Supplementary Material for

Long-range and local circuits for top-down modulation of visual cortical processing

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This PDF file includes:

Materials and Methods
Figs. S1 to S8
Full Reference List
Materials and Methods

Animals

All experimental procedures were approved by the Animal Care and Use Committee at the University of California, Berkeley. Experiments were performed on wild-type (C57) and transgenic mice. The transgenic mice used were PV-Cre (Jackson lab stock #008069), SOM-Cre (#013044), VIP-Cre (#010908), CaMKIIα-Cre (#005359), loxP-flanked-tdTomato (#007914), loxP-flanked-Halo-EYFP (#014539) and loxP-flanked-ChR2-EYFP (#012569) mice. To visualize the interneurons, PV-Cre, SOM-Cre or VIP-Cre mice were crossed with loxP-flanked-tdTomato mice. To inactivate the interneurons, PV-Cre, SOM-Cre or VIP-Cre mice were crossed with loxP-flanked-Halo-EYFP mice. To inactivate the Cg excitatory neurons, PV-Cre mice were crossed with loxP-flanked-ChR2-EYFP mice.

Surgery

Adeno-associated viruses (AAVs) were acquired from the UNC Vector Core (AAV2/2-CaMKIIα-hChR2(H134R)-EYFP and AAV2/2-CaMKIIα-mCherry) and Stanford Neuroscience Gene Vector and Virus Core (AAV-DJ-CaMKIIα-hChR2(H134R)-EYFP). For in vivo experiments, we used AAV2/2 and AAV-DJ; for in vitro experiments, we used AAV-DJ.

Mice (P20-P40) were anesthetized with isoflurane (5% induction and 1.5% maintenance) and placed on a stereotaxic frame. Temperature was kept at 37 °C throughout the procedure using a heating pad. After asepsis, the skin was incised to expose the skull and the overlying connective tissue was removed. A craniotomy (~0.5 mm diameter) was made above the injection site. Viruses or Retrobeads were loaded in a sharp micropipette mounted on a Nanoject II attached to a micromanipulator and then injected at a speed of 60 nL per min. AAV (800 nL) was injected into
the Cg (0.2 mm anterior to bregma and 0.3 mm lateral, at a depth of 0.9 mm). Retrobeads (100-150 nL) were injected into V1 (3.5 mm posterior to bregma and 2.5 mm lateral, at a depth of 0.6 mm) or SC (4.2 mm posterior to bregma and 1 mm lateral, at a depth of 1.8 mm). Histology, in vivo recording, and behavioral experiments were performed > 2 weeks after injection. In vitro recording was performed 1-2 weeks after injection. For recording and behavioral training in awake mice, a custom-designed headplate was implanted right after the virus injection. Small screws and dental acrylic were used to fix the head plate onto the skull. An optic fiber (200 µm in diameter, N.A. 0.39) with 2.5 mm ferrule stick and a protective cap was then implanted on the surface of the Cg to deliver light.

**Histology**

Two weeks after the virus or Retrobead injection, the mice were deeply anesthetized with isoflurane and immediately perfused with chilled 0.1 M PBS followed by 4% paraformaldehyde (wt/vol) in PBS. The brain was removed and post-fixed overnight at 4 °C. After fixation, the brain was placed in 30% sucrose (wt/vol) in PBS solution for 1-2 d at 4 °C. After embedding and freezing, the brain was sectioned into 60 µm coronal slices using a cryostat. After rehydration and permeabilization, slices were incubated with the NeuroTrace fluorescent Nissl stains (1:50) for 20 min, then washed with PBS for 2 hrs and mounted with Vectashield. Fluorescence images were taken under a confocal microscope.
**Visual stimulation**

