**NEUROPHYSIOLOGY**

**Tb**$^{3+}$-doped fluorescent glass for biology

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Optical investigation and manipulation constitute the core of biological experiments. Here, we introduce a new borosilicate glass material that contains the rare-earth ion terbium(III) (**Tb**$^{3+}$), which emits green fluorescence upon blue light excitation, similar to green fluorescent protein (GFP), and thus is widely compatible with conventional biological research environments. Micropipettes made of **Tb**$^{3+}$-doped glass allowed us to target GFP-labeled cells for single-cell electroporation, single-cell transcriptome analysis (Patch-seq), and patch-clamp recording under real-time fluorescence microscopic control. The glass also exhibited potent third harmonic generation upon infrared laser excitation and was usable for online optical targeting of fluorescently labeled neurons in the in vivo neocortex. Thus, **Tb**$^{3+}$-doped glass simplifies many procedures in biological experiments.

**INTRODUCTION**

The optical observation and manipulation of cells in living tissues are widespread in current biological research and have revealed diverse biological properties in cells and intercellular communications. In particular, the real-time optical targeting of specific types of cells benefits single-cell electroporation, single-cell transcriptome profiling, and whole-cell recording because recent technical advances in genetic labeling have enabled the online identification of cells expressing fluorescent proteins (1, 2), but it remains difficult to access fluorescently labeled cells using glass pipettes because the glass is not fluorescent in the visible light range. To visualize glass pipettes, some studies load fluorescent dyes inside the pipettes (3, 4). However, the dyes ejected from the pipette tips often mask fluorescently labeled cells. This problem is especially severe in patch-clamp recordings because positive pressure is continuously applied through the pipettes during cell targeting. Other studies coat the surface of pipette tips with chemical fluorophores (5, 6), quantum dots (7–9), or fluorescent silica nanoparticles (10). To date, these techniques have not become popular, probably because the dyes on the tip surfaces make it difficult to form seals with the plasma membrane of target cells (11) and because the dye-coating procedure adds a laborious step and increases the risk of breaking or soiling the glass tips. Moreover, coated dyes are often subject to rapid photobleaching; otherwise, they would be costly.

To solve these problems, we introduce a new composition of borosilicate glass. Rare-earth ions display unique fluorescence emissions that have sharp peaks in the visible light spectrum. The emissions are independent of the environment (12–14) and are often stable even in glass (15–17). Among rare-earth ions, we focused on terbium(III) (**Tb**$^{3+}$). **Tb**$^{3+}$ has complex energy level structures (Fig. 1A), among which the $^7F_6$→$^7D_{4,5}$→$^2F_2$ radiative transition is theoretically expected to emit green fluorescence at a wavelength of 543 nm upon excitation at a peak wavelength of 484 nm (14, 18). This excitation wavelength is near the highest visibility for human eyes (19, 20) and is also close to that of green fluorescent protein (GFP), which has a peak excitation at 475 nm. Thus, pipettes made of **Tb**$^{3+}$-doped glass are versatile for fluorescence-targeted single-cell manipulations, including electroporation, transcriptome analysis, and patch-clamp recording.

