A luciferase prosubstrate and a red bioluminescent calcium indicator for imaging neuronal activity in mice

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Although fluorescent indicators have been broadly utilized for monitoring bioactivities, fluorescence imaging, when applied to mammals, is limited to superficial targets or requires invasive surgical procedures. Thus, there is emerging interest in developing bioluminescent indicators for noninvasive mammalian imaging. Bioluminescence imaging (BLI) of neuronal activity is highly desired but hindered by insufficient photons needed to digitalize fast brain activities. In this work, we develop a luciferase prosubstrate deliverable at an increased dose and activated in vivo by nonspecific esterase. We further engineer a bright, bioluminescent indicator with robust responsiveness to calcium ions (Ca2+) and appreciable emission above 600 nm. Integration of these advantageous components enables the imaging of the activity of neuronal ensembles in awake mice minimally invasively with excellent signal-to-background and subsecond temporal resolution. This study thus establishes a paradigm for studying brain function in health and disease.
Fluorescence imaging is the standard method for following brain activity in small behaving animals. Genetically encoded fluorescent indicators, including GCaMPs and other Ca$^{2+}$, voltage, and neurotransmitter indicators, allowed the tracking of neuronal activities in specific brain regions and cell types in mammals with the high spatiotemporal resolution for extended periods. Despite the progress, fluorescence neuronal imaging is practically invasive and only reaches a shallow depth. Cranial windows or thinned skulls are often used to access the cortex. Due to tissue absorption and scattering, the imaging depth is ~200 μm for widefield one-photon and a couple of millimeters with multiphoton excitation. To reach deeper brain regions, more invasive procedures, such as implanting optical fibers or gradient-index (GRIN) lenses, are needed.

Bioluminescence, which refers to photon emission from luciferase-catalyzed exothermic oxidation of the corresponding luciferin, is a promising imaging modality for noninvasive in vivo recording. Because bioluminescence needs no excitation, photons emitted from the embedded light sources can travel through several centimeters of mammalian tissue. In addition, compared to fluorescence, BLI has a low background, no photobleaching and phototoxicity, and minimized disturbances of light-sensitive biological components (e.g., the circadian system). Moreover, BLI has excellent compatibility with popular optogenetic and optochemical tools. Macroscopic BLI only requires a dark box with a sensitive camera. Similar setups are already in many laboratories and core facilities; otherwise, they are commercially available or can be constructed at affordable costs.

Commonly used luciferase-luciferin pairs originate from either insects or marine organisms. Insect luciferases are generally slow in catalysis and consume adenosine triphosphate (ATP) for luciferin activation and photon production. In contrast, the oxidation of the coelenterazine (CTZ; Supplementary Fig. 1) luciferin by marine luciferases is ATP-independent. NanoLuc, a marine luciferase variant, exhibits a high photon production rate in the presence of furimazine (FRZ; Supplementary Fig. 1), a marine luciferase variant, exhibits a high photon production rate in the presence of furimazine (FRZ; Supplementary Fig. 1), a synthetic CTZ analog. However, NanoLuc has several unfavorable features for in vivo BLI, including low tissue penetration of its blue emission, and limited substrate solubility and stability. Recent studies have partially addressed these issues by developing additional CTZ analogs and NanoLuc mutants or genetically fusing NanoLuc to long-wavelength-emitting fluorescent proteins (FPs) for redder emission via bioluminescence resonance energy transfer (BRET).

Bioluminescent indicators that change signals in response to neuronal activity are needed for functional imaging. Ca$^{2+}$ is a ubiquitous second messenger, and intracellular Ca$^{2+}$ has been used as a proxy for neuronal activity. Previous studies have used Ca$^{2+}$-sensitive photoproteins, such as aquorin, obelin, and their mutants, for Ca$^{2+}$ detection, but they have drawbacks, including weak light emission and irreversible responses due to slow luciferin recharging. Other studies introduced Ca$^{2+}$-sensory elements into luciferases, including NanoLuc and NanoLuc-FP hybrid reporters, resulting in reversible Ca$^{2+}$ indicators with much-increased light production.

Despite the progress, functional BLI (fBLI) of neuronal activity, which requires fast digitalization, is still hindered by insufficient photons reaching detectors. First, the intrinsic photon production rates of NanoLuc and NanoLuc-based indicators remain several orders of magnitude lower than those achievable in typical fluorescence imaging setups. Another limiting factor is the low amount of marine luciferase substrates that can be systematically delivered to the brain. Moreover, most current bioluminescent Ca$^{2+}$ indicators (Table 1) emit short-wavelength light strongly attenuated by brain tissue, skull, and skin.

To fill the fBLI technical gap, we develop a luciferase prosubstrate for enhanced luciferin delivery and a bioluminescent indicator with redder emission and remarkable brightness and Ca$^{2+}$ responsiveness. We characterize this integrated system for imaging Ca$^{2+}$ dynamics in cultured cell lines, primary mouse neurons, and acute brain slices. Moreover, because of the drastically increased photon flux, the integrated system enables the minimally invasive imaging of deep-brain Ca$^{2+}$ dynamics in awake mice in response to behavioral and disease triggers.

### Results

#### Design and chemical synthesis of luciferase prosubstrates

Our group previously reported teluc, a bright and red-shifted NanoLuc mutant, and its paired DTZ substrate (Supplementary Fig. 1), which showed promising BLI performance in mice. DTZ could be synthesized from inexpensive commercial reagents in two steps with excellent yields. The discrepancy may be caused by expression and assay conditions and the variable oligomerization states of Orange CAMBIs.

#### Table 1 Properties of bioluminescence Ca$^{2+}$ indicators based on NanoLuc or NanoLuc-derived luciferases.

| Construct     | Peak emission (nm) $^b$ | BL/BL0 $^c$ | $K_d$ (nM) $^d$ | Emission fraction $>600$ nm $^e$ | BRET Donor     | BRET Acceptor | $Ca^{2+}$ sensing domain | Reference |
|---------------|-------------------------|-------------|-----------------|---------------------------------|----------------|---------------|-----------------------|-----------|
| GeNLs(Ca$^{2+}$) | 517         | 3.9–5     | 60–520          | 0.017 $^d$                     | NLuc split at residue 66 | mNeonGreen    | CaM, M13              | 25        |
| CalFluxVTN    | 525         | 5$^e$     | 480             | 0.04 $^d$                      | NLuc              | Venus         | TnC                   | 13        |
| LUCI-GECO1    | 515         | 2.6$^a$   | 285             | <0.02                          | NLuc              | ncpGCaMP6s   | CaM, RS20             | 26        |
| GLICO         | 515         | 23         | 230             | <0.02                          | NanoBiT          | GCaMP6f      | CaM, RS20             | 27        |
| RelICO        | 452         | 3.4        | 1.5 × 10$^6$    | <0.02                          | NanoBiT          | R-CEpA1er    | CaM, RS20             | 27        |
| Orange CAMBIs | 586         | 7–8$^f$   | 110–300         | 0.33$^d$                       | NLuc split at residue 133 | tLuc split at residue 133 | mScarlet-I | CaM, M13              | 29        |
| BRIC          | 595         | 6.5        | 133             | 0.54                           | This work        |               |                       | 29        |

