

Supplementary materials

Materials and Methods

Materials

DMEM, glutamine, HBSS, Hank's salts solution, trypsin-EDTA, penicillin/streptomycin solutions were from PanEko (Russia); Minimum Essential Medium (MEM), heat inactivated Fetal Bovine Serum (FBS), Neurobasal medium, Tyrode solution, B27 supplement, Gluta-Max solution, poly D-lysine were from Invitrogen (USA); potassium gluconate, sodium gluconate, bicuculline, nigericin, monensin were from Sigma; Fugene HD was from Promega, Lipofectamine LTX kit with Plus Reagent was produced by Invitrogen. Glass bottom dishes were from Mattek and Fluoridish. HeLa-Kyoto cells were provided by Carsten Schultz, EMBL Heidelberg.

1. Gene design and cloning

Taq polymerase (Evrogen) was used for mutagenic PCR amplifications. The mutant versions of SypHer were obtained in a PCR reaction with Taq polymerase in a high salt reaction mix, leading to random mutations of the gene during the reaction. The original HyPer¹, HyPer-2² and HyPer-3³ plasmids were used as a template DNA for PCR. To introduce a C199S mutation into HyPers, we amplified two fragments using primers: 5'-tccgctagcgccaccatggagatggcaagccag-3', 5'-atcgcgcaaagagtgacatct-3', and 5'agatggcactctttgcgcat-3', 5'-atcaagcttttaaacgctgtt-3' and each HyPer template in separate probe in PCR mix in Taq buffer (Evrogen) added with 640 μM MnSO₄, 200 μM dATP, 360 μM dGTP, 1 mM dCTP and 1 mM dTTP. It was followed by another round of PCR, using the PCR fragments obtained and N- and C-terminal primers 5'tccgctagcgccaccatggagatggcaagccag-3' and 5'-atcaagcttttaaacgctgtt-3' to produce the complete sensor construct. The full-length product was purified by agarose gel electrophoresis and then predigested PCR product was ligated into the target vector pBADHisB (ThermoFisher Scientific) or pC1 (Evrogen)). T4 ligase in Rapid Ligation Buffer (Promega) or T4 ligase (Evrogen) was used for ligation. Final constructs were used for transformation of electrocompetent *E. coli* cells strain BW25113 (4 μg of ligate to 160 μg of cells). Bacteria were plated to 20 dishes Petri dishes with LB-agar containing 50 μg/ml ampicillin. After 24 hours colonies were washed from dishes in 20 ml of SOB with 50 μg/ml ampicillin and diluted to optical density 2.0. After that protein synthesis were stimulated with 0.002% of arabinose for 4 hours.

Bacteria sorted on FACS Aria III cell sorter were plated on dishes and 24 hours later induction of protein synthesis was started by spraying 0.002 % arabinose solution over the bacterial colonies on a Petri dish. After 4 hours incubation at 37°C we visually evaluated the fluorescence intensity of individual colonies under a fluorescent binocular microscope Olympus XD (excitation 420 nm "CFP", 500 nm "GFP", emission 530 nm "GFP") and the brightest colonies were picked out to a new dish. Stimulated cells were sorted with FACS for the brightest fluorescence excited with 405 nm and 488 nm lasers in GFP emission channel into 5 ml of SOC media. Selected bacteria were incubated for 1 hour at 37 °C and plated to fresh LB/ampicillin dish. Induction of protein synthesis was carried out 24 hours after planting bacteria by spraying the bacterial colonies on a petri dish with a 0.002 % arabinose solution, and after 4 hours incubation of the dishes at 37°C we analyzed the fluorescence intensity of individual colonies under a fluorescent binocular microscope Olimpus XD (excitation 420 nm "CFP", 500 nm "GFP", emission 530 nm "GFP") and the brightest colonies were picked out to a new dish. Spectral characteristics of selected

clones were evaluated using a fluorescent spectrophotometer Varian Cary Eclipse. Bacterial cells were transferred from Petri dishes to 2 ml of PBS buffer (25°C) in a plastic spectrofluorimeter cuvette. We detected excitation spectra in the range of 390–510 nm (emission at 530 nm).