Visual stimuli were generated with a Graphics card in a PC running custom written software. The mice viewed a gamma-corrected 7” LCD monitor (maximal luminance: 250 cd/m²) with a refresh rate of 75 Hz. The monitor was placed 10 cm away from the left eye, positioned such that the receptive fields of the recorded neurons were at the center of the monitor. For measuring orientation tuning of V1 neurons, full-field drifting gratings (100% contrast, 2 Hz, 0.04 cycles/°) were presented at 8 directions (separated by 45°) in a pseudorandom sequence. For measuring contrast response, full-field drifting gratings (2 Hz, 0.04 cycles/°) at the preferred orientation of each cell were presented at 11 different contrasts (1%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%) in a pseudorandom sequence. Each trial started with 4 s of drift grating, followed by 2 s of gray screen. Laser stimulation lasted for 3 s, starting from the onset of the drifting grating. Baseline firing rates were measured using separate trials with a gray screen (0% contrast). Each block consisted of 18 trials (16 trials at each orientation plus 2 trials at 0% contrast) in orientation tuning measurement, or 24 trials (22 trials at each contrast plus 2 trials at 0% contrast) in contrast response measurement, with interleaved laser-on and laser-off trials. A total of 3-6 blocks were presented in each experiment. For Halo and ChR2 co-activation experiments, blocks with or without yellow laser were interleaved. To measure orientation discrimination in the behavioral experiment, drifting gratings at 0° and 90° (100% contrast, 2 Hz, 0.04 cycles/°, 4 s per trial) were presented to the left eye (see ‘Behavior’ below).
In vivo recordings and data analysis

Recording experiments were performed >2 weeks after virus injection. For anesthetized experiments, mice were anaesthetized with 5 mg kg$^{-1}$ of chlorprothixene and 0.6-1.2 g kg$^{-1}$ urethane (IP). A custom headplate was fixed to the skull using glue and dental cement right before recording. Mice were then placed onto a custom platform and their temperature was maintained at 37 °C using a feedback-controlled heating pad. Two craniotomies (~1.5 mm) were made above the Cg and V1. In a subset of Cg axon stimulation experiments, 2% lidocaine (1-1.5 μL) was injected into the Cg to block antidromic activation of the cell bodies. For awake experiments, mice were habituated to head fixation within the recording setup for several days before recording. Mice were placed in a custom tube and the headplate was fixed on a holder attached to the air table, which allowed them to move the body inside the tube while the head was fixed. On the day of recording, a craniotomy (~1.5 mm diameter) was made above V1 while the animal was under anesthesia (1.5% isoflurane in O$_2$). The light was delivered through a chronically implanted optic fiber above the Cg.

Cell-attached recordings were made with a Multiclamp 700B amplifier (filtered at 0.5-2 kHz) and sampled at 10 kHz controlled by the Clampex software. Glass pipettes with tip resistance of 4-8 MΩ filled with HEPES buffered ACSF were used. The craniotomy was kept moist with a small bath of ACSF. The reference Ag/AgCl wire was placed in ACSF next to the recording site. For awake experiments, the craniotomy was stabilized with Kwik-Cast after each pipette penetration. Only recordings stable for more than 5 min and with sufficiently high seal resistance (10-100 MΩ) were accepted for analysis. Off-line data analysis was performed using custom software.
To quantify orientation tuning of each neuron, we fitted the firing rate as a function of orientation by the sum of two Gaussian functions with peaks 180° apart:

\[
R(\theta) = a_0 + a_1 e^{-\frac{(\theta - \theta_0)^2}{2\sigma^2}} + a_2 e^{-\frac{(\theta - \theta_0 + 180^\circ)^2}{2\sigma^2}}
\]

where \(R(\theta)\) is the response at orientation \(\theta\), \(a_0\) is the untuned component of the response, \(a_1\) and \(a_2\) are the amplitudes of the two Gaussians, \(\theta_0\) is the preferred orientation, and \(\sigma\) is the standard deviation of the Gaussian function. To quantify the modulation induced by laser stimulation, modulation factor was computed as:

\[
MF = \frac{(a_{1\text{\textsc{laser-on}}} - a_{1\text{\textsc{laser-off}}})}{(a_{1\text{\textsc{laser-on}}} + a_{1\text{\textsc{laser-off}}})}
\]

The fitting error was computed as:

\[
E = \sum_\theta (R_{\text{measure}}(\theta) - R_{\text{fit}}(\theta))^2 / \sum_\theta R_{\text{measure}}^2(\theta)
\]

where \(R_{\text{measure}}(\theta)\) and \(R_{\text{fit}}(\theta)\) are the measured and fitted responses at \(\theta\), respectively. We set a threshold of 0.1 for the fitting error; modulation factor of a cell was included in the analysis only if the fitting error was below the threshold both with and without laser stimulation.

