Recording large-scale, cellular-resolution neuronal activity from freely-moving mice

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Abstract:

To date, recording of cellular-resolution activity from awake mice has required either head fixation under a microscope or attaching a miniaturized device to the skull, both of which inevitably affect mouse behavior. We demonstrate a new approach for recording thousands of neurons in freely-moving, non-restricted mice during several behavioral tasks using the calcium integrator CaMPARI and a simple optical setup without any optical device attached to the mouse during testing.

Main text:

Two-photon laser scanning microscopy (TPLSM) enables longitudinal fluorescence recording from neurons expressing genetically-encoded calcium indicators (GECIs)[1]. Large field of view (FOV) TPLSM systems can access multiple cortical regions in the mouse brain[2-5]; however, functional recording is usually limited to one large plane or a few smaller, axially-shifted planes[2]. For recording neuronal activity from behaving rodents, head fixation is essential, which may result in activation of different neuronal circuits compared to natural behavior[6]. Alternatively, a miniaturized microscopic device may be attached to the mouse skull, allowing head movement during recording[7, 8]. However, the spatial resolution and volumetric recording capabilities are compromised, and the added weight on the head can also affect neuronal activation patterns.

We present a new method for large-scale, volumetric recording in freely-moving mice using the calcium- and light-dependent activity integrator, CaMPARI[9]. CaMPARI permanently changes its fluorescence emission from green to red upon illumination with 400 nm light photoconversion (PC) in a calcium-dependent manner. The second generation of CaMPARI, CaMPARI2, exhibits brighter signal and better recording

sensitivity[10]. Here, we capitalize on a unique property of CaMPARI, which is its ability to separate the recording and readout processes. Recording is conducted by PC, which labels active cells with red, while the readout of this signal can be conducted after the experiment is completed using a TPLSM system (Fig. 1A).

CaMPARI's PC process was initially reported as permanent[9, 10], as each protein undergoes an irreversible conformational change. However, we found that CaMPARI's cellular red-to-green ratio (RGR) returns to baseline levels within days, presumably due to degradation of the red protein and production of new (green) protein. We monitored neurons in the mouse primary visual cortex (V1) after PC and found exponential RGR decay with a half-life of 1.35 days (R^2=0.99; Fig. 1B, Supp. Fig. 1). Two additional PCs, separated by 14 days, demonstrated sequential recordings with CaMPARI (Fig. 1C). We measured CaMPARI's PC rate for different light doses (Supp. Fig. 2.) and found that efficient PC can be conducted with low PC light intensities, similar to room light intensities, given that the exposure time is extended to 15 minutes. Thus, CaMPARI-based recording can be conducted by illuminating a large area, without head fixation and focusing the PC light on the animal head, as previous recordings with CaMPARI did[9, 10].

Simultaneous large-scale volumetric recording under controlled conditions was accomplished by expressing CaMPARI2 in the monocular and binocular visual cortices (V1m and V1b, respectively) and the somatosensory cortices (S1) of 4 mice implanted with cranial windows on each hemisphere. A drifting grating movie was presented to either the right or left eye of lightly-anesthetized mice and was synchronized with PC illumination. The neuronal RGR was read using TPLSM after the recording was completed. As a control, we expressed jGCaMP7s[1] in V1b, V1m, and S1 of one hemisphere (n=2 mice) and recorded the fluorescence changes evoked by visual stimulation to each eye. Example data from CaMPARI2 and jGCaMP7s mice (Fig. 1D) show that the visual regions were more active than somatosensory regions, and that activity levels of the contralateral visual regions were higher than the ipsilateral ones. The activity patterns were similar for both sensors, validating the accuracy of CaMPARI-based recording (Fig. 1E, Supp. Figs. 3-4).

Next, we moved to recording neuronal activity from freely-moving mice by expressing CaMPARI2 in 5 motor and somatosensory regions in one hemisphere (motor caudal front limb, M_{CFA}; motor rostral front limb, M_{RFA}; motor neck-jaw, M_{NJ}; somatosensory forelimbs, S_{FL}; and somatosensory barrel field, S_{BF}; n=6 mice; see Methods for details). The same mice were trained and tested on three tasks, while performing a new task every other week (novel object recognition in the open field (NOR), rotarod (RR), and fear conditioning (FC)), using arenas equipped with a PC light source (Fig. 2A). For all tasks, the last session was conducted with the PC light turned on to photoconvert cells during 15 minutes of recording (Fig. 2B, Videos 1-3). Readout sessions were conducted 24 hours after recording (Fig. 2C, Supp. Fig. 5, and Video 4) and single-cell data was imaged down to a depth of ~300μm under the dura. Comparing RGRs from