**RESULTS**

**Tb**$^{3+}$-doped glass pipettes

We created borosilicate glass containing 3.1 mole percent (mol %) **Tb$_2$O$_3$.** The **Tb**$^{3+}$-doped glass emitted green fluorescence, which was visible to the naked human eye even under room light (Fig. S1). We investigated its transmission spectrum for wavelengths ranging from 200 to 2700 nm (Fig. S2A). **Tb**$^{3+}$-doped glass had an absorption peak at a wavelength of 484 nm, which was not observed in normal borosilicate glass without **Tb**$^{3+}$ (fig. S2B). Because of the popularization of GFP in biology, argon-ion lasers and diode lasers emitting 488 nm are widely used in various experiments, such as fluorescence observation, optogenetic stimulation, and flow cytometry. Upon 488-nm excitation, **Tb**$^{3+}$-doped glass exhibited green fluorescence with a peak wavelength of 542 nm (Fig. 1B). The quantum yield of **Tb**$^{3+}$-doped glass at 488-nm excitation was as high as 68.4%. Similar to the control borosilicate glass, **Tb**$^{3+}$-doped glass was physically stable without crystallization or phase separation even at a high temperature of 750°C for at least 60 min. Grains of the glass had a high hydrolytic resistance at 121°C (table S1). The coefficient of thermal expansion of the glass was 7.5 x 10$^{-6}$ K$^{-1}$, which was also similar to that of the control glass (fig. S3). These features provide **Tb**$^{3+}$-doped glass with plasticity suitable for free molding without loss of fluorescence.

We succeeded in producing glass capillaries (Fig. 1C) and pipettes using conventional micropipette pullers. The molded glass still emitted green fluorescence, and the pipette tips were visible to the eyes using conventional epifluorescence microscopes with xenon or xenon-mercury lamps (Fig. 1D). Scanning electron microscopic inspection did not reveal any aberration of the pipette tips that might affect the quality of electrophysiological recording (Fig. 1E).

The pipette resistance did not differ between the control and **Tb**$^{3+}$-doped glass (P = 0.62, t$_{df}$ = 0.49, n = 48 pipettes, Student’s t test; Fig. 1F). The capacitance of **Tb**$^{3+}$-doped pipettes was slightly higher than that of pipettes without **Tb**$^{3+}$ (P = 8.6 x 10$^{-13}$, t$_{df}$ = 26.9, n = 7

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and 8 pipettes, Student’s t test; Fig. 1G); however, this capacitance was still acceptable for whole-cell patch-clamp recording and did not reduce the recording quality (see below). We found no evidence of photobleaching of Tb³⁺-doped pipettes even under continuous xenon-lamp illumination, in contrast to previously reported dye-coated pipettes and 90th percentiles. The medians and the 25th and 75th percentiles, and the whiskers display the 10th pipette capacitances.

**Fig. 1. Tb³⁺-doped pipettes emit green fluorescence.** (A) Partial energy level diagram of Tb³⁺ [extracted partially from (14)]. (B) Excitation (blue) and emission (green) spectra of 3.1 mol % Tb³⁺-doped glass. (C) Macroscopic color photograph of control (top) and Tb³⁺-doped (bottom) glass capillaries. Photo credit: Yuji Ikegaya, The University of Tokyo. (D) Bright-field monochromatic (top) and fluorescence (bottom) images of the tips of control (left) and Tb³⁺-doped (right) glass pipettes (488-nm laser excitation at 25 mW). Pipettes made of Tb³⁺-doped glass emitted green fluorescence. Photo credit: Kazuki Okamoto, The University of Tokyo and Juntendo University. (E) Scanning electron microscopy image of the tip of a Tb³⁺-doped pipette. Photo credit: Hiroyuki Hioki, Juntendo University. (F) Pipette resistances of control (black) and Tb³⁺-doped pipettes (green). The rectangles show the medians and the 25th and 75th percentiles, and the whiskers display the 10th and 90th percentiles. n = 48 pipettes; Student’s t test. (G) The same as (F) but for pipette capacitances. n = 7 to 8 pipettes, Student’s t test.

**Single-cell gene electroporation using Tb³⁺-doped pipettes**

Using Tb³⁺-doped micropipettes, we first conducted the single-cell electroporation of tdTomato, a red fluorescent protein, into neurons in rat organotypic hippocampal slice cultures. A Tb³⁺-doped pipette containing a pCMV-tdTomato vector and CA1 pyramidal cells that were sparsely labeled with enhanced GFP (EGFP) were simultaneously visualized under an epifluorescence microscope (Fig. 2A, top). An EGFP-positive cell was selected and approached using a Tb³⁺-doped pipette; note that we did not need to switch the light path during the approach, because the pipettes and EGFP have similar fluorescence characteristics and can be visualized using the same optical apparatus. The pipette tips were physically attached to the cells, and electrical pulses were applied for electroporation. After 48 hours, the targeted neurons expressed tdTomato (Fig. 2A, bottom).