$^a$ Data for BRIC were determined in this work. Unless otherwise indicated, other data were reported or calculated from graphs in the initial publications. $^b$ Wavelength for the major or the most red-shifted emission peak. $^c$ Intensity ratio with or without Ca$^{2+}$ at the indicated peak emission wavelength. $^d$ Adapted from Reference 9. $^e$ Originally described as green/blue ratiometric indicators, and the response range (R/R0) was reported to be 11 and 5 for CalFluxVTN and LUCI-GECO, respectively. $^f$ Our determined value is −2.5–3.7 for OCaMBI110. The discrepancy may be caused by expression and assay conditions and the variable oligomerization states of Orange CAMBIs.
Bioluminescent Red Protein (BREP) with increased brain distribution vastly36,37, because P-gp unfavorably group to paclitaxel (Taxol) reduced the interaction with P-gp and (Fig. 1a) is required for substrate oxidation6, C3 derivatizations will injection buffers.

Thus, we chose to use the carboxylate functional group to modify addition installs a carboxylate functional group with a interacts with negatively charged molecules and the succinate ef under understood, the BBB ef issue is further compounded by additional unfavorable factors, such as the rapid clearance and low solubility of these substrates30,35. The literature reported that adding a succinate group to paclitaxel (Taxol) reduced the interaction with P-gp and increased brain distribution vastly36,37, because P-gp unfavorably interacts with negatively charged molecules and the succinate addition installs a carboxylate functional group with a pK_a of ~4. Thus, we chose to use the carboxylate functional group to modify DTZ with the hope of enhancing its hydrophilicity, reducing BBB efflux, and increasing possible dosage via aqueous intravenous injection buffers.

Because the C3 carbonyl group of the DTZ imidazopyrazine ring (Fig. 1a) is required for substrate oxidation6, C3 derivatizations will generate caged substrates resistant to auto- and luciferase-catalyzed oxidation. We designed and synthesized a compound with an extended carboxylate via C3 (see ETZ in Fig. 1a, Supplementary Figs. 1 and 2; computed logD is 1.1 at pH 7). When ETZ is delivered in vivo, nonspecific esterase is expected to hydrolyze the ester bonds, resulting in free DTZ for luciferase-catalyzed bioluminescence (Fig. 1b). We named this compound ETZ for esterase-dependent activation and enhanced in vivo performance (presented below). In addition, for comparison purposes, we synthesized C3-DMA-DTZ (Fig. 1a, Supplementary Figs. 1 and 2; computed logD is 3.8 at pH 7), which contains ester linkages but is partially positively charged at physiological pH.

**Engineering of a BRET-based bioluminescent red protein.** Photons within the optical window (600–950 nm) are less absorbed and scattered by mammalian tissue, thus penetrating tissue deeper38. It is an established strategy to shift the bioluminescence of marine luciferases toward the optical window via BRET to FPs15,16,19,20. Our previous study fused a LumiLuc luciferase to a bright red FP (RFP) mScarlet-I, resulting in a LumiScarlet reporter with ~ 51% of the total emission above 600 nm16. Following the success, we created a similar fusion between teLuc and mScarlet-I and optimized the linker for ~60% of its total emission above 600 nm (Fig. 1c). Although the emission of teLuc is less red-shifted than LumiLuc and has less spectral overlap with mScarlet-I, we observed more efficient BRET in BREP than LumiScarlet due to the shorter
donor-acceptor distance and a possible spatial orientation favoring donor-acceptor dipole coupling in BREP.

**Comparison of luciferase prosubstrates in mammalian cells and for brain imaging in mice.** We next compared DTZ, ETZ, and C3-DMA-DTZ for bioluminescence in cultured mammalian cells. We transiently expressed BREP in human embryonic kidney (HEK) 293 T cells and imaged the cells in 96-well plates upon adding 25 µM of each compound (Supplementary Fig. 4). The initial bioluminescence of ETZ-treated cells was lower than DTZ-treated cells but slightly higher than cells treated with C3-DMA-DTZ. Because the signals of the DTZ and C3-DMA-DTZ groups decayed quickly while ETZ allowed sustained bioluminescence, the integrated signal of the ETZ group during the examined 50 min period was ~2- and 8-fold of the DTZ and C3-DMA-DTZ groups, respectively. The results suggest that ETZ is a promising substrate for mammalian imaging applications.

We further compared the three compounds for brain delivery in mice. We injected ~7,000 HEK 293 T cells transiently transfected with BREP to the hippocampus in anesthetized BALB/cJ mice. After intracranial cell injection, we immediately administered 100 µl buffers containing each substrate at their saturation concentrations via tail vein. ETZ exhibited better solubility than DTZ and C3-DMA-DTZ (Supplementary Fig. 5a) and could be delivered at a dosage of 0.68 µmol per mouse. Mice infused with ETZ showed the most robust and durable bioluminescence (Fig. 1d and Supplementary Fig. 5b). During the 16-min window used in this experiment, the integrated signal of the ETZ group was ~4-fold and ~39-fold higher than the DTZ and C3-DMA-DTZ groups, respectively.

Because there were concerns that endogenous neurons may be different from implanted HEK 293 T cells in compound uptake and esterase activity, we further prepared adeno-associated viruses (AAVs) with the BREP gene driven by the human synapsin 1 (hSyn) promoter and transduced hippocampal neurons in live mice via stereotactic injection. We tested the BREP-expressing mice against a panel of CTZ analogs (Supplementary Fig. 6). FFz (Supplementary Fig. 1) was recently reported as a NanoLuc substrate with enhanced in vivo performance, so we chemically synthesized FFz for comparison. When the compounds were delivered at their saturation concentrations, ETZ again surpassed all others by generating the brightest and most durable emission in mice. In terms of bioluminescence integrated over the experimental period, mice infused with ETZ were ~4.2-, 8.8-, 67.3-, and 122.7-fold of DTZ, C3-DMA-DTZ, FFz, and FRZ, respectively. Therefore, ETZ was confirmed as a superior luciferase substrate for brain imaging in mice.

We further compared the bioluminescence of the BREP-expressing mice infused with the equimolar amount (0.25 µmol) of ETZ and DTZ. Although mice in the ETZ group seemed slightly brighter than those in the DTZ group, there was inadequate statistical power to resolve the difference (Supplementary Fig. 6). Nevertheless, the result unambiguously supports that the enhanced in vivo performance of ETZ from DTZ was primarily caused by the increased substrate delivery enabled by the increased solubility.

In addition, we evaluated the background bioluminescence of ETZ in blank mice or mice with untransfected HEK 293 T cells stereotactically injected into the hippocampus. Corroborating our previous study on DTZ, the background signal from ETZ was negligible compared to authentic bioluminescence signals (Supplementary Fig. 7).