different recording depths showed no depth-dependent reduction of RGR in the recorded mice (Supp. Fig. 6), suggesting diffusive penetration of the PC light into the cortex, beyond the recorded depth. Using the new CaMPARI-based recording, we were able to compare cellular RGRs across different cortical regions and tasks in the same mouse (Fig. 2D), as well as across mice. We found that median NOR activity levels were ~2.5-fold higher than RR and FC (Fig. 2E). When comparing activity across different cortical regions in the same mice while they performed the three tasks, somatosensory regions were more active than motor regions (Fig. 2F). In addition, motor regions showed differential activity levels, where M_{CFA} was significantly more active than M_{RFA} and M_{NJ}, although M_{CFA} and M_{RFA} project to the same limb[11] (Fig. 2G). Activity levels of Layers I and II/III were similar (Supp. Fig. 7), and no significant differences were found between S_{BF} and S_{FL} activity (Supp. Fig. 8).

Overall, we present a new recording method for freely-moving rodents, which acquires simultaneous volumetric activity patterns across multiple brain regions with single-cell resolution. CaMPARI's PC process allows separating the recording and readout processes and therefore, to perform recording sessions without any mechanical device attached to the animal, while activity readout may be performed after the experiment is completed. We recorded from the same mice performing three behavioral and cognitive tasks, and show changes in brain activity patterns across tasks and brain regions, with similar activity levels in recorded cortical layers. Therefore, CaMPARI-based recording in freely-moving mice bypasses existing limitations of state-of-the-art mesoscopic and miniaturized device systems and facilitates longitudinal *in vivo* neuronal activity recordings during non-restricted behaviors in the same animals.

