Remote control of neural function by X-ray-induced scintillation

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Scintillators emit visible luminescence when irradiated with X-rays. Given the unlimited tissue penetration of X-rays, the employment of scintillators could enable remote optogenetic control of neural functions at any depth of the brain. Here we show that a yellow-emitting inorganic scintillator, Ce-doped Gd3(Al,Ga)5O12 (Ce:GAGG), can effectively activate red-shifted excitatory and inhibitory opsins, ChRmine and GtACR1, respectively. Using injectable Ce:GAGG microparticles, we successfully activated and inhibited midbrain dopamine neurons in freely moving mice by X-ray irradiation, producing bidirectional modulation of place preference behavior. Ce:GAGG microparticles are non-cytotoxic and biocompatible, allowing for chronic implantation. Pulsed X-ray irradiation at a clinical dose level is sufficient to elicit behavioral changes without reducing the number of radiosensitive cells in the brain and bone marrow. Thus, scintillator-mediated optogenetics enables minimally invasive, wireless control of cellular functions at any tissue depth in living animals, expanding X-ray applications to functional studies of biology and medicine.
Optogenetics has enabled the elucidation of the causal roles of specific neurons in driving circuit dynamics, plasticity, and behavior1,2. Clinical treatment of neurological diseases may also benefit from optogenetic approaches that can control functions of well-defined neural circuits in precise timings3–7. However, the application of optogenetics to deep brain regions usually requires the invasive implantation of optical fibers tethered to an external light source because the stimulating light (wavelength: ~430–610 nm) used to activate light-sensitive proteins is heavily scattered and absorbed by tissues1. These tethered fiber optics, although widely employed, are known to pose diverse problems, including tissue damage, neuro-inflammation, responses, phototoxicality and thermal effects upon irradiation, as well as physical restriction of animal movement8–11. Recent studies have shown that injectable upconverting nano/microparticles, which emit visible light in response to tissue-penetrating near-infrared (NIR) light irradiation, can be used for minimally invasive actuation of neurons deep in the brain12–16. However, even NIR light penetrates only up to several millimeters of tissue. Furthermore, the low upconversion yields of these particles demand high-energy NIR illumination which can cause abrupt tissue heating and photodamage15,16. Non-optical forms of energy delivery to control the activities of specific neuronal populations using magnetothermal17 and ultrasonic18 stimulation have also been explored; however, these approaches are associated with a significantly reduced time resolution compared with optogenetics and are currently restricted by limited compatibility with free behavior.

Here, we report the development of an X-ray-mediated, wireless optogenetic technology, which is practically unconstrained by tissue depth. We employ a scintillator that can absorb the energy of incoming X-ray particles and release it in the form of visible luminescence called scintillation. Scintillators are widely used in various particle detectors, such as X-ray security and computed tomography (CT) scanners. The concept that scintillators could serve as optogenetic actuators has already been proposed19,20. However, it has not yet been proven whether scintillation induced by X-rays can effectively manipulate neuronal functions in behaving animals by activating light-sensitive proteins. Also, it is currently unknown whether scintillators are biocompatible and can be safely implanted in living animals. We found that an inorganic scintillator, Ce-doped Gd₃(Al,Ga)₅O₁₂ (Ce:GAGG), emits yellow scintillation21,22 and thereby can effectively activate red-shifted excitatory and inhibitory opsins. Using Ce:GAGG microparticles, we were able to activate a specific neuronal population in freely moving mice, driving-related behaviors with X-ray irradiation that does not harm radiosensitive cells. Our analysis also shows the biocompatibility of Ce:GAGG microparticles. Overall, this work demonstrates that X-rays can be used to control the function of cells at any tissue depth, expanding the range of X-ray applications in biology and medicine.

Results

Ce:GAGG luminescence activates red-shifted opsins. When irradiated onto a mouse head (Fig. 1a and Supplementary Fig. 1), X-rays readily penetrated through the head skin, skull, and brain tissue, whereas most of the energy derived from NIR and visible light did not reach deep into the brain due to absorption and scattering by tissue. Moreover, X-ray irradiation did not increase the temperature of tissues, whereas NIR illumination with a conventional optogenetic stimulation protocol caused striking tissue heating (Supplementary Fig. 2). These results highlight the distinct advantages of using X-ray-induced scintillation for remote optogenetic control of neural circuits deep in the brain. In this study, we utilized single scintillator crystals of Ce:GAGG which emit yellow luminescence in response to UV or X-ray radiation21,22 (Fig. 1b). These crystals were transparent and non-deliquescent (Fig. 1b). UV-induced photo-luminescence (PL) and X-ray-induced radio-luminescence (RL) of a Ce:GAGG crystal has essentially the same spectrum21,22 (peak wavelength: 520–530 nm; Fig. 1b) because both PL and RL are based on the 5d-4f transitions of Ce³⁺. The RL light yield of Ce:GAGG is reported to be 46,000 photons/MeV21,22.