**Single-cell RNA sequencing using Tb³⁺-doped pipettes**

We next performed quantitative single-cell RNA sequencing (Patch-seq) (21–23) from parvalbumin (PV)—positive GABAergic neurons in acute slices of the mouse primary motor cortex of PV-GFP transgenic mice (24). A PV-GFP–positive cell was patch-clamped by optical targeting with a Tb³⁺-doped pipette (Fig. 2B, top), and RNA was extracted by applying suction (top). Scale bar, 50 μm. Transcripts per million (TPM) of GFP-positive and GFP-negative cells (bottom). Gray dots indicate all detected gene transcripts. Red dots are representative unique gene transcripts, Praluβ and Gad2 (GAD65) for GFP-positive cell versus Me2c and Slc17a7 (VGLUT1) for non-GFP-positive cell. Photo credit: Kazuki Okamoto, The University of Tokyo and Juntendo University.

In vitro patch-clamp recordings using Tb³⁺-doped pipettes

We conducted patch-clamp recordings from primary cultures of hippocampal neurons that were sparsely labeled with EGFP using a Tb³⁺-doped pipette loaded with red fluorescent Alexa Fluor 594 (Fig. 3A). The targeted cells were filled with Alexa Fluor 594 when successfully held by the pipette in the whole-cell configuration. The same method was also applicable to acute brain slice preparations (Fig. 3B), in which neurons are located deeper in less transparent tissues than cultured neurons. We patched CA1 pyramidal cells in acute hippocampal slices using Tb³⁺-doped pipettes. These neurons...
exhibited normal action potentials in response to brief current injection under the current-clamp configuration. They also exhibited normal spontaneous excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs) under the voltage-clamp configuration. To examine the noise level in the signal recorded using Tb3+-doped pipettes, we monitored miniature EPSCs in the presence of 1 μM tetrodotoxin (fig. S5A). Neither the sizes nor the frequencies of the miniature EPSPs differed between Tb3+-doped pipettes and conventional pipettes without Tb3+ (fig. S5 and C), nor did their signal-to-noise ratio differ (fig. S5D). Tb3+-doped pipettes were used for long-term stable recordings; neither the waveforms (fig. S6A) nor the amplitudes of evoked EPSCs changed for 60 min (P = 0.34, J = 0.41, n = 4 cells, Jonckheere-Terpstra trend test; fig. S6B). Tb3+-doped pipettes were also useful for recordings from neurites (Fig. 3C). After neurons were intracellularly loaded with Alexa Fluor 488 via somatic whole-cell patch-clamp pipettes, dendrites visualized with green fluorescence were targeted using Tb3+-doped pipettes at 488-nm excitation under a spinning-disk confocal microscope. Upon somatic activation, backpropagating action potentials were recorded from the targeted dendrites (Fig. 3C).

**In vivo patch-clamp recordings using Tb3+-doped pipettes**

We next characterized the nonlinear multiphoton excitation of Tb3+-doped glass (Fig. 4A). We illuminated the glass with infrared light at wavelengths that were approximately double the single-photon excitation peak (484 nm) of Tb3+. The Tb3+-doped pipettes emitted green fluorescence that was captured using a photomultiplier tube through a 495- to 540-nm band-pass filter. The 495- to 540-nm emission peaked at an excitation wavelength of 975 nm (Fig. 4B), which suggests that the 975-nm laser excited Tb3+-doped glass through a two-photon absorption process. However, unexpectedly, we could not observe the shapes of the pipette tips (Fig. 4A, middle). This was due to the raster scanning microscopy and the long lifetime of the green fluorescence of Tb3+-doped glass (τ1/2 = 2.8 ms; Fig. 4B, inset). The signal at a pixel, which is detected by the GaAsP photomultiplier tube, corresponds to the focal point of two-photon excitation. However, the laser scanning speed in two-photon microscopy when cells are observed is typically several microseconds per pixel, and the afterglow of the Tb3+ green light led to artifactual signal in pixels where the Tb3+-doped pipette did not exist when the specimens were raster-scanned.