**Engineering and initial characterization of a bioluminescent red indicator for Ca2+.** We further engineered a bioluminescent Ca2+ indicator from BREP. Inspired by Orange CaMBIs, we similarly inserted CaM and M13 between residues 133 and 134 of teLuc in BREP, resulting in a prototype showing a 2.5-fold (BL/BL0) Ca2+-dependent bioluminescence increase (Supplementary Fig. 8). Next, we performed six rounds of error-prone PCRs and screened the libraries for high bioluminescence brightness and Ca2+ responsiveness. The effort led to a Bioluminescent Red Indicator for Ca2+ (BRIC) with a 6.5-fold (BL/BL0) response (Fig. 2a, b, Supplementary Fig. 9, and Table 1). BRIC, at its Ca2+-bound condition, retained ~46% of the brightness of BREP. Using the purified protein, the dissociation constant (Kd) of BRIC to Ca2+ was determined to be ~133 nM (Fig. 2c). The response magnitude increased as pH changed from 5.5 to 7 and was relatively stable at pH > 7 (Fig. 2d). When compared in parallel, BRIC was more responsive to Ca2+ than OCaMBI110 (Supplementary Fig. 10a, b and Table 1).

To evaluate BRIC for imaging cellular Ca2+ dynamics, we transiently expressed BRIC in human cervical cancer HeLa cells, in which histamine can evoke Ca2+ waves. As expected, we observed single-cell bioluminescence oscillations in response to histamine-evoked bioluminescence microscopy (Fig. 2e and Supplementary Movie 1). Furthermore, we transduced cultured primary mouse neurons with BRIC AAVs harboring the hSyn promoter and successfully detected Ca2+ influx after high K+ depolarization (Fig. 2f and Supplementary Movie 2). In contrast, the control BREP-expressing neurons showed little bioluminescence increase.

In addition, we compared BRIC with OCaMBI110 in HeLa cells and cultured mouse neurons. Under fluorescence channels, we observed extensive puncta in cells overexpressing OCaMBI110 (Supplementary Fig. 10c) but not in BRIC-expressing cells. In addition, the cells with fluorescent puncta showed little bioluminescence and were unresponsive to histamine or high K+. Although the exact reason is unknown, the observed puncta may be OCaMBI110 oligomers because each OCaMBI110 molecule contains two copies of dimeric CyOFP1 (Supplementary Fig. 10d)19. Moreover, with punctum-containing cells excluded from analysis, the response magnitude of BRIC was still higher than OCaMBI110 in both HeLa cells and cultured neurons (Supplementary Fig. 10e, f).

Further, we examined BRIC for imaging Ca2+ influx in primary mouse neurons induced with ionomycin, a Ca2+-selective ionophore (Supplementary Fig. 1)42. The bioluminescence of cultured neurons in Ca2+-containing buffers increased upon the addition of ionomycin. The responses depended on extracellular Ca2+ concentrations, as higher Ca2+ caused faster and more pronounced intensity changes.

**Imaging of the bioluminescent Ca2+ indicator in the brain in live mice.** Because ETZ exhibited enhanced bioluminescence in the brain in live mice and BRIC performed well in cultured cells, we next examined the integration of ETZ and BRIC for in vivo BLI. We started by investigating the brightness of BRIC and ETZ in the brain in live mice. We stereotactically injected BRIC and OCaMBI110 AAVs (adjusted to the same viral titer) into the hippocampus of BALB/cJ mice (Fig. 3a) and compared the brightness of the two indicators on day 19 post viral administration. We examined OCaMBI110 with either FRZ or the recently reported FFz substrate. Each substrate was intravenously administered to anesthetized animals at their saturation concentrations (Supplementary Fig. 12a). Using an EMCCD camera, we followed the signals of BRIC with 500-ms exposure for 1 h. The starting bioluminescence of BRIC in the presence of ETZ was ~168- and 29-fold higher than OCaMBI110 in the presence of FRZ and FFz, respectively (Fig. 3b and Supplementary Fig. 12b). In addition, the BRIC signals were consistently higher than the background during periods much longer than
OCaMBI110 with either substrate (Fig. 3c and Supplementary Fig. 12c). Regarding the signals integrated over time, BRIC was ~153.7- and 22.0-fold of OCaMBI110 in the presence of FRZ and FFz, respectively (Fig. 3d). Furthermore, we prepared acute brain slices from BRIC-expressing mice and observed bioluminescence rise in response to high K\(^+\), confirming the activity of BRIC in the brain tissue (Supplementary Fig. 13 and Supplementary Movie 3).

We finally examined BRIC for monitoring Ca\(^{2+}\) dynamics in awake mice (Fig. 4a). First, we administered the virus to the basolateral amygdala (BLA) region (Supplementary Fig. 14) responsible for fear processing\(^{43}\). Upon the intravenous injection of ETZ, head-fixed mice were subjected to BLI in a dark box. As expected, we detected rapid bioluminescence changes in BRIC-expressing mice, which were statistically different from BREP-expressing control mice (Fig. 4c and Supplementary Movie 5). However, there were noticeable response variations between individual BRIC-expressing animals. We reason that the differences may reflect biological variations since the extent of KA-induced seizures and calcium responses could be different in individual animals, as well as technical limitations since KA-induced seizures are known to be intermittent and some mice were probably not imaged right during the occurrence of seizures\(^{44,45}\).
GraphPad Prism software does not provide extract we further engineered a bioluminescent Ca\^{2+} cells and brain slices. Finally, we combined the prosubstrate and imidazopyrazine ring, leading to the ETZ prosubstrate exhibiting resistance to auto-oxidation, increased aqueous solubility, and much-reduced light output of Renilla luciferase in live mice bearing brain tumors compared to the equimolar amount of CTZ\textsuperscript{30}. However, these previously explored C-3 caged groups were typically hydrophobic, unfavorably reducing substrate solubility in aqueous solutions. Also, a prosubstrate strategy was recently used to deliver the amide derivatives of firefly luciferins into the mouse brain for fatty acid amide hydrolase (FAAH)-dependent activation\textsuperscript{52,53}.

BREP is the most red-shifted marine luciferase-FP fusion protein that retains high brightness. The construct uses mScarlet-I as the BRET acceptor. Because existing far-red and near-infrared FPs that are more red-shifted than mScarlet-I have much-reduced fluorescence quantum yields, it is not yet possible to use the FP fusion strategy to further red-shift bioluminescence without remarkably harming brightness. Alternatively, one may covalently conjugate far-red or near-infrared synthetic dyes to luciferase variants via self-labeling protein tags (e.g., Halo tag)\textsuperscript{18,54,55}.

BIRC is a high-performance and red-shifted bioluminescent Ca\^{2+} indicator. We compared it with the current benchmark, OCaMBI110. BRIC showed larger responses to Ca\^{2+} as purified proteins and in HeLa cells and primary neurons. In addition,
BRIC was less prone to aggregation. When applied in mice, the BRIC signals in the presence of ETZ were much brighter and lasted much longer than OCaMBI110 with either FRZ or FFz. The integration of BRIC with ETZ allowed us to image Ca\(^{2+}\) dynamics in awake mice minimally invasively with excellent signal-to-background and subsecond temporal resolution. However, despite the enhancement, BRIC is still suboptimal since its response to physiological Ca\(^{2+}\) changes (e.g., from \(\sim 50\) nM to \(\sim 1\) µM) is limited to \(~2\)-fold. We plan to further optimize the practical dynamic range of this indicator in future.