SypHer3s were cloned to pQE-30 vector for expression in XL1Blue E.coli strain with BamHI and HindIII enzymes and to eukaryotic pC1 vector (Evrogen) with NheI – HindIII sites. SypHer3s-IMS was produced by replacing the EGFP-coding region of IMS-EGFP by SypHer3s-coding DNA using restriction enzymes BamHI and NotI. To produce SypHer3s mito, we replaced sequence of Hyper in d-mitoHyPer pC1 plasmid (Evrogen) to SypHer3s one using Bam HI and Afe I sites.

Vector pLU-camkII-synaptophysin-tdTomato with α -Calcium/calmodulin-dependent kinase II neuronal promoter was created by inserting tdTomato into previously created in our lab pLU-camkII-synaptophysin-SypHer2⁴ using NheI and BsrGI sites with following primers: 5'-gactacgtagctagcatggtgagcaagggcgagg-3' and 5'-acagctatgtacattactatacagctcgccatgccg-3'

Protein expression and purification

To purify SypHer line probes competent E.coli XL1Blue cells were transformed with pQE30 plasmid containing SypHer, SypHer2 or SypHer3s gene. Single colonies selected on ampicillin were picked and used to inoculate 200 ml LB/ampicillin medium (1 mg ml⁻¹). Bacterial cultures were shaken at 200 rpm for 24 h at 37 °C and 12 h at rt. Bacteria were harvested by centrifugation at 4,500 × g for 30 min (4°C). The pellets were resuspended in 7 ml of PBS, lysed by B-Per Reagent (Thermo Fisher Scientific) for 20 min at rt and clarified by centrifugation at 18,000 × g at 4 °C for 40 min. Proteins were purified from the cell-free extracts by metalaffinity chromatography (Talon Metal Affinity Resin, Clontech). A 2 ml of the extract was applied to a column filled with 3.0 ml resin equilibrated with PBS. The column was washed with 30 ml of PBS and then the protein was eluted with PBS containing 200 mM imidazole. The purified protein was applied to a 10 ml Sephadex G-25 column (GE Healthcare) equilibrated with PBS to remove imidazole.

SDS-PAGE

The purity of the proteins used in experiments is 85-90% according to gel electrophoresis picture what is standard to Talon resin purification protocol. SDS-PAGE of the protein samples was performed using a 10-25% gradient gel according to ⁵. Gels staining was done according to the standard Coomassie G250 staining protocol (Suppl. Fig 1.)

Molecular weight of the SypHers monomers in denaturative environment is the same for all SypHers and 53.2 kDa, as it was expected from gene sequence of The SypHer3s, confirming data from gel filtration analysis.

Spectroscopic characterization of the probe

Equal aliquots of SypHer, SypHer2 and SypHer3s variants were diluted in 2 ml PBS and their absorption spectra were measured in the range of 200–800 nm to determine molar extinction coefficients of proteins. The molar extinction coefficients were determined using the formula $\epsilon_i = \text{Abs}_i \cdot C_{\text{prot}}(M) \cdot l(\text{cm})^{-1}$ where, Abs_i—the optical density at the absorption maximum and l—the optical path length. Concentrations of proteins were determined by Bradford and microbiuret protein assay.

To determine the quantum yield of fluorescence of the probe, the emission spectra of the fluorescence excited at 495 nm were measured and compared with the proteins with known QY (EGFP). QY for each protein was determined by using the formula $QY = QY1 \cdot (Abs1 \cdot Em2 - Abs2 - 1 \cdot Em1 - 1)$ where Abs1 and Em1—the values of the absorption and emission of standard (EGFP: Abs488 Em507), Abs2 and Em2—the values of the absorption (Abs495) and emission (Ex 410 nm and 490 nm) for SypHer probes, QY1—the quantum yield of fluorescence of standard (QY(EGFP)=0.6, Abs488 = 56 000 cm⁻¹*M⁻¹), QY—the quantum yield of fluorescence of SypHer3s probe.