On the other hand, another bright signal at 1300-nm excitation (Fig. 4A, bottom) was observed through a 410- to 450-nm band-pass filter (Fig. 4C). Spectrometric inspections revealed that the emission intensity peaked at a wavelength of 431 nm, which corresponds to nearly one-third of the 1300-nm excitation wavelength (Fig. 4D). Given that the energy levels in Tb3+ are not compatible with two-photon or three-photon excitation by a 1300-nm excitation wavelength (Fig. 1A), the signal likely arose from third harmonic generation (THG). Consistent with this idea, the emission intensity approximated a cubic curve as a function of the excitation intensity (scaling exponent = 3.0, y intercept = 10−2.9; Fig. 4E). The THG had little afterglow, and the pipette tips were visualized by raster scanning (Fig. 4A, bottom). THG is also intrinsic in normal Tb3+-free glass, but the THG of Tb3+-doped glass was stronger than that of control borosilicate glass without Tb3+ [Tb(−); 2.9 ± 0.3 arbitrary units (a.u.), Tb(+); 9.4 ± 2.5 a.u., P = 3.8 × 10−4, t9 = 5.9, n = 5 pipettes, Student’s t test; Fig. 4, F and G]. By taking advantage of the strong THG signal of Tb3+-doped pipettes, we conducted in vivo whole-cell patch-clamp recordings using a multiphoton laser microscope. Layer 2/3 pyramidal cells in the primary motor cortex that had been transfected with adeno-associated virus carrying the tdTomato gene were optically targeted. The cells and Tb3+-doped pipettes were simultaneously visualized using dual-laser irradiation at 1040 and 1300 nm, respectively.
and THG of Tb3+-doped glass provide a platform applicable for multipurpose applications in biological research. Glass pipettes are an irreplaceable material for patch-clamp recordings (25). The composition of the glass has changed little in the past 30 years because the composition of the glass has a notable impact on pipette formation and recording quality (26, 27). Thus, glass pipettes for the patch-clamp technique were not developed, despite significant advances in biological technology. Rather, glass materials have been independently developed in the engineering field. Rare-earth ion doping is a useful technology for optics. Tb3+-doped glass has also been invented for optical purposes to manipulate visible lasers (16). In this work, we proposed a new application of fluorescent glass and improved the targeted patch-clamp technique.

The method of patch clamping has undergone many innovations and developments, including planar patch clamping (28, 29), pipette cleaning (30), and automated patch clamping (31–33). However, “targeted” patch clamping is still difficult to automate, mainly because it requires an automatic image recognition system, in which the tip of the pipette provides a clue as to its location (34, 35). In this respect, Tb3+-doped pipettes may support automation and human experimenters.

The emissions of rare-earth ions derive from the activity of 4f electrons, which are shielded by the 5s5p6-closed shells and are independent of other orbitals. Thus, their fluorescence is stable and is minimally affected by their ligands and environments. Probably because of this stability, Tb3+-doped glass displayed similar excitation and emission to Tb3+ solution and other Tb3+ compounds. We observed two-photon excitation of Tb3+-doped glass, but the long lifetime of its emission may limit its biological application (Fig. 4B, inset). The f-f transition of rare-earth elements is prohibitive and requires intersystem crossing, which may extend the fluorescence lifetime of rare-earth ions (36). Thus, we could not observe the tips of fluorescent pipettes under 975-nm excitation using raster scanning two-photon microscopy (Fig. 4A, middle). The lifetime of emission varies with the codoping of another rare-earth ion, but it is difficult to maintain the emission wavelength of 543 nm for lifetimes longer than 1 ms (37). The reduction in lifetime is thought to be on the order of milliseconds and is insufficient to enable raster scanning in two-photon microscopy.