**In vivo optical stimulation**

Optical activation of ChR2 was induced by blue light. A blue laser (473 nm) was connected to an optic fiber (200 µm in diameter) and controlled by a stimulator. The tip of the optic fiber was placed near the cortical surface, and its position was precisely controlled by a micromanipulator and read out from a DR1000 Digital Readout. To activate the Cg or Cg axons in V1, we used pulse
trains (10 Hz, 5 ms) of laser at a power of 3-5 mW at the fiber tip. To silence Cg by activating PV+ neurons in PV-ChR2 mice, we used square pulses of laser at the same power.

Optical activation of Halo was induced by yellow light. A yellow laser (593 nm) was connected to an optic fiber (600 μm in diameter). The yellow light covered the whole area stimulated by blue light. For SOM-Halo and VIP-Halo experiments, we used yellow light at a power of 5-8 mW. For PV-Halo experiments, we used a lower power of 1-3 mW to avoid epileptic discharge. The light (blue and/or yellow) started at the same time as the visual stimulus and lasted for 3 s.

**Behavior**

We trained head-fixed mice on a go/no-go visual discrimination task. The detailed method has been described previously (27). Briefly, mice were trained to discriminate between drifting gratings at 0° and 90° (100% contrast). After training, laser stimulation was applied in 50% of randomly selected trials, and d’ was analyzed separately for trials with and without laser stimulation. We used 5 AAV2/2-CaMKIIα-hChR2(H134R)-EYFP injected mice for the experimental group and 3 AAV2/2-CaMKIIα-mCherry injected mice for the control group in behavioral testing.

Hit and False alarm (FA) rates were quantified as followings:

\[
\text{Hit rate} = \frac{\text{number of Hits}}{\text{number of Hits} + \text{number of Misses}}
\]

\[
\text{FA rate} = \frac{\text{number of FAs}}{\text{number of FAs} + \text{number of CRs}}
\]

Based on the Hit and FA rates, orientation discriminability (d’) was quantified by:

\[
d’ = \text{norminv}(\text{Hit rate}) - \text{norminv}(\text{FA rate})
\]

where norminv is the inverse of the cumulative normal function (43, 44). Higher d’ values indicate better performance in visual discrimination.
Slice preparation and recording

Mice were anaesthetized with 5% isoflurane. After decapitation, the brain was dissected rapidly and placed in ice-cold oxygenated HEPES buffered ACSF (in mM: NaCl 92, KCl 2.5, NaH₂PO₄ 1.2, NaHCO₃ 30, HEPES 20, glucose 25, sodium ascorbate 5, thiourea 2, sodium pyruvate 3, MgSO₄·7H₂O 10, CaCl₂·2H₂O 0.5 and NAC 12, at pH 7.4, adjusted with 10 M NaOH), and coronal sections of primary visual cortex (V1) were made with a vibratome. Slices (350 μm thick) were recovered in oxygenated NMDG-HEPES solution (in mM: NMDG 93, KCl 2.5, NaH₂PO₄ 1.2, NaHCO₃ 30, HEPES 20, glucose 25, sodium ascorbate 5, thiourea 2, sodium pyruvate 3, MgSO₄·7H₂O 10, CaCl₂·2H₂O 0.5 and NAC 12, at pH 7.4, adjusted with HCl) at 32 °C for 10 min and then maintained in an incubation chamber with oxygenated standard ACSF (in mM: NaCl 125, KCl 3, CaCl₂ 2, MgSO₄ 2, NaH₂PO₄ 1.25, sodium ascorbate 1.3, sodium pyruvate 0.6, NaHCO₃ 26, glucose 10 and NAC 10, at pH 7.4, adjusted by 10 M NaOH) at 25 °C for 1-4 hr before recording (45).

