experiments the lateral stimulation pattern shown in Figure 2A was repeated 3 times at the soma position, then the laser focus was moved 6 µm up or down and stimulation was repeated 3 times per position.

To perform a mapping experiment, we targeted C1V1-EYFP expressing neurons across different focus planes of the mapping volume, repeating stimulations between 5 to 10 times per plane, while recording whole cell currents from the patched neuron. To identify connected neurons, we offline identified EPSCs occurring in response to each stimulation. Considering that maximal latencies of evoked APs in calibration experiments were observed around 70 ms, only EPSCs occurring within 100 ms from the start of stimulation were considered to potentially result from the stimulation of a connected neuron. We selected a single EPSC per stimulation, such that the variation of delays across repeats in the same plane was lowest. We discarded EPSC sets with a standard deviation of delay higher than 18 ms (see results and Figure 2C) and with success rate equal or lower than 0.2 to reduce interferences from background activity or stimulation of neighboring neurons. Positions of targeted cells were confirmed offline. Only targets at which we could clearly detect a cell were considered for further analysis.

To reduce false negatives, we obtained the fluorescence of each targeted neuron automatically within a 5x5 µm square centered at the target positions. For each map, we chose the connected neuron displaying the longest EPSC delays compared to the other observed connections. We then compared this delay to the AP delays obtained in calibration data (Fig. 2G), adding 9 ms to the AP delays, as this was the mean time between AP peak and EPSC peak observed in confirmation experiments (Fig. 5). From these calculations, we assigned a normalized fluorescence intensity value to the connected neuron, based on the linear fit of AP delay and normalized fluorescence in calibration data. Next we compared the absolute fluorescence of all targeted neurons to the absolute fluorescence of the connected neuron. With this, we were able to assign normalized fluorescence values to all targeted neurons. We excluded those with less than 0.15 normalized fluorescence from analysis, as calibration neurons beyond this threshold did not fire APs upon optical stimulation (Fig. 1F).

For the axial calibration data set (n=9 cells), the AP delay increase in dependency on the z position was fitted to a parabolic function with a cubic term:

$$\Delta Delay = a+b(z-z_{min})^2+c(z-zmin)^3$$

Where ΔDelay is the observed AP delay increase, z corresponds to the axial z position and z_{min} to the z position where the delay was shortest. The constants where fitted to the following values considering their ±95% confidence interval: a = (-2 ±2) 10^{-1} ms, b= 0.1±0.1 ms ·μm^{-2} and c= (1.3 ±14) 10^{-3} ms ·μm^{-3}. The cubic term in the parabolic function was used to account for the slight asymmetry in the delay increase dependence on z position. We evaluated the similitude of the EPCSs delays from targeted cells to the calibration function using a χ^2 -test. χ^2 was calculated using the following formula:

$$\chi^2 = \sum_{i=1}^{n} \frac{(O_i - C_i)^2}{\sigma_i^2}$$

Where *O* is the EPCSs delay observed, *C* is the calibration delay and σ the standard deviation of calibration delay. Then we calculated the associated p-value for a χ^2 cumulative distribution function with n-2 degrees of freedom in χ^2. We assumed the position of the targeted cell within ± 6 µm of the point of lowest delay in the calibration function. Targeted cells with $p < 0.1$ where discarded as possible connections (See Figure 4 with examples of different fittings obtained).

In the cases where we observed neurons located on top of each other, we fitted the data to two calibration functions, each one centered at each position identified (n=3 cases, see Figure 4C). When two calibration curves overlapped, we always considered the calibration function with lowest delay value at that z position. Then, as we did for single calibration curve, we evaluated the similitude between the function with two calibration curves and EPSC delay data by calculating the associated p-value, now considering n-4 degrees of freedom. Stimulations with $p < 0.1$ where considered as not featuring two connected cells. The discarded ones were tested further for similitude to single calibration function considering each of the soma locations identified. The soma location resulting in the higher p-value was chosen as the connected neuron.

For analysis of synaptic current peak amplitudes, the values obtained when targeting the soma directly as those within 6 µm axial distance were averaged. Correlation coefficients were obtained using MATLAB.

Data were acquired and alayzed using PackIO (Watson et al., 2016), Ephysviewer (Watson et al., 2016), MiniAnalysis (Synaptosoft, NJ), IgorPro (Wavemetrics, OR) and custom-written MATLAB code (Mathworks, MA).

References

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