We first sought opsins that could be effectively activated by the PL of Ce:GAGG. DNA plasmids encoding different opsins were transfected into HEK 293 cells and photocurrents induced by PL illumination were measured (Fig. 1c). For depolarizing opsins, the yellow PL of Ce:GAGG (1.8 mW/cm²) elicited the largest photocurrents in cells expressing the red-shifted opsin ChRmine23 (2.28 ± 0.31 nA, n = 11; Fig. 1d). Regarding inhibitory opsins, the anion channelrhodopsin GtACR125 showed the strongest activation (267.2 ± 80.2 pA, n = 12; Fig. 1e) in response to the PL of Ce:GAGG. PL-irradiation of GFP-expressing cells induced undetectable currents (Fig. 1d, e). Thus, Ce:GAGG PL can activate red-shifted opsins that are used for optogenetic control of neurons. Furthermore, the yellow PL of Ce:GAGG could also induce significant activation of the enzyme rhodopsin BeGCC125 (Supplementary Fig. 3), suggesting that the scintillation of Ce:GAGG could be used for the activation of various light-sensitive proteins.

Bidirectional control of neuronal activities by Ce:GAGG luminescence. We next examined the intensity of Ce:GAGG luminescence required to actuate neuronal activities. Cre-dependent adeno-associated virus (AAV) vectors were injected into the ventral tegmental area (VTA) of DAT-IRæ-Cre mice to induce the specific expression of ChRmine in dopamine (DA) neurons (Fig. 2a and Supplementary Fig. 4a, b). In acute slice preparations, 1-s pulses of the yellow PL of Ce:GAGG elicited depolarizing photocurrents in DA neurons in a PL intensity-dependent manner (Fig. 2b). Irradiation with 3.3 μW/cm² elicited action potentials (APs) in 10 out of 15 DA neurons which were current-clamped to −60 mV, and the rate of PL-evoked APs plateaued at approximately 15 μV/cm² (Fig. 2c, d). When the cells were held at around ~40 mV to exhibit spontaneous APs, 1-min PL illumination (Fig. 2e, f), and 1-s pulsed PL illumination (5–20 Hz; Supplementary Fig. 5) increased the AP rate at an even lower intensity (1.7 μV/cm²). Essentially the same results were obtained in ChRmine-expressing neurons of the medial septum (Supplementary Fig. 6). In VTA-DA neurons expressing inhibitory soma-targeted GtACR125 (stGtACR1; Supplementary Fig. 4c, d), hyperpolarizing photocurrents were induced by PL illumination (Fig. 2g), which attenuated AP generation at 1.7 μV/cm² and maximally suppressed spiking at around 15 μV/cm² PL (Fig. 2h–k). Thus, the activity of ChRmine-expressing or stGtACR1-expressing neurons can be modulated by illumination with Ce:GAGG PL at intensities of a few microwatts.

Ce:GAGG microparticles enable neuronal actuation in vivo. Having identified scintillator-opsin combinations that modulate neuronal activity, we next examined the ability of X-ray-induced RL of the Ce:GAGG crystal to activate neurons in vivo. We first pulverized the Ce:GAGG crystal into particles with an average size of 2.3 μm (named scintillator microparticles, SMPs; Fig. 3a), to be injected into the brain. The SMPs injected in vivo (50 mg/ml, 600 nl) formed clusters with an average diameter of 119 ± 13 μm (n = 12 mice). The intensity of RL emitted by SMPs, which was proportional to the dose rate of X-irradiation (Supplementary Fig. 7), can reach ~2 μW/cm² near the injection site at the VTA.
with X-irradiation onto the mouse head at a rate of 1.0 Gy/min (Fig. 3b). We next induced the expression of ChRmine in VTA-DA neurons using AAV injections and injected the SMPs (50 mg/ml, 600 nl) at the same location as the AAV injection (Fig. 3c). X-irradiation for a total of 5 min (1-min pulses every 2 min, five times, at 0.5 or 1.0 Gy/min) induced c-Fos expression in a larger fraction of ChRmine-expressing neurons compared to control conditions (Fig. 3d, e and Supplementary Fig. 8). The fraction of c-Fos-positive cells increased as the dose rate of X-irradiation increased. Thus, the SMPs injected in vivo can activate neurons in an X-ray-dependent and opsin-dependent manner.