Whole-cell recordings were made at 30 °C in oxygenated solution (in mM: NaCl 125, KCl 4, CaCl₂ 2, MgSO₄ 1, NaH₂PO₄ 1.25, sodium ascorbate 1.3, sodium pyruvate 0.6, NaHCO₃ 26 and glucose 10, at pH 7.4). EPSPs were recorded using a potassium based internal solution (in mM: K-gluconate 135, KCl 5, HEPES 10, EGTA 0.3, MgATP 4, Na₂GTP 0.3, and Na₂-phosphocreatine 10, at pH 7.3, adjusted with KOH, 290-300 mOsm). EPSCs and IPSCs were recorded using a cesium-based internal solution (in mM: CsMeSO₄ 125, CsCl 2, HEPES 10, EGTA 0.5, MgATP 4, Na₂GTP 0.3, Na₂-phosphocreatine 10, TEACl 5, QX-314 3.5, at pH 7.3, adjusted with CsOH, 290-300 mOsm) and isolated by clamping the membrane potential of the recorded neuron at the reversal potential of inhibitory and excitatory synaptic currents, respectively. For measuring the monosynaptic inputs from Cg axons to V1 neurons (fig. S5), TTX (1 μM) and 4-aminopyridine
(100μM) were bath applied to block action potentials and permit direct depolarization of axon terminals by ChR2 activation with 5-ms pulses of blue light (29). The resistance of patch pipette was 3-5 MΩ. The cells were excluded if the series resistance exceeded 40 MΩ or varied by more than 20% during the recording period. Data were recorded with an amplifier filtered at 2 kHz and digitized at 10 kHz. Recordings were analyzed using custom software.

**Optical stimulation in slices**

To activate ChR2, we used mercury arc lamp gated by an electromagnetic shutter coupled to the epifluorescence light path and bandpass filtered at 419-465 nm, which minimized co-activation of Halo. Pulse trains of blue light (10 Hz, 5 ms) were delivered through a 40× 0.8 NA water immersion lens at a power of 1-2 mW. The size of the light spot was controlled by a calibrated aperture on a microscope. To activate Halo, a 600 μm optic fiber was coupled to a yellow laser (593 nm) and controlled by a stimulator. The optic fiber was mounted on a micromanipulator and placed < 1 mm from the recorded area under the 40× objective. We used square pulse of yellow light at a power of 5 mW.

**Rabies-virus-based retrograde monosynaptic tracing**

Glycoprotein-deleted (ΔG) and EnvA-pseudotyped rabies virus (RV-ΔG-tdTomato+EnvA) was used for retrograde monosynaptic tracing from different types of V1 neurons (46, 47). TVA receptor and rabies glycoprotein, which are required for virus infection and trans-synaptic spread, respectively, were expressed in Cre-positive neurons by co-injection of AAV2/2-EF1α-DIO-TVA-EGFP and AAV2/2-EF1α-DIO-Glycoprotein (400 nL) into V1 of CaMKIIα-Cre, PV-Cre, SOM-Cre and VIP-Cre mice. RV-ΔG-tdTomato+EnvA (400 nL) was injected two weeks after AAV injection. The histology experiments were performed 7 days after rabies virus injection.
AAV preparation followed previously reported protocol (48). To construct AAV-EF1α-DIO-TVA-EGFP and AAV-EF1α-DIO-Glycoprotein, TVA and EGFP linked by the 2A ‘self-cleaving’ peptides or rabies glycoprotein was cloned into pAAV-MCS in an antisense direction flanked by a pair of canonical loxP sites and a pair of lox2272 sites. AAV particles (AAV2/2) were produced by co-transfection of packaging plasmids into HEK293T cells, and cell lysates were fractionated by iodixanol gradient ultracentrifugation. Viral particles were further purified from the crude fraction by heparin affinity column, desalted and concentrated with centrifugal filter (100K). The genomic titer of AAV2/2-EF1α-DIO-TVA-EGFP (4.4× 10^{13} gc/mL) and AAV2/2-EF1α-DIO-Glycoprotein (2.2 × 10^{12} gc/mL) was determined by quantitative PCR. TVA and rabies glycoprotein were subcloned from the AAV-TRE-HTG plasmid from L. Luo (49).