However, the THG of Tb3+ is short-lived enough to be applicable to photomultiplier-based raster scanning imaging. THG is one of the nonlinear optic phenomena that occur at high densities of photons. Among rare-earth ions, Gd3+ and Yb3+ have been used as ingredients in nonlinear optical crystals (37, 38), but few studies have characterized the nonlinear optics of Tb3+-doped materials. As pulse lasers at 800 nm are reported to induce the three-photon excitation and fluorescence of Tb3+ (39, 40), we observed the fluorescence of Tb3+-doped pipettes upon excitation at 800 nm (Fig. 4B); at

(Fig. 5A). Current injection-induced action potentials (Fig. 5B) and spontaneous membrane fluctuations (Fig. 5C) were recorded under the current-clamp configuration.

**DISCUSSION**

We invented Tb3+-doped glass that emitted green fluorescence strong enough to be recognized by the naked human eye. The glass had similar physical properties to conventional borosilicate glass (except for the fluorescence) and did not exhibit apparent photobleaching or cytotoxicity. Thus, pipettes made of Tb3+-doped glass enabled online fluorescence manipulations, including optical targeting of single-cell electroporation, single-cell RNA sequencing, and electrophysiological recordings. In addition, the glass emitted THG upon three-photon excitation and was applicable for in vivo manipulations. We thus believe that the single-photon green fluorescence and THG of Tb3+-doped glass provide a platform applicable for multiple purposes in biological research.
this wavelength, however, the emission was so weak that we could not observe the tips of the pipettes.

Even without Tb\(^{3+}\), normal borosilicate glass exhibits THG, but its intensity is insufficient for targeted patch-clamp recordings because some biological structures, such as the myelin sheath and blood vessels, emit intrinsic THG at 1300-nm excitation (41, 42) and cause background noise. Tb\(^{3+}\) strengthened the THG of glass to a level suitable for practical use; note that 1300 nm is a zero-dispersion wavelength in glass, which enhances the cubic nonlinear optical effects (Fig. 4, F and G) (43, 44). We observed a strong THG signal in vivo in the neocortical layer 2/3 parenchyma against background THG noise. Near-infrared light of 1300 nm can reach deep brain tissue with little absorption by hemoglobin (45, 46). For example, the three-photon imaging of hippocampal neurons is implemented with 1300-nm excitation (41). However, under our conditions, the THG wavelength of Tb\(^{3+}\) was 431 nm and was susceptible to absorption and diffusion in brain tissues. The problem will be solved with longer-wavelength excitation. Laser wavelengths over 1300 nm have not yet prevailed for multiphoton microscopy, but a 1700-nm laser is already used to observe deep brain tissue (42). Therefore, we expect technical advances in infrared lasers to expand the application of Tb\(^{3+}\)-doped pipettes.

Last, other rare-earth ion doping could also be useful for biological experiments. For instance, Eu\(^{3+}\) and Eu\(^{2+}\)-doped glass emit red and blue fluorescence, respectively (47, 48). Therefore, fluorescent pipettes are available at different wavelengths. In addition to patch-clamp pipettes, rare-earth ion-doped glass can be molded into any shape. For example, glass fibers and nanoparticles made of Tb\(^{3+}\)-doped glass may irradiate a broad area of a deep brain region and may be usable as an optogenetics tool. We believe that the fluorescence emitted by Tb\(^{3+}\)-doped and other rare-earth ion-doped glass can be exploited by new ideas to open new frontiers in biological research.