We further assessed the biocompatibility of the scintillator. The bioactivity of the scintillator was evaluated by measuring the number of surviving cells after 7 days in culture. The number of surviving cells was not different between the opsin-expressing and GFP-expressing groups (Fig. 3d, e and Supplementary Fig. 8). The fraction of c-Fos-positive cells increased as the dose rate of X-irradiation increased. Thus, the SMPs injected in vivo can activate neurons in an X-ray-dependent and opsin-dependent manner.

Bidirectional change of behaviors induced by scintillator-mediated optogenetics. We finally tested whether the scintillator-mediated activation of neurons in vivo could induce behavioral changes. Transient activation and inhibition of DA neurons in the VTA are sufficient for behavioral conditioning27-29. We therefore induced the expression of excitatory ChRmine or inhibitory stGtACR1 in VTA-DA neurons through viral injections and bilaterally injected SMPs in the VTA (Fig. 5a). The conditioned place preference (CPP) test was performed by placing the mice into a test chamber with two compartments, only one of which was irradiated with either X-ray pulses (50 ms, 10 Hz, 10 times every 30 s) through an X-ray chopper wheel (Pulsed conditioning) [PC]; Fig. 5b, c and Supplementary Fig. 11) or continuous X-ray irradiation (Free moving conditioning [FC]; Fig. 5d, e and Supplementary Fig. 11). The initial place preference was not different between the opsin-expressing and GFP-expressing control mice. After PC, however, mice expressing ChRmine had a significantly higher preference for the X-ray
conditioned compartment than control mice (Fig. 5f, g), whereas those expressing stGtACR1 had a lower preference for the conditioned compartment after FC (Fig. 5h, i). The CPP score of GFP-expressing mice was similar to that of control mice conditioned without X-irradiation (Fig. 5f), suggesting that X-irradiation itself has no effect on place preference. Thus, scintillator-mediated remote optogenetics can be used for bidirectional neuronal actuation deep in the brain of mice, resulting in behavioral changes.

The total dose of X-irradiation during the behavioral tests is far below the threshold for acute neuronal and vascular dysfunction in the brain. X-irradiation corresponding to the FC (~7 Gy) did not change locomotor behavior (Supplementary Fig. 12) and blood–brain barrier function (Supplementary Fig. 13). In a long-term observation, all the mice that experienced the FC survived for at least 8 weeks after radiation with no significant difference in body weight from non-X-irradiated control mice at the same age (Supplementary Fig. 14a). These results suggest that scintillator-mediated optogenetics can be used for days-long behavioral experiments in mice even with a relatively high dose of radiation. On the other hand, the total body X-irradiation is known to damage radiosensitive cell populations, depending on its dose.
cumulative dose. A high-dose X-irradiation reduced the number of immature neurons in the hippocampal dentate gyrus (Fig. 6a, b) through apoptotic cell death of neuronal precursor cells \(^{32,33}\) (Supplementary Fig. 15). Consistent with previous reports \(^{32,33}\), hippocampal neurogenesis was partially impaired, and the number of immature neurons was not recovered at 8 weeks after the FC radiation (Fig. 6a, b; Supplementary Fig. 14b–f). However, the PC, which causes considerably less radiation dose (~0.5 Gy), induced neither loss of immature neurons (Fig. 6a, b) nor apoptosis (Supplementary Fig. 15) in the hippocampus. Although a high-dose X-irradiation caused an overall reduction in the number of bone marrow cells \(^{34}\) (Fig. 6c) without specificity of cell-types (Supplementary Fig. 16), the PC rather increased the number of the total bone marrow cells (Fig. 6c) presumably due to radioresistance effects \(^{35}\). Increasing the total radiation dose to ~1.5 Gy (three times the PC dose) significantly reduced the number of immature neurons in the hippocampus (Fig. 6a, b), whereas four times (~2 Gy) or less PC did not reduce the number of total bone marrow cells (Fig. 6c). Thus, reducing the radiation dose to less than two times of PC will allow for the safer application of scintillator-mediated optogenetics.