Our current approach macroscopically records concerted changes in a neuronal population that expresses the BRIC indicator. The spatial resolution is limited by scattering when photons travel through brain tissue, skull, and skin. Also, photons reaching the detector are still scarce; when using the experiment setups presented here, the temporal resolution is in the second and subsecond ranges. Although the overall duration of the rising and extinction of intracellular Ca\(^{2+}\) signals after the action potential is likely within this time range, the kinetics of Ca\(^{2+}\) concentration changes is much faster than the achieved temporal resolution\(^5\)\(^6\). Thus, interested users should be aware that the recorded signals are undersampled over time. Considering these reasons and the fact that fluorescent Ca\(^{2+}\) indicators, such as GCaMPs, have been extensively optimized\(^5\)\(^6\), fluorescence remains the choice of imaging modality for fiber photometry or single-cell-resolution recording through cranial windows. On the
other hand, the method presented here offers technical simplicity, reduced invasiveness, and deep-brain imaging capability. The approach complements, but cannot replace, fluorescence or electrical recording.

This study tested ETZ in both BALB/cJ and C57BL/6 J strains of mice and used intracranial injection to deliver viral vectors. Recently, AAV vectors with engineered capsids have been reported to cross the BBB of C57BL/6 J mice and marmoset.\textsuperscript{27,28} We are currently exploring these AAV vectors for peripheral gene delivery and transduction of specific neuronal populations in transgenic Cre mice with the C57BL/6 genetic background. We expect the effort to lead to an utterly noninvasive strategy for recording the activity of neuronal ensembles in small mammals. Also, we had to apply baseline corrections for time-lapse intensity quantification. A monoeponential decay model was adequate for baseline corrections in those experiments spanning short periods, although a more complex decay was evident during the more extended 60-min period shown in Fig. 3c. Further, BRIC is an intensimetric indicator, and we used head-fixed awake mice for macroscopic BLI. Unavoidable motion on blood flow changes in animals may cause intensity instability, resulting in potential artifacts when recording small Ca\textsuperscript{2+} changes. Future developments may allow two-channel BLI of the mouse brain with high sensitivity, enabling ratiometric correction for baseline decays, blood flow changes, and animal motions.

In addition, a recent study described the use of FRZ and a NanoLuc-derived bioluminescent voltage indicator for imaging cortical activity in mice.\textsuperscript{31} To gain enough signals, a cranial window was used for bioluminescence collection and continuous substrate delivery to the cortex. Furthermore, a GaAsP image intensifier was placed in front of the EMCCD to boost signals. Another recent preprint described a NanoLuc-derived bioluminescent glutamate indicator that has been tested in cultured cells.\textsuperscript{39} We expect our results and strategies presented here to assist in the further development of these bioluminescent indicators for deep-brain in vivo imaging with minimally invasive procedures. Furthermore, as much-enhanced BLI tools, ETZ, BREP, and BRIC are expected to find diverse applications and generate broad impacts beyond neurobiology.

Methods

Ethical statement. The study complies with all relevant ethical regulations, and all animal experiments were conducted according to the approval (Protocol #1496) and guidelines of the University of Virginia Institutional Animal Care and Use Committee. BALB/cJ mice (C57BL/6 J mice (C500651) and C57BL/6 J mice (C500664) were purchased from the Jackson Laboratory and bred under standard conditions. Mice were housed in a temperature-controlled room (~23 °C) with a 12 h/12 h dark-light cycle and ~50% humidity. Animals were randomly allocated to experimental groups with a balance of females and males at the age of ~8 weeks.

Reagents and general methods. Unless otherwise stated, all chemicals were purchased from MilliporeSigma, Fisher Scientific, or VWR and used without further purification. Kainic acid monohydrate (KA) was purchased from Cayman Chemical. DNA (500 bp). Orange CamBRL110 (Addgene #124994) and Addgene #59829 were gifts from M.Z. Lin and L.L. Looger, respectively. pAdDeltaF6 (Addgene #112867), PAAV229n (Addgene #112865) were gifts from J.M. Wilson. Synthetic DNA oligonucleotides (Supplementary Table 1) were purchased from Integrated DNA Technologies or Eurofins Genomics. Restriction endonucleases and PhosIn High-Fidelity DNA Polymerase were purchased from Thermo Scientific. Products of PCR and restriction digestion were purified with preparative agarose gel electrophoresis, followed by gel extraction. DNA sequences were analyzed by Eurofins Genomics. Gibson assembly was performed using a homemade kit by following a procedure from Addgene. Small-scale plasmid DNA preparation was performed using Miniprep kits from SYL Labs. Large-scale plasmid DNA preparation was performed using alkaline lysis followed by isopropanol precipitation, PEG 8000 precipitation, and phenol/ chloroform extraction. Merck Gedura Si 60 silica gel was used for normal-phase column chromatography. MassLynx (Version 4.2) was used to run a Waters Prep 150 mm. Aprotinin (10000 UI/ml) and 1 M HCl were added to the buffer to increase the molarity of the buffer. A 25-mL vial with an XBridge BEH Amide/Phenyl OBd Prep Column (130 Å, 5 μm, 30 mm x 150 mm) for preparative reverse-phase HPLC purification. Lyophilization was performed on a 12-port Labconco freeze dryer with an Edwards RV3 vacuum pump. All NMR spectra were collected on a Bruker Avance III 600 MHz NMR spectrometer with the Bruker TopSpin IconNMRS (Version 3.5pl4) software, and data were further analyzed using MestReNova (Version 12.0.3). Reference values for residual solvents were taken as 7.27 (CDCl3) or 3.30 (methanol-d4) ppm for 1H-NMR, and 77.2 (CDCl3) or 49.0 (methanol-d4) ppm for 13C-NMR. Splitting patterns of NMR active peaks are reported as s (singlet), d (doublet), t (triplet), dd (doublet of doublets), dt (doublet of triplets), and m (multiplet). The BMG Labtech CLARIOStar Plus Reader Software (Version 5.70 R2) was used to control the CLARIOStar Plus microplate reader, and results were automatically exported to the BMG Labtech MARS Data Analysis Software (Version 3. 42 RS) for analysis. Agilent MassHunter Workstation Data Acquisition (Version B.09) was used to acquire high-resolution mass spectra (HR-MS) on an Agilent 6545 Q-TOF LC/MS system via direct infusion. Agilent MassHunter Quantitative Analysis Navigator (Version B.08) was used to analyze HR-MS data. The UVP VisionWorksLS software (Version 8.6) and µManager (Version 2) were used to control a UVP BioSpectrum dark box and cameras for macroscopic BLI, while Leica LAS X (Version 3.5.7) or MicroManager (Version 0.20) was used to acquire microscopic images. The ChemAxon LogD Predictor (https://disco.chemaxon.com/calculators/demo/plugins/logd/) was used to predict the LogD values of the specified compounds at pH 7.