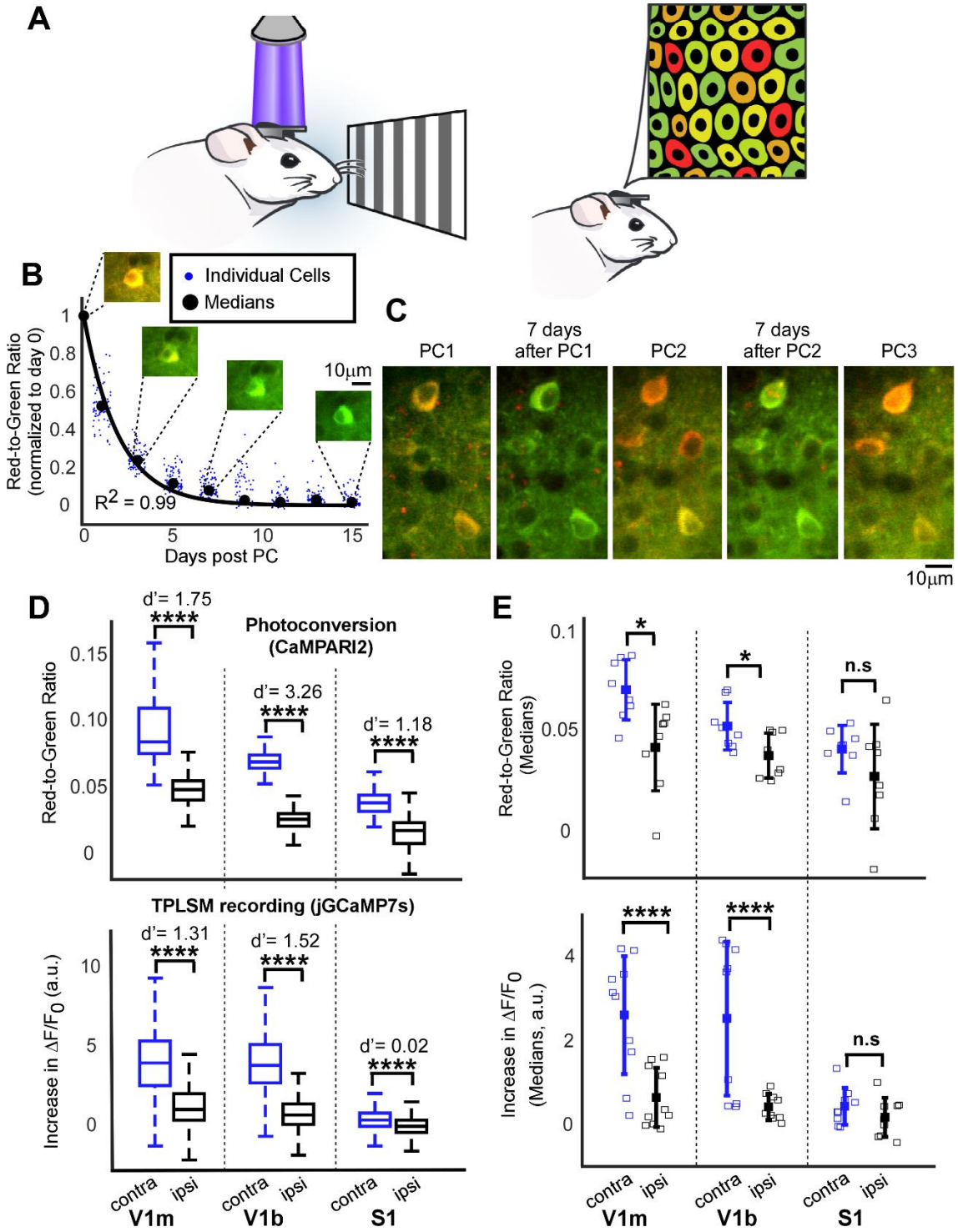

Figure 1: Simultaneous recording from multiple brain regions. (A) Schematics of the visual-evoked activity recording setup. A drifting grating movie was presented to the mouse eye while PC light illuminated its two cortical hemispheres (left). Following PC, cellular RGR was recorded using TPLSM (right). **(B)** RGRs from 73 photoconverted cells

(n=2 mice) were normalized to each cell's post-PC RGR (Day 0) and were monitored for 15 days. Median values were fit with an exponential curve ($\tau_{1/2}$=1.35 days). Example images are presented above the respective days. **(C)** Example images of repeated PCs in the same neurons. **(D)** Example data from one mouse expressing CaMPARI2 (top) showing simultaneous recording from V1m, V1b, and S1 in both hemispheres (111-256 cells/region, median=193). Activity levels are summarized by boxplots (horizontal lines show medians, boxes show the 25th–75th percentiles, whisker length is the shorter of 1.5 times the 25th–75th range or the extreme data point). Bottom, example data from a jGCaMP7s mouse showing similar distribution of increased fluorescence (930-2085 cells/region, median=1433). Sensitivity indices (d') and significance levels (Wilcoxon Ranksum test) across contralateral regions are plotted. **(E)** Summary of median activity levels measured with CaMPARI2 (top; n=4 mice, mice were recorded twice with stimulation for each eye; 81-1455 cells/regions, median=353; *, *$p < 0.05$, paired t-test*). Recording from jGCaMP7s-expressing mice showed similar increases in fluorescence during stimulation (150-2085 cells/regions, median=634; see Methods).