**METHODS**

**Tb\(^{3+}\)-doped glass capillaries**

Tb\(^{3+}\)-doped glass consisted of 62.6 mol % SiO\(_2\), 4.9 mol % Al\(_2\)O\(_3\), 9.8 mol % B\(_2\)O\(_3\), 14.0 mol % Na\(_2\)O, 5.5 mol % MgO, 3.1 mol % T\(_2\)O, and 0.1 mol % CeO\(_2\). The starting materials were 99.9% pure SiO\(_2\), Al\(_2\)O\(_3\), B\(_2\)O\(_3\), Na\(_2\)CO\(_3\), MgO, T\(_2\)O, and CeO\(_2\). Approximately 300-g batches of the materials were fully melted within a platinum crucible in an electronic furnace at 1500°C. For the first 1 hour, the glass melt was stirred at 1500°C. Then, the melt was poured onto a steel plate, annealed at 630°C for 1 hour, and gradually cooled at a rate of 0.5°C/min in an electric furnace. The glass block was cut into a cylindrical shape (outer diameter: 9.0 mm, inner diameter: 5.1 mm, and length: 100 mm). This tube-shaped glass was pulled at 550° to 650°C to obtain glass capillaries (outer diameter: 1.5 mm, inner diameter: 0.85 mm, and length: 100 mm). The source of Tb\(^{3+}\) was Tb\(_2\)O\(_3\), which contained Tb\(^{3+}\) and Tb\(^{4+}\). By the addition of CeO\(_2\) in batches, we improved the quantum yield of Tb\(^{3+}\)-doped glass from 48.8 to 68.4%. The final Tb\(^{3+}\) concentration in the glass was 3.1 mol %, which corresponded to 1.5 \times 10^{21} ions/cm\(^3\).

**Terbium-doped glass properties**

The excitation and emission spectra were measured through a 0.69-mm-thick glass plate using an F-4500 spectrofluorometer (Hitachi High-Technologies). The transmittance spectra were measured through 1.00-mm-thick glass plates using an ultraviolet-visible/NIR spectrophotometer (V-770, JASCO). The quantum yield was measured in a glass block (10 cm by 10 cm by 10 cm) using Quantaurus-QY (Hamamatsu Photonics). To obtain the image shown in Fig. 1D, the tips of the pipettes were coated with osmium with an osmium plasma coater (OPC80T, Filgen) and observed with a scanning electron microscope (S-4800, Hitachi High-Technologies). Thermomechanical analysis (TMA; TD-5000SA, Bruker) was performed on glass rod samples measured at a heating rate of 5°C/min from room temperature to 680°C. The coefficient of thermal expansion was calculated on the linear section of the curve (range from 50° to 350°C). The glass transition temperature was determined as a change in the slope of the curve. The sag temperature (glass viscosity: approximately \(10^{10}\) dPa-s) was determined by TMA as the point at which the glass rod started to sag (loading weight: 5 g). The dielectric constant was measured with 0.7 mm thickness using a Precision LCR meter (E4980A, Agilent) and the dielectric test fixture (16541B, Agilent). The Tb\(^{3+}\)-doped pipettes were pulled using a P-1000 micropipette puller (Sutter Instrument). The pipette capacitance was measured on the surface of a Sylgard bead (49).