RV-ΔG-tdTomato was amplified in B7GG cells and pseudotyped using BHK-EnvA cells in a manner similar to that previously described by (50). EnvA pseudotyped rabies virus was titered (1.2 × 10^8 IU/mL) using HEK293-TVA cells. RV-ΔG-tdTomato was a gift from B. Lim. B7GG cells, BHK-EnvA cells and HEK293-TVA cells were gifts from E. Callaway.
Supplementary Figures and Legends

Fig. S1. Retrograde tracing from V1 and SC and anterograde tracing from Cg. (A) Fluorescence images of Retrobeads-labeled neurons in Cg, corresponding to the line drawings in Fig. 1, B and D. (B) Distribution of neurons retrogradely labeled from V1 in different cortical areas. In the frontal area only Cg and the immediately adjacent M2 were labeled. (C) Distribution of Cg axons in different cortical areas, based on data from Allen Mouse Brain Connectivity Atlas, http://connectivity.brain-map.org/ (51). Among sensory cortices, visual areas receive 94% of the Cg projections.
Fig. S2. Effects of laser stimulation on contrast-response function in ChR2-expressing and control mice. (A) Schematic of experimental setup. (B) Contrast-response function (measured at the preferred orientation of each cell) in ChR2 mice. Left, population contrast-response function with (blue) or without (black) laser stimulation. The functions of each cell were normalized by its firing rate at 100% contrast without laser stimulation. Right, Slope of contrast-response function of each neuron in laser-on vs. laser-off trials ($P < 10^{-4}$, paired $t$-test). Each circle represents one cell. Red cross, mean ± SEM. (C) Left, population orientation tuning curves with (blue) or without (black) laser stimulation in control mice (not injected with AAV). Right, Modulation factors in control mice (mean ± SEM., -0.014 ± 0.005, $P = 0.6$, $t$-test). Each circle represents one neuron. (D) Similar to (B), for control mice ($P = 0.4$, paired $t$-test). Error bars, mean ± SEM.
Fig. S3. Activation of Cg axons in V1 increases the slope of contrast-response function at the site of activation. (A) Schematic of experimental setup. (B) Left, population contrast-response function with (blue) or without (black) laser stimulation. Error bars, mean ± SEM. Right, Slope of contrast-response function of each neuron in laser-on vs. laser-off trials ($P = 0.002$, paired $t$-test). Red cross, mean ± SEM.
Fig. S4. Center-surround modulation of visual cortical responses induced by Cg axon stimulation after blocking antidromic spiking of Cg neurons. (A) Schematic of experimental setup. (B) Lidocaine injection into Cg abolished local field potential (LFP) responses recorded in Cg evoked by light stimulation in V1. (C) Modulation factor vs. stimulation location in V1 in the presence of lidocaine in Cg. At 0 µm, 0.15 ± 0.04 (mean ± SEM), $P = 8 \times 10^{-4}$, $n = 20$; 200 µm, -0.19 ± 0.03, $P = 9 \times 10^{-6}$, $n = 24$; 400 µm, -0.01 ± 0.02, $P = 0.53$, $n = 19$. 
**Fig. S5. Disynaptic inhibition of pyramidal neurons evoked by Cg axon stimulation.** (A) Schematic of slice experiment, with whole-cell recording from layer 2/3 pyramidal neurons and focal activation of Cg axons in layer 1. (B) The enlarged initial part of light-evoked EPSC (red) and IPSC (blue) in an example L2/3 pyramidal neuron. Cyan bar, duration of light stimulation (5 ms). (C) IPSC with (black) or without (blue) AMPA type glutamate receptor antagonist (CNQX, 10 µM). Cyan dots, 5-ms light stimulation (10 Hz). (D) EPSCs and IPSCs in a layer 2/3 pyramidal neuron at different stimulus locations. (E) Normalized excitatory (red) and inhibitory (blue) input strengths (measured by total charge) vs. stimulus location for all recorded L2/3 pyramidal neurons (n = 42). Each input was normalized by its strength at 0 µm. At 200 µm, excitatory input was significantly weaker than that at 0 µm (P = 3×10^{-15}, t-test), inhibitory input was significantly stronger (P = 0.01, t-test).
**Fig. S6. Subtypes of V1 neurons receiving direct excitatory inputs from Cg.