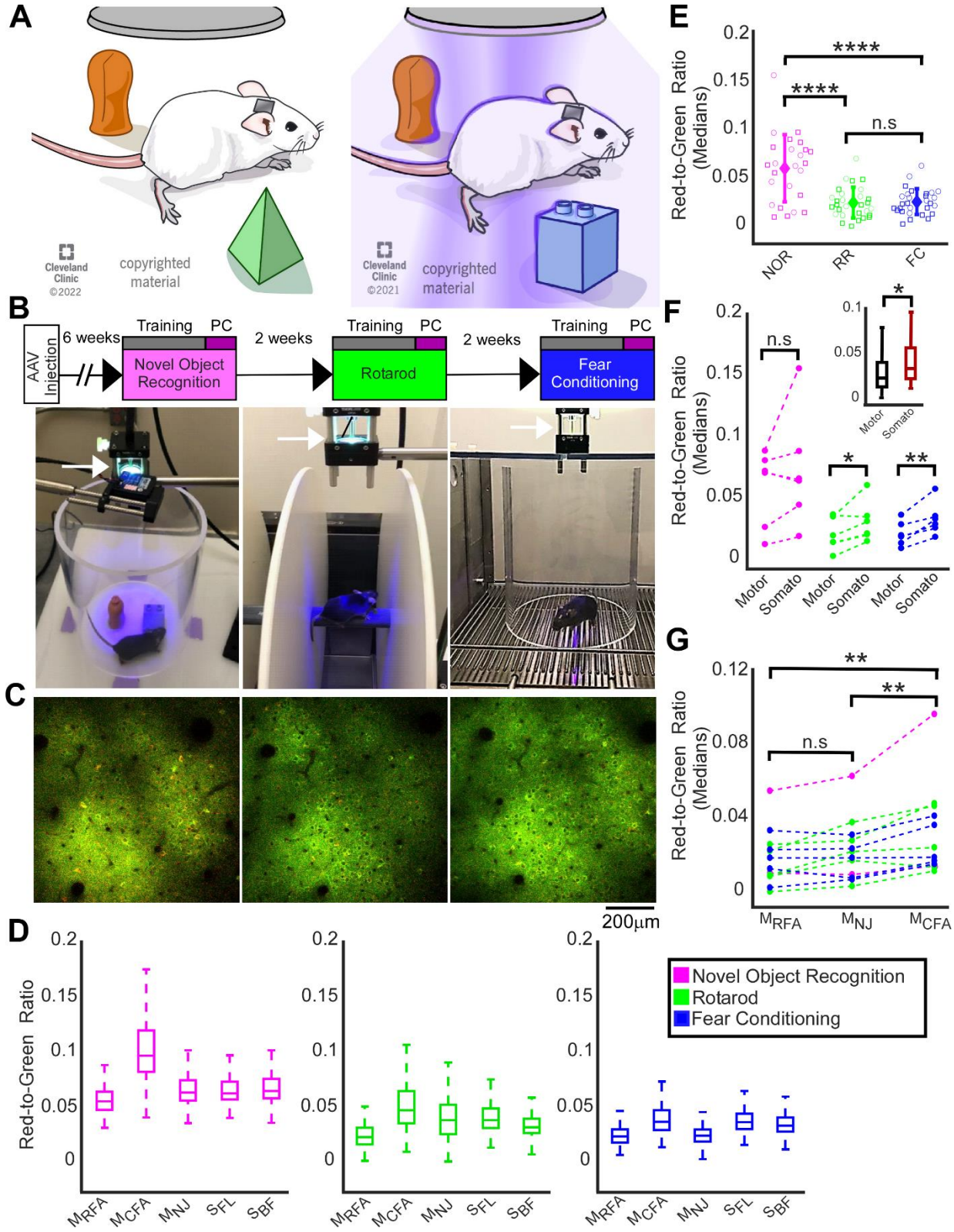

Figure 2: Recording in freely-moving mice during behavioral and cognitive tasks. (A) Schematics of the experimental setup (left)*:* a mouse trains on a task inside an arena without PC illumination. Following the training period, the PC light is turned on to record

cortical activity (right). RGR readout is done 24 hours after the experiment is completed in anesthetized mice using TPLSM. Each mouse was recorded every other week while performing three different tasks. **(B)** Experimental timeline with images taken during novel object recognition (left), rotarod (middle), and fear conditioning (right) tests. The PC light source is indicated by arrows. **(C)** Post-PC images after the three tasks performed in the same mouse 14 days apart. **(D)** RGR from all recorded cells in the five recorded cortical regions (same mouse as in **C**; NOR: 204-819 cells/region, median 547; RR: 166-847 cells/region, median 495; FC: 229-1315 cells/region, median 874). **(E)** Median activity levels (n=6 mice) during NOR were significantly higher than those during RR and FC tests (squares - motor, circles - somatosensory regions; mean ± s.d.; NOR: 42-906 cells/region, median 240; RR: 93-847 cells/region, median 251; FC: 85-1315 cells/region, median 321, **** $p < 0.0001$, Wilcoxon Ranksum test; n.s., non-significant). **(F)** Somatosensory regions were more active than motor regions across all tasks (inset), for individual tasks, increase was significant for the RR and FC task (n=6 mice; $p = 0.26$ *for NOR*, $p = 0.036$ *for RR,* and $p = 0.006$ *for FC;* paired t-test). **(G)** Activity levels in M_{CFA} were significantly higher than in M_{NJ} and M_{RFA} (p=0.006 and 0.002 respectively; n =5 mice, 89-902 cells/region, median 266; paired t-test).

Acknowledgements:

The authors would like to thank Drs. Eric Schreiter and Benjamien Moeyaert and the HHMI Janelia GENIE project for developing and sharing the CaMPARI sensor. We thank Dr. Stefanie Kaech Petrie for her assistance with conducting the TPLSM recording experiments. We thank Drs. Christopher Nelson, Dimitrios Davalos, and Shy Shoham for helpful comments and suggestion to improve the manuscript. JR and HD were supported by grant R21AG065914 from the NIH.