**Animal experiment ethics**

Experiments were performed with the approval of the Animal Experiment Ethics Committee at the University of Tokyo (approval no. P29-3, P29-9) and according to the University of Tokyo guidelines for the care and use of laboratory animals.
Primary culture preparation
Primary cultures were used for in vitro patch-clamp recordings. Dissociated hippocampal cells were prepared from postnatal 0-day-old C57BL/6j mice (50). The hippocampi were dissociated in pre-warmed Hanks’ balanced salt solution and treated with 0.25% trypsin/EDTA at 37°C for 15 min. Next, the tissue was treated with 0.01% deoxyribonuclease I (Sigma-Aldrich) at room temperature for 5 min and washed with Hanks’ balanced salt solution, which was then replaced with Neurobasal plating medium containing 2% B2 supplement, 0.5 mM glutamine, 25 μM glutamate, 0.1% penicillin, 0.1% streptomycin, 1 mM Hepes, and 10% horse serum. The tissues were triturated with Pasteur pipettes and filtered through a 40-μm pore cell strainer (Corning). The dissociated cells were plated onto 13-mm coverslips coated with poly-d-lysine at a cell density of 8.0 × 10⁶ cells per well in culture medium in 24-well plates and were incubated at 37°C in a humidified 5% CO₂ atmosphere. The medium was changed every 3 days. The cells were treated with a solution containing 130 mM CsMe-Cl, 7.0 mM MgSO₄, 2.4 mM CaCl₂, 26 mM NaHCO₃, and 10 mM glucose. The brains were sliced horizontally or coronally at a thickness of 300 μm using a vibratome (VT1200S, Leica) in ice-cold, oxygenated modified aCSF consisting of 222.1 mM sucrose, 27 mM NaHCO₃, 1.4 mM sodium phosphate buffer, 2.5 mM KCl, 1.0 mM CaCl₂, 7.0 mM MgSO₄, and 0.5 mM ascorbic acid, as previously described (51). Slices were maintained for 30 min at 35°C in oxygenated aCSF and then incubated for at least 30 min at room temperature before use.

In vitro electrophysiology
All recordings were performed at 33°C to 35°C. Whole-cell recordings were obtained from hippocampal CA1 pyramidal cells or layer 5 pyramidal cells in the somatosensory cortex using a MultiClamp 700B amplifier and a Digidata 1550A digitizer controlled by pCLAMP10 software (Molecular Devices). Glass pipettes (3 to 6 megohms) were filled with a solution containing 130 mM CsMe-SO₄, 140 mM CsCl, 10 mM Hepes, 10 mM Na₂-phosphocreatine, 4.0 mM MgATP, 0.3 mM Na₂GTP or 120 mM K-gluconate, 5.0 mM KCl, 1.0 mM MgCl₂, 10 mM Hepes, 0.2 mM EGTA, 10 mM Na₂-phosphocreatine, 2.0 mM MgATP, and 0.1 mM Na₂GTP.

Single-cell RNA sequencing
Glass pipettes (3 to 5 megohms) were filled with 1 μl of solution containing 120 mM K-gluconate, 5.0 mM KCl, 1.0 mM MgCl₂, 10 mM Hepes, 0.2 mM EGTA, 10 mM Na₂-phosphocreatine, 2.0 mM MgATP, and 0.1 mM Na₂GTP. While cells were held in the whole-cell voltage-clamp mode, a moderate negative pressure (approximately −50 mPa) was applied for 3 to 8 min. After the cytosol was obtained by this method, the intrappetite solution was pressure-ejected into 100 μl of lysis buffer (LB1 in NucleoSpin RNA Plus XS, Clontech), and total RNA was isolated with NucleoSpin RNA Plus XS (Clontech). Isolated total RNAs were converted to complementary DNA and amplified with the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Clontech). The final sequencing libraries were constructed with the Nextera XT DNA Library Prep Kit (Illumina) and the Nextera XT Index Kit (Illumina). The pooled sequencing library was analyzed on a NovaSeq 6000 (Illumina). Reads were aligned to the mouse genome (mm10 assembly; Ensembl Genome Browser) using STAR (v2.7.3a). Read counts were calculated using RSEM (v1.3.3).