General information for chemical synthesis. Synthetic schemes and compound numbering information are shown in Supplementary Fig. 2. NMR and HR-MS spectra for key compounds are presented in Supplementary Figs. 15–18. The reagents used in the chemical syntheses are listed below. Furimazine (FRZ) and FFz were synthesized following reported methods.\textsuperscript{19,39,40,147}

4-Oxo-4-(prop-2-yn-1-yloxy) butanoic acid (Compound 2). NHS (920.0 mg, 8.0 mmol, 0.4 equiv.), DMAP (260.0 mg, 2.0 mmol, 0.1 equiv.), TEA (840.0 mg, 8.0 mmol, 0.3 equiv.) and propargyl alcohol (7.4 mL, 60 mmol, 3.0 equiv.) were separately added into a solution of sucinic anhydride (2000.0 mg, 20.0 mmol, 1.0 equiv.) dissolved in 50 mL anhydrous toluene. The mixture was heated to reflux overnight under Ar. After cooling down to room temperature, the mixture was filtered, and EtOH was removed with rotovap concentration. The residue was further purified with silica gel chromatography (EtOAc/hexane = 1/5, v/v) to give compound 2 (2028.0 mg, 65%).

2-Amino-3,5-diphenylpyrazine (Compound 3). Compound 3 was synthesized by modifying a previous method.\textsuperscript{29} 2-Amino-3,5-dibromopyrazine (3000.0 mg, 12.0 mmol, 1.0 equiv.), phenylboronic acid (5800.0 mg, 48.0 mmol, 4.0 equiv.) and bis(benzonitrile)dichloro palladium (1700.0 mg, 2.4 mmol, 0.2 equiv.) were dissolved in 1,4-dioxane (50 mL). The mixture was heated at reflux overnight under Ar. After cooling down to room temperature, the mixture was filtered, and EtOH was removed with rotovap. The residue was acidified to pH 4–5 with HCl (1 N) and washed three times with EtOAc. The organic layer was combined and dried with anhydrous Na2SO4. After filtration and rotovap concentration, the residue was further purified with silica gel chromatography (EtOAc/hexane/aetic acid = 1/4/0.005, v/v/v) to afford compound 3 (2075.0 mg, 70%).

Diphenylirizane (DTZ). DTZ was synthesized by modifying a previous method.\textsuperscript{6} N-HCl (5.4 mL, 50.0 equiv.) was added to 5 mL of 1,4-dioxane in a 60-ml Ace pressure tube (Sigma-Aldrich #Z568767) containing compound 3 (150.0 mg, 0.61 mmol, 1.0 equiv.) and 1,1-dioxy-3-phenylacetone (534.0 mg, 2.4 mmol, 4.0 equiv.). The tube was sealed, and the mixture was maintained at 120 °C with stirring overnight. Next, the mixture was cooled down to room temperature before the solvent was removed under reduced pressure. The crude was re-dissolved in 15 mL MeOH, and next, with purification with Waters preparative RPLC-MS (acetonitrile/water = 30:70 to 98:2, 20 mL/min). Product fractions were combined and lyophilized to give DTZ (126.0 mg, 55%).
reaction system with stirring at room temperature over 15 min. Subsequently, DTT (101.7 mg, 0.25 mL, 1.0 equiv.) was quickly added into the reaction system through a “neck” of the flask. The system was washed with acetone and the reaction mixture was stirred for additional 20–30 min. The progress of the reaction was monitored with TLC (hexane/EtOAc = 3:1). After completion of the reaction, the mixture was cooled down to −20 °C to precipitate the DCC by-product. After filtration and concentration in vacuo, the residue was further purified with silica gel chromatography and elution with a mixture of MeOH/H2O (4:1, 80%).

1H NMR (600 MHz, CDCl3) δ 8.99–8.84 (m, 2H), 8.24 (s, 1H), 8.16 (dd, J = 8.4, 1.2 Hz), 7.60–7.55 (m, 2H), 7.54–7.46 (m, 3H), 7.42–7.37 (m, 1H), 7.36–7.29 (m, 4H), 7.26–7.22 (m, 1H), 4.75 (d, J = 2.5 Hz, 2H), 4.24 (s, 2H), 2.79–2.74 (m, 2H), 2.73–2.64 (m, 2H), 2.66 (t, J = 2.5 Hz, 1H).13C NMR (151 MHz, CDCl3) δ 171.7, 147.0, 138.1, 136.8, 136.4, 131.3, 133.3, 133.4, 130.8, 129.8, 129.2, 128.8, 128.7, 128.5, 128.4, 128.3, 126.4, 126.3, 109.7, 77.2, 75.5, 73.5, 52.6, 34.3, 28.8, 28.1. ESI-MS (m/z): [M + H]+ calcd. for C27H22N8O5, 516.19; found, 516.18.

2-(4-(((2-Benzyl-6,8-diphenylimidazo[1,2-a]pyrazin-3-yl)oxy) methyl)oxy) methyl) 4-(2-(Dimethylamino)ethoxy) methyl) 3.0 equiv.) were consequently added into a solution of succinic anhydride (2000 μg/mL ampicillin and 0.02% (w/v) L-arabinose. Cells were grown at 30 °C, 250 rpm for 48 h, harvested by centrifugation, and resuspended in 300 μL of 100 mM NaCl and 30 mM Tris HCl, pH 7.4. The mixture was shaken for 2 s before the bioluminescence spectrum was recorded using the equipped red-sensitive PMT. The instrument was set to scan from 400 to 700 nm with 5-nm intervals. Three technical repeats were performed to derive the average spectrum. The BRIC library construction and screening. The CaM-M13 fragment was PCR-amplified from pCDNA3-1-Orange_CaMBl110 with oligos pBad_FW2_CaM and pBad_RW2_M13. Next, pBAD-BREP was amplified with either pBad_FW1,mScarlet and pBad_RW1_teLuc, pBad_FW1_mScarlet and pBad_RW1_teLuc,myc or pBad_RW3 teLuc(134) and pBad_RW3 teLuc(168) to generate two fragments. The three fragments, along with pBAD/HisB predigested with Xho I and Hind III, were assembled in a four-part Gibson assembly reaction. The product was used to transform E. coli DH10B cells (Thermo Fisher). Cells were allowed to grow on 2 × YT agar plates supplemented with 100 μg/mL ampicillin and 0.02% (w/v) L-arabinose at 37 °C overnight. Red colonies were inoculated and used to inoculate cultures in 5 mL 2 × YT broth supplemented with 100 μg/mL ampicillin and 0.2% (w/v) L-arabinose. Cells were grown at 30 °C, 250 rpm for 4 h, harvested by centrifugation, and resuspended in 300 μL of B-PER at 4 °C for 30 min. After centrifugation, cell lysates were prepared. 5 μL of the supernatant was diluted with 185 μL of a Ca2+–free buffer (30 μM MOPS, 100 mM KCl, 10 mM EGTA, pH 7.2) and a Ca2+–containing buffer (30 mM MOPS, 100 mM KCl, 10 mM CaEGTA, pH 7.2) expected to provide 39 μM free Ca2+. Next, DTZ was dissolved in the in vitro assay buffer described above to the concentration of 300 μM, and 10 μL of the DTZ solution was injected into each well via a reagent injector in a BFM Labtech CLARIOstar Plus microplate reader. The final concentration of DTZ was thus 25 μM. The bioluminescence spectra and intensity of each well were measured from 400 to 700 nm with 5-nm intervals. Mutants with high brightness and Ca2+ responsiveness were selected.