**Discussion**

We have here demonstrated the feasibility of a scintillator-mediated optogenetic technology that allows full wireless control of neuronal activity in behaving animals. Our electrophysiological recordings (Fig. 2; Supplementary Figs. 5 and 6), c-Fos-induction

![Figure 3](image-url)
Fig. 4 CeGAGG microparticles were non-cytotoxic and biocompatible. **a** Top, dissociated hippocampal neurons cultured with a CeGAGG crystal (upper). Bottom, the survival rates of dissociated neurons cultured with or without a CeGAGG crystal (n = 7 dishes for each group, F_{1(2,4)} = 2.18, p = 0.15; two-way ANOVA). **b, c** Left, representative confocal images of immunoreactivity against NeuN (green) at the injection site of vehicle or SMPs, and at the ventral tip of an implanted optical fiber with a diameter of 200 μm or 400 μm, at one week (1 w) (b) or four weeks (4 w) (c) after surgery. The trace of SMPs or optical fiber is outlined by a dashed line. Blue: DAPI. Right, an average number of NeuN-positive cells counted in a 100 μm × 100 μm square near the injection/implantation traces. 1 w: Vehicle, n = 3 mice; SMPs, n = 3 mice, p = 0.633, 200-μm fiber, n = 3 mice, p = 0.022; 400-μm fiber, n = 3 mice, p = 0.0016. 4 w: Vehicle, n = 3 mice, SMPs, n = 3 mice, p = 0.106; 200-μm fiber, n = 4 mice, p = 0.0277; 400-μm fiber, n = 3 mice, p = 0.0121; Dunnett’s multiple comparison test vs. the vehicle group, two-sided. Neuronal loss was found near the optical fiber traces, but not around implanted SMPs. **d** Mean number of NeuN-positive cells in a 100 μm × 100 μm square at different distances from the injection/implantation traces at 1 w or 4 w after surgery. 1 w: n = 3 mice for each group; 4 w: Vehicle, n = 3 mice, SMPs, n = 3 mice, 200-μm fiber, n = 4 mice, 400-μm fiber, n = 3 mice. 1 w-Vehicle, F_{1(2,6)} = 0.114, p = 0.894; SMPs, F_{1(2,6)} = 0.643, p = 0.559; 200-μm fiber, F_{1(2,6)} = 4.7, p < 0.0001; 400-μm fiber, F_{1(2,6)} = 4.83, p = 0.0563; one-way ANOVA. 4 w-Vehicle, F_{1(2,6)} = 3.34, p = 0.106; SMPs, F_{1(2,6)} = 1.11, p = 0.390; 200-μm fiber, F_{1(2,9)} = 9.88, p = 0.0054; 400-μm fiber, F_{1(2,6)} = 3.80, p = 0.0857; one-way ANOVA. 200-μm fiber: 1 w, 0.1–0.2 mm, ***p = 0.0001; 1 w, 0.2–0.3 mm, ***p = 0.0001; 4 w, 0.1–0.2 mm, **p = 0.0072; 4 w, 0.2–0.3 mm, **p = 0.0072; Dunnett’s multiple comparison test vs. the 0–0.1 mm group, two-sided. Open circles and lightly colored lines indicate individual data. N.S., not significant. Values are mean ± SEM.

experiments (Fig. 3), and behavioral experiments (Fig. 5) collectively suggest that scintillation of CeGAGG particles could bi-directionally manipulate the activities of opsins-expressing neurons in vivo by X-irradiation. The scintillator particles were biocompatible and injectable and remained at the injection site for long periods without notable cytotoxicity (Fig. 4), serving as minimally invasive optogenetic actuators. The thermal effects on neuronal activity were negligible using this technology (Supplementary Fig. 2), a significant advantage over conventional10,11 and NIR-mediated15,16 optogenetics. Besides, scintillator-mediated optogenetics does not require chronic implantation of large devices such as power modules15,36,37, which would be another advantage over existing LED-based wireless optogenetic methods. Using a careful radiation dose setting, this technology can be safely applied in a variety of rodent behavioral experiments such as those with more naturalistic, context-rich settings, which are normally hindered by the tethered fiber optics, or the large implant on the head in other wireless optogenetic technologies15,36. By employing flexible biomaterials containing scintillator particles, it might be possible to control nerve activities in the spinal cord and peripheral nervous system of freely moving rodents with less invasive procedures than practiced in conventional37,38 or NIR-mediated39 optogenetics. Given the unlimited tissue penetration of X-rays, scintillator-mediated optogenetics may also be applied to larger animals, including monkeys.