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Methods:

All experimental and surgical procedures were performed following the set guidelines and protocols approved by the Lerner Research Institute and Oregon Health & Science University Institutional Animal Care and Use Committees (IACUCs) and Institutional Biosafety Committees (IBCs), and were consistent with the ARRIVE guidelines. Mice were group-housed in standard vivarium conditions until the start of the study. The vivarium was maintained at 20-21°C and food (in LRI facility: Teklad 2918 regular diet, Envigo;in OHSU facility: PicoLab Rodent Diet 20, no. 5053; PMI Nutrition International) and water were available *ad libitum.* Lights were kept on a 12-hour light: 12-hour dark cycle.

Surgical procedure and virus injection

For detecting visual responses in mice, 8- to 10-week-old C57BL6/J wild-type mice were used. The mice were anesthetized using isoflurane (3% for induction, 1.5% during the surgery) and placed on a heating pad. Each mouse was injected with local pain medication (bupivacaine 0.5%) and the skull bone above the two cortical hemispheres was exposed. A 3x5 mm^2 craniotomy was drilled (Omnidrill35, World Precision

Instruments) over an area covering the monocular and binocular primary visual (V1m and V1b, respectively) and primary somatosensory cortices (S1) in both hemispheres. An adeno-associated virus (AAV) solution expressing the CaMPARI2 sensor (*hsyn*-NES-his-CaMPARI2-WPRE-SV40, Addgene catalog number 101060) was injected into two locations, separated by ~600μm, in each cortical region (50 nL of ~1× 10^{12} GC/mL solution, 3 injection depths per location, 200μm, 400μm, and 600μm under the pia) using an automated injection pump (Fusion 200 touch Syringe Pump, Chemyx, and Micro-2T, WPI) and a pulled and beveled micropipette (P-1000 and BV-10, respectively, Sutter Instruments). Injection coordinates were chosen according to brain atlas coordinates[12]: 2.2 mm lateral and 0.2 mm anterior to Lambda (V1m), 2.8 lateral and 0.2 mm anterior to Lambda (V1b), and 2.5 mm lateral and 3.4 anterior to Lambda (S1). Cortex buffer[13] was used consistently to keep the brain wet during the time of surgery and injections. Following the viral injection, a cranial window (two glued layers of rectangular glass, Tower Optical Corporation) was placed carefully, and a custom-made metallic head bar was attached using dental cement (Contemporary Ortho-Jet, Lang Dental). Animals were injected with Buprenorphine (0.1mg/kg) and Ketoprofen (5mg/kg, immediately, 24, and 48 hours after the surgery) for post-operative care and were allowed a minimal recovery time of 3 weeks before the start of experiments.

For recording activity from awake behaving mice, a similar surgical procedure was used, but the AAV was injected into 5 locations in the mouse left motor and somatosensory cortices, according to microstimulation study[11] and mouse brain atlas coordinates[12]: 1) 1.25/2 mm lateral/anterior to Bregma (rostral forelimb area of the motor cortex, M_{RFA}); 2) 1.25/0 mm (caudal forelimb area of the motor cortex, M_{CFA}); 3) 2/1.5 mm (neck and jaw regions of the motor cortex, M_{NJ}); 4) 2/0 mm (forelimb region of the primary somatosensory cortex, S_{FL}); and 5) 2.5/-1.75 mm (somatosensory barrel field cortex, close to the border with the trunk region, S_{BF}).

Recording of visual stimulus-evoked activity

Mice were lightly anesthetized (0.5% isoflurane), held on a 37°C heating pad, and injected with Chlorprothixene Hydrochloride (IM, 30 μl of 0.33 mg/ml solution, Santa Cruz). PC of the CaMPARI2 signal started at least 30 min after the Chlorprothixene Hydrochloride injection, and after verifying that the mouse was responsive to pain but not voluntarily moving. The visual stimulation was presented to the mouse's right or left eye and generated using the psychophysical toolbox[14, 15] in Matlab (Mathworks) on an LCD monitor (30x36 cm^2 display, located 15 cm in from of the mouse right eye, tilted 45° with respect to the nose line, and covered with a blue plexiglass to minimize contamination into the recording channels) that subtended an angle of ±50° horizontally and ±45° vertically. The visual stimulus consisted of a drifting grating moving in 1 of 8 directions for 4 sec, followed by 8 sec of gray display. This stimulation cycle was repeated 5 times. PC light was delivered for 1 sec during the presentation of the drifting grating, 1.5 sec after