2-Benzyl-6,8-diphenylimidazo[1,2-a]pyrazin-3-yl (2-(dimethylamino)ethyl) succinate (C3-DMAD-TZ). Compound 5 (240.0 mg, 1.33 mmol, 1.0 equiv.) was added to the reaction mixture. The resulting mixture was stirred for additional 80 min, then filtered, concentrated, and purified with silica gel chromatography (MeOH/DCM = 1/10, v/v) to give C3-DMAD-TZ (25.1 mg, 15 %).1H NMR (600 MHz, methanol-d4) δ 8.69–8.64 (m, 2H), 8.51 (s, 1H) [Xho I, 1.5]; 7.92 (s, 1H) [Hind III, 2 Randox Kit (Agnitent Technologies) was used for enzyme-protein (EP)-PCR-based random mutagenesis. Oligos pBAD_FW EP and pBAD_RW EP were used for these reactions. PCR products were inserted into pBAD/HisB between Xho I and Hind III via Gibson assembly. The DNA libraries were used to transform E. coli DH10B cells, which were cultured on 2 × YT agar plates supplemented with 100 μg/mL ampicillin and 0.02% (w/v) L-arabinose at 37 °C overnight. Red colonies were inoculated and used to inoculate cultures in 5 mL 2 × YT broth supplemented with 100 μg/mL ampicillin and 0.2% (w/v) L-arabinose. Cells were grown at 30 °C, 250 rpm for 48 h, harvested by centrifugation, and resuspended in 300 μL of B-PER at 4 °C for 30 min. After centrifugation, cell lysates were prepared. 5 μL of the supernatant was diluted with 185 μL of a Ca2+–free buffer (30 μM MOPS, 100 mM KCl, 10 mM EGTA, pH 7.2) and a Ca2+–containing buffer (30 mM MOPS, 100 mM KCl, 10 mM CaEGTA, pH 7.2) expected to provide 39 μM free Ca2+. Next, DTZ was dissolved in the in vitro assay buffer described above to the concentration of 300 μM, and 10 μL of the DTZ solution was injected into each well via a reagent injector in a BFM Labtech CLARIOstar Plus microplate reader. The final concentration of DTZ was thus 25 μM. The bioluminescence spectrum and intensity of each well were measured from 400 to 700 nm with 5-nm intervals. Mutants with high brightness and Ca2+ responsiveness were selected.

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high-purity OCaMBI110 protein due to proteolysis. To address this issue, an additional Strept-tag II sequence was appended to the C-terminus of the reading frame, such that the OCaMBI10-S tag was followed by 8 normal-saline buffer volumes. All procedures were performed with the identical procedure described above. To purify the protein, the N-terminal His-tagged protein in cell lysates was first enriched with Ni-NTA agarose beads, and then the eluate was applied to a Strept-Tactin Superflow high-capacity column (IBA Lifesciences). Finally, the eluate from the Strept-Tactin column was further subjected to size-exclusion chromatography. The bioluminescence intensity at 595 nm was used to assess the purity of the protein.

**Spectroscopic characterization of BRIC and OCaMBI10.** Protein concentrations were determined with the Pierce 660 nm Protein assay using bovine serum albumin (BSA) standards. The Ca²⁺-containing and Ca²⁺-free buffers mentioned above were used to record the emission spectra for Ca²⁺-free and Ca²⁺-saturated states. Final protein and substrate concentrations were 50 nM and 25 μM, respectively. The Ca²⁺ affinity was determined as previously described using a series of buffers made from mixing the Ca²⁺-containing and Ca²⁺-free buffers. The bioluminescence intensity ratios at 595 nm in the presence and absence of Ca²⁺ were plotted against the pH values.

**Evaluation of luciferase substrates in BREP-expressing HEK 293 T cells.** pBAD-BRE was amplified with oligos pDNA3_FW_HindIII and pDNA3_RV_Xhol via PCR. The fragment was inserted into pDNA3 to afford pDNA3-BREP, pDNA3-BRE was next used to transfect HEK 293 T cells (purchased from ATCC, Cat. # CRL-3126) with a described procedure. Cells were collected 20 h post-transfection, and resuspended in and loaded with 1×PBS (pH 7.2), ~70,000 cells in 100 μL of total volume were placed in individual wells in a 96-well plate. The compounds (DTZ, ETZ, and C3-DMA-DTZ) were dissolved in 1×PBS (pH 7.2) to gain 50 μM concentrations. Next, 100 μL of the substrates were added to BREP-expressing HEK 293 T cells in the 96-well plate. BLI was performed immediately with a UVP Biospectrum dark box, a Computar Motorized ZOOM lens (M621212MP3), and an Andor iXon Life 888 EMCCD camera. The camera was first set to the "Photon Counting" mode using OptAcquire pre-settings, and the gain was next adjusted down to 500. Other parameters were: camera binning 1 × 1; camera sensor temperature −70 °C; 100 ms exposure time with acquisitions every 10 s. The lens was controlled using the UVP VisionWorksLS software with aperture set to 100%, zoom set to 0%, and focus set to 0%. Anesthetized mice were placed 21 cm away from the front of the lens with no emission filter used. Imaging settings were acquired with the mManager software and processed in the Fiji version of ImageJ 2.1. Image stacks were first subtracted for background by setting the rolling ball radius to 100 pixels. Next, the region of interest (ROI) was selected based on bioluminescence from the mouse brain, and the intensity value integrated over the ROI was extracted for further analysis. Data were plotted and statistical analysis was performed in GraphPad Prism. After the software-based background subtraction, the images were left with residual background, so the ROI was moved away from the mouse brain region to evaluate residual background, which was further used to subtract signals for calculating the integrated bioluminescence intensity (area under the curve).