Scintillator-mediated optogenetics would also be advantageous for a combination of neuronal manipulations with electrophysiology or imaging because tethered optic fibers or implanted devices often limit the physical space for simultaneous recording. For example, large-scale Ca^{2+} imaging from the cerebral cortex in head-restrained40 or freely moving41,42 rodents can be combined with X-ray-mediated, wireless manipulation of the activities of specific neurons in subcortical structures, which is otherwise practically impossible. In chronic electrophysiology or endoscope imaging, the implantation of a microdrive or a miniature microscope on the skull makes it difficult to be combined with optogenetics apart from photo-stimulation around the recording site. However, with scintillator-mediated optogenetics, it would be much easier to manipulate neuronal activities at any target region by injecting AAV and SMPs before implantation.

The total radiation dose of our PC procedure (~0.5 Gy), which is roughly equivalent to the dose of a single perfusion CT
Fig. 5 Scintillator-mediated wireless optogenetics drove conditioned place preference and aversion in freely behaving mice. a Schematic of the experiment. Inset: an epi-fluorescence image showing SMPs (dashed outline) injected at dorsal VTA. Green: ChRmine-eYFP, Blue: DAPI. Similar results were obtained in 21 mice. b CPP test chamber for “Pulsed conditioning” (PC). The X-ray shieldings and the X-ray wheel chopper are made of lead (Pb). c Time course of CPP test with PC. The gate between two compartments is open for pre-tests and post-tests but closed during PC. d CPP test chamber for “Free moving conditioning” (FC). e Time course of CPP test with FC. The gate between the two compartments is open throughout the test. f Quantification of CPP with PC (n = 9 mice for no radiation control [No rad.] group, 6 mice for hrGFP group, 8 mice for ChRmine group). Pre-test (top), F(2,20) = 1.48, p = 0.252; post-test (bottom), F(2,20) = 19.0, p < 0.0001; one-way ANOVA. Post-test (bottom): No rad. vs. hrGFP, p > 0.9999; No rad. vs. ChRmine, ***p < 0.0001; hrGFP vs. ChRmine, ***p = 0.0001; Bonferroni’s multiple comparison test, two-sided. g Representative tracking data (left) and the corresponding heat maps (right) for mice before (Day 0) and after (Day 5) PC, as extracted from oblique-view movies. h Quantification of CPP with FC (n = 10 mice for hrGFP group, 9 mice for stGtACR1 group). Pre-test (top), p = 0.243; post-test (bottom), p = 0.0435; Mann–Whitney U test, two-sided. I Same as g, but for mice before (Day 0) and after (Day 3) FC. N.S., not significant. Open circles indicate individual data. Values are mean ± SEM.

scan, is more than 100 times lower than the standard dose of radiotherapy for brain tumor treatment (50–70 Gy30,31). Our experiments on radiation toxicity revealed that the maximal radiation dose for safer experiments using free moving mice would be around 1 Gy. This dose level corresponds to 2400 pulses of 50 ms optogenetic stimuli, which enables repetitive interrogations of neural functions in single animals. Blockade of neuroinflammation is ameliorated impairment of neurogenesis caused by radiation45, which may increase the applicability of our technology for longer-term experiments. Although we have shown that scintillator-mediated optogenetics can be applied to behavioral experiments at a non-toxic cumulative dose, increased safety in the use of this technology can be achieved by focal X-irradiation of the brain, which prevents radiation exposure to other organs. In experiments using head-restrained animals, simple shielding would enable such focal X-irradiation. Stereotactic focusing of small radiation beams on specific brain regions, as was achieved in gamma knife surgery46, may provide the further safer application of this technology.

Since Röntgen's discovery in the late 19th century47, X-rays have been widely used for medical imaging and cancer therapy. However, X-rays have never been used to control the physiological functions of cells in living animals, as we have shown here. The development of scintillator-mediated optogenetics thus...
expands the application of X-rays to functional studies of biology and medicine. Biomedical technologies that use visible light for genome editing48 or control of intracellular signaling49 would benefit from wireless applications targeting deeper tissues, which is now possible with scintillator-mediated approaches.