the grating appeared, using an X-Cite Fire lamp (Excelitas) and a 400/40 nm bandpass filter (Brightline, Semrock) with 100 mW output at the sample plane. For recording of visual-evoked activity, the PC light covered a ~12 mm diameter circle that included the two cranial windows in it, with an intensity of ~0.9 mW/mm^2. After PC was completed, CaMPARI2 signal was recorded using a two-photon microscope (Bergamo II, Thorlabs) with 1040 nm excitation light (Insight X3, Spectra-Physics). Images were acquired using ThorImage 4.1 software (Thorlabs) with 15 frames per second and 1024×1024 pixels covering an area of 585×585 μm^2 of layer II/III neurons. Green and red CaMPARI2 signals were recorded simultaneously (525/50 nm and 607/70 nm filters, respectively, separated by a 562 nm dichroic filter, Semrock) using 2 GaAsP PMTs (PMT2100, Thorlabs).

For measuring the decay of red CaMPARI signal over time, we monitored the same V1 neurons immediately after the PC, and during the subsequent days until the red signal returned to its baseline value.

For measuring visual-evoked fluorescence changes with jGCaMP7s[1], we used the same TPLSM system used for the CaMPARI recording (30 frames per sec acquisition with 512x512 pixels and FOV size of 585x585 μm^2). The same drifting grating movie (4 sec of drifting grating followed by 4 sec of blank display) was presented to either the right or the left eye of the mice, and activity was measured from V1b, V1m, and S1 regions of the left cortical hemisphere to sequentially acquire ipsilateral and contralateral activity data.

Recording of cellular activity from freely-moving animals

Following the cranial window implantation, mice were shipped from the Lerner Research Institute to Oregon Health and Science University. Following a quarantine and recovery period of 4 weeks, mice were randomly divided into 2 groups. Each week, one group was trained for 2-3 days on one of three behavioral tasks (see below). Then, we illuminated the mouse cranial windows with PC light during the last trial of a given behavioral test to induce PC. A broadband light source (XCite Xylis, Excelitas) with a 400/40nm bandpass filter (FBH400-40, Thorlabs) and lightly focused by a 100 mm achromat lens (AC254-100-A, Thorlabs) was placed 20 cm above the arena to illuminate a circular cross-section of 15.25 cm in diameter in which the CaMPARI PC occurred. The light intensity was 485 mW, or ~2.65 mW/cm^2. Mice were placed inside a plastic enclosure (16.5 cm diameter; TAP plastics) on matte white plastic flooring to keep them inside the illuminated region, and 15 min of PC illumination were used for each recording to elicit sufficient PC signal (based upon previous experiments we conducted with other mice).

Activity readouts were acquired using two-photon microscopy 24 hours after the PC during the behavioral and cognitive tests. Mice were anesthetized with isoflurane (4% induction, 1.5% maintenance) and put on a heating pad with core body temperature

monitored by a rectal thermometer. Imaging was conducted with a Zeiss LSM 7 multiphoton microscope (Zeiss instruments), which utilizes a femtosecond pulsed Ti:Sapphire laser (Chameleon Ultra II, Coherent), two BiG.2 GaAsP detectors, and Zen imaging software (Zeiss). The laser was tuned to 1040 nm excitation wavelength at 16mW when recording from the brain surface and up to 24mW when imaging 300μm under the pia. Distilled water was placed on the cranial window to image with a 20x/1.0 water immersion objective (Zeiss, 421452-9880). We acquired volumetric data of layer I and II/III neurons (z-stack of ~100 images typically from the brain surface, 512 x 512 pixels, 425 μm field of view (FOV) size, 3μm step size between adjacent images) with green and red channels (500-550nm and 575-610nm, respectively, with a 560nm dichroic filter) from all identified CaMPARI2-injected spots in all animals.

Behavioral testing

1) Exploratory behavior, measures of anxiety, and object recognition were assessed in an open field for a total of 4 consecutive days. Days 1 and 2 included exposure to the open field without objects for 5 min/day. Introduction of two identical objects occurred on day 3, and day 4 consisted of replacing one of the familiar objects with a novel one and 400nm illumination over the entire arena. Both days with objects consisted of 15-minute trials. Behavior was recorded with Ethovision 15 XT software. Camera and PC light guide suspensions over the arena were built with Thorlabs mechanical component.