**Preparation of Adeno-Associated Viruses (AAVs).** Indicator genes were amplified from their corresponding pDNA3/3.1 plasmids and inserted into a pAAV-hSyn vector to generate pAAV-hSyn-BRIC, pAAV-hSyn-BREP, and pAAV-hSyn-OCaMBI110. Next, individual transfer plasmids, along with pAd-ΔDeltaF6 and pAAV293a, were used to transfect HEK 293 T cells to pack viruses. A pAAV-hSyn vector to generate pAAV-hSyn-BRIC, pAAV-hSyn-BREP, and pAAV-hSyn-OCaMBI110 intracranial cell injection. Bioluminescence images were acquired with a BLI system composed of a UVP Biospectrum dark box, a Computar Motorized ZOOM lens (M621212MP3), and a QSI 628 Cooled CCD camera. The settings were: camera binning 4 × 4; high gain; camera sensor temperature −15 °C; 10 s exposure time with each acquisition every 60 s. The lens was controlled using the UVP VisionWorksLS software with aperture set to 100%, zoom set to 0%, and focus set to 0%. Anesthetized mice were placed 21 cm away from the front of the lens with no emission filter used. To minimize biological variables, each mouse was sequentially tested with three substrates, and the next substrate was administered after the signal of the previous substrate faded out. A total of three mice were used, and the substrate injection order was rotated for each mouse to control the bias. To further evaluate the compounds with endogenous neurons in mice, 500 μL of BREP AAV (~1 × 10^15 GC/mL) was delivered to each side of the hippocampus of 8-week-old BALB/c mice via intracranial stereotactic injection at a flow rate of 100 μL min^-1 using the coordinate described above. Three weeks later, mice were used for brightness comparison. ETZ (6.8 mM), ETZ (2.5 mM), DTZ (2.5 mM), C3-DMA-DTZ (4.5 mM), FxZ (6.0 mM), and FRZ (3.0 mM) were dissolved in 1×PBS (pH 7.2) to gain 50 μM concentrations. Next, 100 μL of the substrates were added to BREP-expressing HEK 293 T cells in the 96-well plate. BLI was performed immediately with a UVP Biospectrum dark box, a Computar Motorized ZOOM lens (M621212MP3), and an Andor iXon Life 888 EMCCD camera. The camera was first set to the "Photon Counting" mode using OptAcquire pre-settings, and the gain was next adjusted down to 500. Other parameters were: camera binning 1 × 1; camera sensor temperature −70 °C; 100 ms exposure time with acquisitions every 10 s. The lens was controlled using the UVP VisionWorksLS software with aperture set to 100%, zoom set to 0%, and focus set to 0%. Anesthetized mice were placed 27 cm away from the front of the lens with no emission filter used. Imaging settings were acquired with the mManager software and processed in the Fiji version of ImageJ 2.1. Image stacks were first subtracted for background by setting the rolling ball radius to 100 pixels. Next, the region of interest (ROI) was selected based on bioluminescence from the mouse brain, and the intensity value integrated over the ROI was extracted for further analysis. Data were plotted and statistical analysis was performed in GraphPad Prism. After the software-based background subtraction, the images were left with residual background, so the ROI was moved away from the mouse brain region to evaluate residual background, which was further used to subtract signals for calculating the integrated bioluminescence intensity (area under the curve).

**Characterization and comparison of BRIC and OCaMBI110 in HeLa cells.** pBad-BRIC was used for PCR to amplify oligos pDNA3_FW_HindIII and pDNA3_RV_Xhol via PCR. The fragment was inserted into pDNA3 to afford pDNA3-BRIC. HeLa cells (purchased from ATCC, Cat. # CCL-2) were transfected with 3 μg of the plasmid pDNA3-BRIC or pDNA3.1-Orange_CaMBI110 using a described procedure. Cells were allowed to grow at 37°C in a 5% CO2 incubator for 16 h. Cells were rinsed twice with 1 × PBS and placed in 1 × PBS 15 min before imaging. Images were acquired on an inverted Leica DMi8 microscope equipped with a Photometrics Prime 95B Scientific CMOS camera. 40 μM DTZ or 40 μM FRZ was supplemented for bioluminescence imaging. Imaging settings were: 40× oil immersion objective lens (NA 1.2), no filter cube, 2 × 2 camera binning, 1 s exposure with 0 s interval, camera sensor temperature −20 °C, 12-bit and high sensitivity mode. Histonase was dissolved in 1 × PBS and added during time-lapse imaging to a final concentration of 100 μM. Image stacks were processed as described in the previous section, except that ROIs were selected for individual cells and the mean of intensity values over the ROI was extracted for further analysis. Models for the decay law were fitted to each individual AAW signal. For the exponential model: Y = (Y_plateau)*exp(-K*X) + Plateau. Data were plotted and statistical analysis was performed in GraphPad Prism.
transduction were used for quantitative comparison. Growth medium was replaced with 0.8 mL of the luminescence imaging buffer (0.49 mM MgCl₂, 2 mM CaCl₂, 0.4 mM MgSO₄, 0.44 mM K₂HPO₄, 5.3 mA CaCl₂, 4.2 mM NaHCO₃, 0.34 mM Na₂HPO₄, 138 mM NaCl, 10 mM HEPES pH 7.2, 15 mM D-glucose, and 0.1 mM sodium pyruvate) supplemented with 100 μM of DTZ or FRZ. Images were acquired on an inverted Leica DMi8 microscope equipped with a Photometrics Prime 95B Scientific CMOS camera. During time-lapse imaging, 0.2 mL of the high k⁺ stimulation buffer (0.49 mM MgCl₂, 2 mM CaCl₂, 0.44 mM MgSO₄, 0.44 mM KH₂PO₄, 143.2 mM KCl, 4.2 mM NaHCO₃, 0.34 mM Na₂HPO₄, 10 mM HEPES pH 7.2, 15 mM D-glucose, and 0.1 mM sodium pyruvate) was added to depolarize cells. Instrumental settings and data analysis were identical to those described for the HeLa cell experiment, except that the exposure time was 2 s. To use BRIC to image ionomycin-induced Ca²⁺ influx, neurons on day 4 after BRIC AAV transfection were placed in 1 mL of the luminescence imaging buffer supplemented with 100 μM DTZ. During time-lapse imaging, 1 μL of 10 mM ionomycin was added to gain a final concentration of ~10 μM. The luminescence imaging buffer described above contains 2 mM Ca²⁺. In the experiment with 10 mM extracellular Ca²⁺, the luminescence imaging buffer was additionally supplemented with 8 mM CaCl₂. Instrumental settings and data analysis were unchanged from the high k⁺ depolarization experiment.

Comparison of BRIC and OcAMBI110 brightness in the hippocampus in mice. 500 nL AAV (~1 × 10¹³ GC/mL) was delivered to each side of the hippocampus of 8-week-old BALB/c mice via intracranial stereotactic injection at a flow rate of 100 nL/min. The slices were imaged during the following day after viral injection. Image exposure was utilized for brightness comparison. 100 μL ETZ (6.8 mM) in the in vivo injection buffer was intravenously delivered into the anesthetized BRIC-expressing mouse. Subsequently, BLI was performed with a UVP BioSpectrum dark box, a Computar Motorized ZOOM lens (M6Z1212MP3), and an Andor iXon Life 888 EMCCD camera. Mice were placed 21 cm away from the front of the lens. The imaging data for high K⁺-induced Ca²⁺ dynamics in the hippocampus was adequate for histamine-induced Ca²⁺ dynamics in HeLa and in vivo imaging of footshock- and KA-stimulated mice. The imaging data for high K⁺-induced Ca²⁺ dynamics in cultured neurons and brain slices required a more complicated correction. Because high K⁺ induced a relatively large, concerted intensity increase, the baseline was not properly identified when the whole stack was used for monoeponential fitting. Instead, the mean intensity values of each image in the stack were extracted and exported to Microsoft Excel. Data points during the expected peak responses were excluded, and monoeponential fitting was applied to the remaining data points. Finally, the offline-derived decay parameters were used to correct the whole image stacks in Fiji.