There have been many potential candidates for scintillator materials that could be used in X-ray-based optogenetics19,20. The Ce:GAGG crystal has been one of the scintillators with the highest light yield21,22. However, the Ce:GAGG crystal is also a fluorescent material with a relatively broad emission/excitation spectrum22, which hampers simultaneous imaging and neuronal manipulations in the same neuronal populations. Another limitation of the current technique is that the intensity of the RL emitted by Ce:GAGG microparticles in vivo is not high enough to instantaneously induce action potentials in neurons with millisecond temporal precision. Clearly, our study is therefore only a first step toward establishing safe and efficient X-ray-based optogenetics for controlling cellular functions. This study demonstrates the feasibility of the use of X-rays for functional studies, providing evidence for bi-directional modulation of neuronal functions in behaving animals by X-ray irradiation. Future improvements in light yields of scintillators, engineering of opsins-bound scintillator nanocrystals, and combination with focused X-irradiation will all contribute to allowing control of cellular functions over larger volumes of tissue with less risk of radiation toxicity.

**Methods**

**Scintillator preparation.** The single crystal Ce:GAGG was synthesized using the conventional Czochralski method21,22. For electrophysiology (Figs. 1 and 2, and Supplementary Figs. 5 and 6) and other measurements (Supplementary Figs. 2, 3, and 9), the crystal was fabricated into 3–8 mm rectangular blocks 0.5–1 mm thick. For injection of the crystals in the mouse brain, we pulverized the Ce:GAGG crystals into particles using a planetary ball mill. These particles were further crushed in an agate mortar and collected in ethanol. The ethanol solution containing the particles was sonicated for 10 min, and smaller particles were obtained after evaporation of the remaining vehicle. SMPs (Supplementary Fig. 7) were measured with a spectrophotometer (QE-Pro, Ocean Optics). RL emission spectra under X-ray irradiation (70 kV, 1 mA) were measured through an optical filter fabricated from UV-cut goggles (SSUV filter). The photodiode sensor (1 cm × 1 cm; PD300-1W, Ophir) placed over the recording chamber was used to convert the RL intensity for electrophysiological recordings to luminescence intensity (in watts). For 3D simulation of the RL intensity (Fig. 3b), we assumed that the average RL intensity at the surface of a spherical aggregate of the SMPs corresponds to the measured RL power (Supplementary Fig. 7). RL emission spectra under UV illumination (340 ± 10 nm, LAX-103, Asahi Spectra) were measured using a spectrometer (QE-Pro, Ocean Optics). RL emission spectra under X-ray irradiation (70 kV, 1 mA) were measured through an optical fiber using a CCD spectrometer (DU-420-BU2, Andor)22.

**Plasmids.** For expression in HEK293 cells, all plasmids encoding opsin and fluorescent proteins were constructed by subcloning into an empty pCMV vector unless otherwise noted. pCMV-PsChR-Venus and pCMV-C1V1-Venus were obtained from H. Yawo (Tokyo University). RhoCtChR-Ts-eYFP and ChRm1ne-eYFP were isolated from pAAV-CaMKIIa-DIO-bRedAches-Ts-eYFP and pAAV-CaMKIIa-DIO-ChRm1ne-eYFP, respectively, both of which were gifted by K. Deisseroth (Stanford University). A full-length gene encoding BeGCl1 (accession number KF309499) was synthesized after human codon optimization and inserted into the pCwP-N1 vector. For AAV production, pAAV-E1aΔ-DIO-ChRm1ne-eYFP-WPRE was obtained from K. Deisseroth (Stanford University) and pAAV-hSyn1-S10tGtACR1-FusionRed was obtained from Addgene (#105678).
Cell culture and transfection. For electrophysiological recordings in cultured cells, expression vector plasmids encoding opsins or hrGFP were transfected into HEK 293 cells using FuGene HD Transfection Reagent (Promega) with 2 μg plasmid and the pGloSensor-42F cGMP vector (Promega) using Lipofectamine 2000 (Thermo Fisher Scientific). After transfection, the cultured neurons were maintained in the medium in an incubator at 37 °C with 5% CO2/95% air for 24–36 h before recordings.

Dissociated hippocampal neurons were prepared from embryonic (E17.5) mice. Isolation of the hippocampal formation was performed using a steel dental drill. The hippocampal formation was then transferred into cold dissection buffer containing (in mM): 87 NaCl, 25 NaHCO3, 25 D-glucose, 2.5 KCl, 1.25 NaH2PO4, 0.5 CaCl2, and 7 MgCl2, and then washed three times with HBSS. The tissues were then dispersed by pipetting in a Neurobasal medium (Thermo Fisher Scientific). After removing aggregated cells by filtration, the hippocampal cells were seeded on a coverslip (12 mm diameter) coated with poly-L-lysine in DMEM (Sigma-Aldrich) and incubated for 4 h at 37 °C. The culture medium was subsequently replaced by Neurobasal medium supplemented with 0.5 mM GlutaMAX (Thermo Fisher Scientific), 2% (vol/vol) B-27 (Thermo Fisher Scientific), 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The cultured neurons were maintained in the medium in an incubator at 37 °C with 5% CO2 and 95% air.