2) Sensorimotor performance on the rotarod was tested for three consecutive days containing three trials each. Days 1 and 2 consisted of a standard rotarod protocol, with a starting speed of 5 RPM that was increased by 1 RPM every 3 seconds. On the third day, parameters were adjusted to a starting speed of 4 RPM, a maximum speed of 12 RPM, and an increase of 1 RPM every 60 seconds. This ensured that the animals remained on the rod for 5 minutes per trial (3x trials per day) and allowed for full 15-minute illumination while on the rod. Experimenters noted the speed at which the rod was turning when the animal fell off and the duration of time that the animal was able to stay on the rod before falling.

3) Contextual fear memory over two days was tested using a Med Associates mouse fear conditioning system for use with optogenetics (MED-VFC-OPTO-USB-M, Med Associates). During the training day, the animal was placed in the plastic arena (described above) that was located inside a white LED-lit (100 lux) fear conditioning chamber with a metal grid floor. Animals were habituated to the arena for a 300-s baseline period, followed by a 2-s, 0.7mA foot shock, administered a total of 4 times with an interval of 60 s. The following day, animals were placed in the same plastic enclosure within the fear conditioning chamber, and contextual fear memory recall was tested for 15 minutes under illumination. Movement in the chamber and the percentage of freezing were automatically determined by the Med Associates VideoFreeze automated scoring system (MED-SYST-VFC-USB2, Med Associates).

Data analysis

Data analysis was performed using custom MATLAB scripts. For visual stimulation experiments and depending upon the specific experiment, cell bodies were segmented for each FOV using either a semi-automatic algorithm[16], Suite2P softeware[17], or the CellPose software[18]. Both of the latter options allow for automatic segmentation with experimenter quality control. Suite2P was used for segmenting experiments where dynamic recording with jGCaMP7s was used, Cellpose was used to segment data from PC experiments with CaMPARI2, and semi-automatic segmentation was used when one of the automatic approaches yielded poor results. We compared the performance of the three segmentation methods on identical data and found that they produced similar results except for the number of detected cells, which was higher for Suite2P (Supp. Fig. 9). For each recording day, we calculated and corrected the effect of dark current values for the green and red PMT channels by recording images for each channel with the same exposure parameters used for the experiments, but with the laser turned off. Since CaMPARI's green signal penetrates the red channel[9], we also calculated and corrected the red-to-green contamination ratio by recording CaMPARI images before PC (pre-PC) and measuring the contamination ratio. Finally, since the angle of the cortical windows on each hemisphere was different with respect to the microscope's optical axis, we measured a weak component of red channel auto-fluorescence for each hemisphere that was corrected by finding the intercept of the linear regression line of the green and red signals from cells from the same hemisphere before PC (Supp. Fig. 10). Following these corrections, we calculated the post-PC RGR for all cells in a brain region, and used the median value for comparisons across regions.

For light dose calculations, we photoconverted the same brain region several times on the same day and measured the RGR from all cells after each PC. The light dose was calculated as the PC light intensity divided by the illumination cross-section and multiplied by the illumination time.

For identifying possible decay of RGR when imaging deeper into the tissue, we calculated the median RGR ratio for all identified cell within each FOV from each brain region of all recorded mice. We grouped median values from all mice for each brain region, as well as grouped all brain regions from all mice to search for a depth-dependent reduction. For each brain region we restricted the compared depth to the value from which we had data from all mice. For comparing all mice, we evaluated data down to a depth of 240μm, and excluded specific brain regions, which could not be imaged down to this depth. One-way ANOVA test showed no significant difference across the median RGR values acquired at different depths.

For calculating visual-evoked fluorescence changes measured by jGCaMP7s, we calculated the accumulative fluorescence changes above the baseline level (averaged over 0.66 sec before the onset of visual stimulus) during all appearances of the drifting grating stimulation. This value was divided by 2 and then we subtracted the accumulated change of fluorescence during the 2 sec before the appearance of each grating stimulation.

For recording from freely-moving mice, we first averaged every 3 adjacent images (spanning 6 μm in the z-axis) and skipped the next 2 images, in order to generate a set of images with minimal overlap of cells. We performed the same signal corrections as described above and calculated the RGR for all recorded cells and the median values for comparing across brain regions, mice, and tasks.