BRIC responses in the acute hippocampal slices. Acute brain slices were prepared 3 weeks post viral delivery, which is described in the previous section. Freshly extracted mouse brains were pre-cooled and sliced to be 350 μm thickness in an ice-cold ACSF buffer (2.5 mM CaCl₂, 119 mM NaCl, 1.3 mM MgSO₄, 26 mM NaHCO₃, 1 mM NaH₂PO₄, 2 mM CaCl₂, and 10 mM glucose, 95% O₂/5% CO₂). Next, the brain slices were incubated in ACSF with 37 °C for 30 min and then perfused with 2.0 mL of the luminescence imaging buffer (described above for neuron experiments) supplemented with 100 μM of DTZ. Images were acquired on a Scientifica SliceScope Pro 1000 equipped with a Photometrics Prime 95B Scientific CMOS camera. Imaging settings were: 4× objective lens (NA 0.1), no filter cube, 2 × 2 camera integration with 0.1 integration time, sensor temperature −15 °C; 12-bit high sensitivity mode. During time-lapse imaging, 0.5 mL of the high k⁺ stimulation buffer (described above for neuron experiments) was slowly added using a perfusion pump at a rate of 11.9 μL min⁻¹. This ~42 s process minimized the motion of the brain slice. BREP-expressing slices were prepared and imaged using the same procedures. Image processing and data analysis was identical to those described for the HeLa cell experiment.

In vivo imaging of brain activities in awake mice. 500 nL of the virus was at a flow rate of 100 nL min⁻¹ delivered to each side of the basolateral amygdala (BLA) of 8-week-old BALB/c mice and each side of the hippocampus of 8-week-old C57BL/6 mice via intracranial stereotactic injection. The coordinate for BLA was: 8-week-old BALB/cJ mice via intracranial stereotactic injection at a dosage of 11.9 μL min⁻¹. If no seizure was observed within 2 hours, a second dose KA (5 mg per kg body weight) was delivered via i.p. injection. Finally, when an evident tremor was observed, 100 μL ETZ (6.8 mM) in the in vivo injection buffer was administered to the mouse via tail vein. Next, BLI was performed with the head-fixed animal as described above. Data analysis was identical to those described for the HeLa cell experiment, except that ROIs were selected for biologist luminescence from the brain.

Evaluation of the background signal of ETZ in mice. 100 μL ETZ (6.8 mM) in the in vivo injection buffers was delivered to 8-week-old, anesthetized BALB/c mice via tail vein. BLI was subsequently performed with a UVP BioSpectrum dark box, a Computar Motorized ZOOM lens (M6Z1212MP3), and an Andor iXon Life 888 EMCCD camera. Mice were placed 21 cm away from the front of the lens with no emission filter. Other imaging conditions and data analysis procedure were identical to the descriptions in the above section. The same procedure was used to examine mice with untransfected HEK 293 T cells intracranially injected into the hippocampus.

General procedure to correct time-lapse images for baseline decays. The Fiji version of the ImageJ 2.1 was used for image processing. Image stacks were first subtracted for background by setting the rolling ball radius to 100 pixels. Next, an average image of each stack was used to generate a binary mask, which was subsequently applied to the image stack. Thus, the information for pixels within the ROI was extracted and the intensity values for other background pixels were set to 0. This conversion was necessary so that the baseline signals were not amplified during the subsequent baseline decay correction. Next, the whole image stacks were subjected to "photobleaching correction". This procedure essentially fitted the image stacks with a monoeponential model: $Y = (Y_0 - Plateau) \times e^{-(X - Plateau)}$, and the intensity of each image in the stack was rescaled. This correction procedure was adequate for histamine-induced Ca²⁺ dynamics in HeLa and in vivo imaging of footshock- and KA-stimulated mice. The imaging data for high K⁺-induced Ca²⁺ in cultured neurons and brain slices required a more complicated correction. Because high K⁺ induced a relatively large, concerted intensity increase, the baseline was not properly identified when the whole stack was used for monoeponential fitting. Instead, the mean intensity values of each image in the stack were extracted and exported to Microsoft Excel. Data points during the expected peak responses were excluded, and monoeponential fitting was applied to the remaining data points. Finally, the offline-derived decay parameters were used to correct the whole image stacks in Fiji.

Statistics & reproducibility. Fiji (Image) Version 2.1 was used to analyze microscopic images. Imaging background was typically subtracted by setting the rolling ball radius to 300 pixels and more detailed procedures for imaging processing are presented in the above sections. Microsoft Excel (Version 15.21.1), GraphPad Prism (Version 8), and Affinity Designer (Version 1.10.4) were used to generate figure legends, and default settings in GraphPad Prism were used for these calculations. The significant confidence interval was set at 95%. The exact P values are provided in figures or figure legends, and default settings in GraphPad Prism were used for these calculations. The exact confidence interval was set at 95%. The exact P values are provided in figures or figure legends, except when P is less than 0.0001 because GraphPad Prism does not provide exact P values below 0.0001.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The plasmids pCDNA3-BREP (#172337), pCDNA3-BRIC (#172338), pAAV-H5m-BREP (#172341), pAAV-H5m-BRIC (#172341), and pBAC-BRIC (#172343) and their sequence information have been deposited to Addgene. All key data and experimental methods are presented in the main text or the supplementary materials. Source data are provided with this paper. Protein structures (Entries 2BBM, 7MJ3, and 5LK4) used for creating graphs can be accessed from the RSCB Protein Data Bank. Source data are provided with this paper.

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Author contributions

H.A. conceived the project. X.T. synthesized compounds, engineered and characterized BRIC in vitro and in cultured cells, and prepared virus. Y.Z. performed in vivo viral injection. X.L. prepared primary neurons. X.T., X.L. and Y.Z. performed in vivo brightness comparison of substrates and indicators. Y.Z. and X.T. imaged brain slices and live animals. T.W. prepared some brain slices and conducted intravenous injections of substrates. Y.X. developed and recorded the emission of BREP. H.A., X.T., Y.Z. and Y.X. analyzed data and prepared figures. H.A., X.T., Y.Z. and Y.X. wrote the manuscript.

Competing interests

Although there is no current plan to patent ETZ and BRIC, HA was an inventor of a patent (US Application # 15/694238) about DTZ and telLuc awarded to the University of California. Also, the University of Virginia filed a patent application (US Application # 17/434351) covering BREP, and HA and YX were listed as inventors. The remaining authors declare no competing interests.

Additional information

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