Acute slice preparation. The mice were anesthetized with 5% iso-flurane in 95% O2 and 5% CO2. The mice were then decapitated, and the brain was isolated and cut into m-thick horizontal sections on a vibratome in the ice-cold dissection buffer.

In vitro electrophysiology. Whole-cell patch-clamp recordings from cultured HEK 293 cells or neurons in acute brain slices were performed using an IPA amplifier (Sutter Instruments) at RT. Fluorescently labeled cells were visually identified using an upright microscope (BX51WI; Olympus) equipped with a scientific-grade complementary metal-oxide-semiconductor (sCMOS) video camera (Wondershare Filmora scrn). The ears of mice were tracked of video data, which were then converted to a time course for each mouse using the SutterPatch software.

Conditioned place preference test. More than 2 weeks after AAV injection, mice were bilaterally injected with SMPs (50 mg/ml in Ringer’s solution) and used for behavioral tests at >1 week after SMP injection. Conditioned place preference for stGtACR1-expressing mice were performed in a two-compartment aversive conditioning chamber (CAB; Asepta) under white LED lights at the dark period of the 12:12-h light/dark cycle. We used two types of test chambers. Both chambers had two compartments with different floor textures, only one of which was irradiated with X-rays. To restrict the X-ray irradiation (X-irradiation) to one side, the other compartment was shielded with lead (except for a small hole to allow Entry/Exit). Therefore, the stimulus of X-irradiation only in one compartment (150 kV, 3 mA; 50 ms duration, 10 pulses at 10 Hz, every 30 s), which we call “Pulsed conditioning” or “PC” (Fig. 5c). On the sixth day, the mice were placed in Chamber I and freely explore for 10 min without X-irradiation. For CPP tests using stGtACR1-expressing mice and corresponding control mice, the mice were habituated to Chamber I (15 min/day, three sessions). On the first day of the tests, mice were placed in the test chamber and allowed to freely explore for 10 min without X-irradiation. On the following 4 days, the mice were locked in either of the two compartments for 15 min each and received pulsed X-irradiation only in one compartment (150 kV, 3 mA; 50 ms duration, 10 pulses at 10 Hz, every 30 s), which we call “Free moving conditioning” or “FM” (Fig. 5e). On the fourth day, the mice were placed in Chamber II for 10 min without X-irradiation. On the first day and last day of the tests, the mice were placed in an oblique angle of an AC380P18 (Jasco) video camera which was illuminated by ambient white LED light. Movies taken by the camera were displayed on a computer screen using Acamap and recorded online using Wondershare Filmora scrn. The ears of mice were tracked offline using DeepLabCut2D. After completion of the CPP tests, the mice were perfused with 4% PFA and the brain was post-fixed overnight. In some cases, the brains were sectioned coronal sections (section thickness: 80 μm) and the SMP injection sites were observed. The traces of the SMP injections were found at ±0–200 μm away from the dorsal edge of VTA. In some cases, these injected SMPs were found along the injection track as well (Fig. 5a).

Intracranial cannula placement. The ears of mice were tracked of video data, which were then converted to a time course for each mouse using the SutterPatch software.