Slice culture preparation and electroporation
Organotypic slice cultures were used for single-cell electroporation. Entorhinal-hippocampal organotypic slices were prepared from 7-day-old Wistar/ST rats as previously described (52, 53). Rat pups were anesthetized by hypothermia and decapitated. The brains were removed and placed in ice-cold oxygenated Gey’s balanced salt solution supplemented with 25 mM glucose. The tissues were sliced horizontally at a thickness of 300 μm using a vibratome (DTK-1500, Dosaka). The slices were placed on Omnipore membrane filters (JHWP02500, Millipore) and incubated in 5% CO₂ at 37°C. The culture medium—which was composed of 50% minimal essential medium, 25% Hanks’ balanced salt solution, 25% horse serum, 0.1% penicillin, and 0.1% streptomycin—was changed every 3.5 days. Phosphate-buffered saline containing AAV-hSyn-EGFP (1.4 × 10¹¹ vg/ml) was dripped onto the slices on DiV 1. Experiments were performed on DiV 12 to 19. For electroporation, pipettes were filled with intracellular solution including pCMV-tdtTomato vector (100 ng/μl; gift from Koyama R) and attached to target cells. Stimulation was 200 electric pulses with a duration of 1 ms and an amplitude of 0.4 V at 200 Hz (54).

Virus injection and surgical procedures in vivo
Postnatal 8-week-old mice (male, C57BL/6j) were anesthetized by an intramuscular injection of ketamine (74 mg/kg) before the surgery. After the mice were anesthetized, a 2 × 4 mm craniotomy was performed over the left forelimb primary motor cortex (approximately 0.2 mm anterior and 1.0 mm lateral to the bregma) as previously described (55). Before virus injection, a pulled glass pipette (broken and beveled to a 25- to 30-μm outer diameter; Sutter Instruments) and a 5-μl Hamilton syringe were back-filled with mineral oil (Nacalai Tesque) and front-loaded with virus solution. Layer 2/3 of the left forelimb primary motor cortex was stereotaxically targeted, and a total of 1 μl of AAV1-CaMKII-Cre (1.6 × 10¹¹ vg/ml) and AAV1-CAG-Flex-tdTomato (4.8 × 10¹² vg/ml) was injected at 0.02 μl/min using a syringe pump (KDS310). After injection, the pipette was maintained in place for 10 min and then slowly withdrawn. A 2-mm by 4-mm glass coverslip (#4 thickness; Matsunami Glass) was pressed onto the brain surface, and its edges were sealed with dental cement. One week after virus injection, the mice were anesthetized with 1% isoflurane and used for recording and imaging. Before the experiment, the glass window was replaced with 3% agarose (w/v). A 3-mm by 3-mm glass coverslip (#1 thickness; Matsunami Glass) was pressed onto the agarose surface, and its edges were sealed with a silicone elastomer (Kwik-Cast; World Precision Instruments).

Multiphoton imaging and in vivo electrophysiology
Multiphoton images were acquired with an FVMPE-RS system (Olympus), a 25× water immersion objective lens (XLPLN25XSMP2, Olympus), and a broadly tunable laser with a pulse width of 120 fs and a repetition rate of 80 MHz (InSight DS+ Dual, Spectra Physics) as previously described (56). The signals passed through emission
filters of 410 to 455 nm or 495 to 540 nm (V30-FVG, Olympus) and were collected using a GaAsP photomultiplier tube (Hamamatsu Photonics). The frame acquisition rate was 1 frame/s, with two galvanometric scanning mirrors for the x and y axes. The pixel dwell time was 2 μs, and the size of the imaging fields was 512 × 512 pixels (0.994 μm per pixel for pipette imaging and 0.249 μm per pixel for in vivo imaging). To take pipette images, pipettes were filled with Milli-Q, and the images were z-stacked. For whole-cell recordings, pipettes were filled with intracellular solution containing 135 mM K-glucuronate, 4.0 mM KCl, 10 mM Hepes, 0.3 mM EGTA, 10 mM Na2-phosphocreatine, 4.0 mM MgATP, 0.3 mM Na2GTP, and 0.2% biocytin.

**THG measurement**

The light source was a regeneratively amplified femtosecond laser (Andor Technology) with a charge-coupled device camera (DU420LC-BU2, Andor Technology) with an oscilloscope with less than 0.5 mW average power through the ND filter. The THG signals collinear to the measurement was performed at room temperature.

**REFERENCES AND NOTES**

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/doi/10.1126/sciadv.abb8299

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