** (A) Cg axons (green) in V1 of PV-tdTomato (left), SOM-tdTomato (middle) and VIP-tdTomato (right) mice. Red, PV+, SOM+ or VIP+ neurons expressing tdTomato. Scale bar, 200 µm. (B) Schematic of experimental setup for slice recording in V1. (C) Example blue light-evoked EPSPs recorded from pyramidal (Pyr), PV+, SOM+ and VIP+ neurons. (D) Normalized EPSP amplitudes in different cell types (normalized by EPSP amplitude from the most superficial L2/3 Pyr neuron recorded on the same day). EPSP amplitude in VIP+ neurons was larger than those in any other group ($P < 0.03$, t-test). (E-G) Similar to (B-D), but in the presence of TTX (1 μM) and 4-AP (100 μM). In this experiment, TTX (which blocks sodium channels and therefore action potentials of V1 neurons) was used to eliminate polysynaptic responses evoked by Cg axon stimulation. However, since TTX also greatly attenuates the monosynaptic responses, 4-AP (which blocks the potassium channels and thus allows direct depolarization of axon terminals by ChR2 activation) was used to boost the monosynaptic inputs from Cg axons (29). Blue dot, 5-ms blue light pulse.
Fig. S7. Monosynaptic innervation of different cell types in V1 by Cg neurons detected by rabies virus-based circuit tracing. (A) Schematics for the AAV vectors (used for cell type-specific expression of TVA receptor and rabies glycoprotein) and rabies virus. (B) Schematic of experimental procedure. (C) Injection sites in V1 of CaMKIIα-, PV-, SOM- and VIP-Cre mice. (C1) Left, starter cells (yellow) and AAV-transduced cells that did not receive RV (green) were found in V1 of a CaMKIIα-Cre mouse. Scale bar, 100 µm. Right, fluorescence image for the small white square in the left panel. Arrowheads point to a starter neuron. Scale bar, 10 µm. (C2 to C4) Similar to (C1), but with AAV and rabies virus injected into V1 of PV-, SOM- or VIP-Cre mice. (D) Transsynaptically labeled tdTomato+ neurons (red) in Cg. (D1) Left, Cg neurons innervating CaMKIIα+ excitatory neurons in V1. Scale bar, 200 µm. Right, enlarged image (rotated 90°) for the white rectangle in the left panel. Scale bar, 20 µm. (D2 to D4) Similar to (D1), but for Cg neurons innervating PV+, SOM+ and VIP+ interneurons in V1 respectively. Dashed lines, estimated borders between layers.
Fig. S8. Dual retrograde tracing of Cg neurons projecting to different V1 locations. (A) Sites of injection of red and green Retrobeads (at the same location, 0 μm) in V1 of a representative experiment. (B) Images of Retrobeads-labeled Cg neurons. Red/green arrowheads, neurons labeled with red/green beads; white arrowheads, double labeled neurons. (C and D) Similar to (A and B), but the red and green beads were injected in V1 ~200 μm apart from each other. (E) Quantification of the overlap between Cg neurons projecting to the two V1 locations (0 μm, n = 3 mice; 200 μm, n = 3 mice). Shown are mean ± SEM of the percentages of double labeled cells across mice. The low degree of overlap between Cg neurons projecting to the two locations 200 μm apart was not due to a low efficiency of retrograde labeling, since injection of red and green beads at the same V1 location revealed a high degree of overlap.
References and Notes


21. The projection to superior colliculus arises only from deep layers (Fig. 1D), similar to that in primate FEF.


30. I. R. Wickersham, D. C. Lyon, R. J. Barnard, T. Mori, S. Finke, K. K. Conzelmann, J. A. Young, E. M. Callaway, Monosynaptic restriction of transsynaptic tracing from single,


37. Based on a magnification factor of 10 μm per degree in mouse V1 (42), 200 μm of cortical distance corresponds to 20° of visual angle. A previous study (26) showed that for most neurons, the preferred size of visual stimulus was <15° in radius, and stimuli beyond this radius suppressed neuronal responses. This suggests that surround suppression for top-down modulation and bottom-up processing occur on similar spatial scales. The same inhibitory circuits could also contribute to decreased receptive field similarity and signal correlation between V1 neurons over ~200 μm (42).