Immunostaining. For immunostaining of brain slices, we performed transcardial perfusion and post-fixation overnight using 4% paraformaldehyde (PFA). The fixed brains were sectioned into coronal slices on a vibratome (section thickness: 80 μm) or using a cryostat (after immersion of the fixed brain in 30% sucrose solution for >2 days at 4 °C; section thickness: 40 μm). The slices were washed three times with a blocking buffer containing 1% bovine serum albumin (BSA) and 0.2% Triton-X in phosphate buffered saline (PBS) and then incubated with primary antibodies (anti-tyrosine hydroxylase, rabbit polyclonal, 1:1000, Merck Millipore; anti-Iba1, rabbit monoclonal, 1:5000, Wako; anti-GFAP, mouse monoclonal, 1:1000, Merck Millipore; anti-NeuN, mouse monoclonal, 1:500, Merck Millipore; anti-mouse serum albumin, goat polyclonal, 1:1000, Abcam; anti-doublecortin, rabbit polyclonal, 1:1000, Millipore; anti-c-Fos, rabbit polyclonal, 1:1000, Millipore; anti-choline acetyltransferase, rabbit polyclonal, 1:1000, Millipore). Slices were then incubated with secondary antibody conjugated to Alexa Fluor 488 (Invitrogen, 1:200) at 4 °C. After washing buffer overnight at 4 °C. Only for immunostaining of NeuN, the slices were incubated with the primary antibody for ~35–40 h at 4 °C. The slices were then stained and visualized with a confocal microscope (Zeiss LSM 710) using appropriate filters.
Data analysis. The number of HEK 293 cells in 35-mm culture dishes (Supplementary Fig. 9) was counted by randomly selecting 100 μm × 100 μm squares surrounding the CeGAGG crystal for the dishes containing the crystal or anywhere on the coverslip for control dishes. The mean cell density was calculated as the average of the cell densities of three sites per dish.

To estimate the survival rate of dissociated hippocampal neurons (Fig. 4a), we randomly selected three 0.2 mm × 1.0 mm rectangles per dish, surrounding the CeGAGG crystal (one of the longer sides of the rectangle was attached to the edge of the crystal) for the dishes containing the crystal or anywhere on the coverslip for control dishes. The dissociated neurons within these sites at DIV1 were monitored at DIV2, 4, and 7.

To quantify the glial accumulation in epi-fluorescence images (Supplementary Fig. 10a, b), binary images were obtained by thresholding. A 100 μm × 100 μm square was drawn around the trace of the SMPs or the optical fiber (one of the sides of the square was attached to the edge of the trace; three sites per image), and the number of fluorescent cells exceeding the threshold in these squares was calculated using ImageJ. Likewise, the average number of NeuN-positive cells (Fig. 4b–d) in 100 μm × 100 μm squares drawn around the SMP/optical fiber trace in confocal images (three sites per image) was calculated using ImageJ.

To analyze animal movements in the CPP tests using a Python package of DeepLabCut23, the left and right ears of mice were manually annotated using 20–80 frames per movie to train a deep neural network. The animal trajectories and heat maps (Fig. 5g, i) were generated using custom-made Matlab programs. An estimated error position with a low likelihood (<0.8) was omitted and replaced with a pixel value obtained using linear interpolation of neighboring values. The middle pixel coordinate between the tracked left and right ears was considered the animal trajectory, assuming that the coordinate represented the location of the mouse head. The heat maps shown in Fig. 5g, i indicate the probability of the presence of the animal trajectory within each 100 × 100-pixel image. In the CPP tests, two mice that showed a substantially biased preference for one of the two compartments (>85%) on the first day were excluded from the analysis. All values are expressed as mean ± SEM. Statistical tests were performed using GraphPad Prism or Igor Pro. The normality of data distribution was routinely tested. Analyses of two-sample comparisons were performed using unpaired or paired t-tests when at least one of the samples in every two-sample comparison was not normally distributed. Tests for two-sample comparison were two-sided. Statistical analyses for multiple comparisons were carried out using one-way or two-way ANOVA followed by Bonferroni’s multiple comparison tests or Dunnett’s multiple comparison tests vs. the control unless otherwise noted.

Data availability
The data used to generate figures that support the findings of this study are freely available in the Open Access CERN database Zenodo: https://zenodo.org/communities/ty-lab-data with DOI hyperlink: https://doi.org/10.5281/zenodo.4964867. Source data are provided with this paper.

Code availability
The Matlab codes used to generate Figs. 3b and 5i are freely available on GitHub: https://github.com/juniti-y/Matsubara_et_al_Nat_Commun_2021. Received: 10 May 2021; Accepted: 30 June 2021; Published online: 22 July 2021

References
1. Yihzar, O., Fenno, L. E., Davidson, T. J., Mogri, M. & Deisseroth, K. Optogenetics in neural systems. Neuron 71, 9–34 (2011).
2. Kim, C. K., Adhikari, A. & Deisseroth, K. Integration of optogenetics with complementary methodologies in systems neuroscience. Nat. Rev. Neurosci. 18, 222–235 (2017).
3. Kravitz, A. V. et al. Regulation of parkinsonian motor behaviours by optogenetic control of basal ganglia circuitry. Nature 466, 622–626 (2010).