To determine the apparent pKa buffers with pH values from 3.0 to 11.5 were prepared. Calibration of SypHer3s in vitro signal upon pH (3.0 – 10.55) measured in buffers containing 130 mM KCl, 30 mM NaCl, 0.5 mM MgCl₂, 0.2 mM EGTA and (pH 3.0-4.5) 30 mM HCl – NaH₂C₆H₅O₇ or (pH 5.0-7.5) 15 mM KH₂PO₄ – Na₂HPO₄ or 20 mM (pH 8.0-11.0) Na₂B₄O₇ – HCl/NaOH. The pH titration of oxidized protein was performed by adding 15 µl of concentrated protein in PBS into 2 ml of each of the buffers described above. The fluorescence of probe in each buffer condition was recorded using a spectrophotometer Varian Cary Eclipse.

Cell culture and transfection

HeLa-Kyoto (EMBL collection) were seeded into 35-mm glass bottom dishes (MatTek or Fluoridish) and cultured in DMEM with 10% FBS at 37 °C in a 5% CO₂ atmosphere. After 24 h, cells were transfected by a mixture of 2 ng DNA and 6 µl FuGene HD transfection reagent in 100 µl OptiMEM solution per one dish. After 12 h, cell medium was replaced by fresh DMEM medium.

Mixed (neuronal and glial) mouse cortex primary embryonic neuronal cell culture was prepared as indicated in ⁶, with the protocol for animal handling approved by the ethical committee of the Institute of Bioorganic Chemistry RAS. Pregnant C57Bl/6 mice were used at E17 and embryos were anesthetized in cold HBSS on ice, brains were extracted, hemispheres separated from meninges and cortexes collected in 15 ml ice cold HBSS. The cortexes were incubated in 0.05% trypsin/EDTA solution for 15 minutes at 37°C, washed 4 times in rt DMEM supplemented with 10% FBS and 20 mM glutamine, and carefully resuspended with a 1 ml tip for 8 times. Cells were seeded on poly-D-lysine covered 35 mm glass bottom dishes, at 15*10⁴ cells in 100 µl of DMEM supplemented with 10% FBS and 20 mM glutamine. After 40 min after plating, medium was added with for 2 ml Neurobasal medium supplemented with B27, 10% FBS and 20 mM Gluta-Max.

Neuronal culture were transfected on the 4th day after plating with Lipothectamine LTX by a mixture of 2 ng DNA (or 1:1 mix of plasmids in total amount with 2 ng) and 4 µl of Plus Reagent and 5 µl of Lipofectamine LTX in 100 µl OptiMEM per dish. Every 3rd day 30% of media was replaced for fresh neurobasal media with 10% FBS, 10 mM B-27 and 20 mM Gluta-MAX.

Intracellular pH calibration was performed as described in ⁷. Each calibration buffer contained 5 µM nigericin, 5 µM monensin, 130 mM potassium gluconate, 20 mM sodium gluconate, 0.5 mM MgCl₂, 0.2 mM EGTA, and 30 mM Tris (pH 8.0–9.0) or HEPES (pH 6.8 - 7.8) or MES (pH 5.0 - 6.6). For each pH point, cell dishes were washed 2 times and incubated for 4 min in a corresponding calibration buffer at 37°C.

Fluorescent imaging

For imaging 24-48 hours after transfection HeLa-Kyoto cells were placed into an environmental chamber in 2 ml Hank's (HBSS) or MEM solution supplemented with 20 mM HEPES (or NaHCO₃/CO₂ buffer) at 37°C and imaged using a Leica CTR6500 wide-field microscope equipped with a 20x air

objective and HCX PL APO lbd. BL or 63x 1.4NA oil objective. For 5% CO₂ in media cell dishes lids were sealed using vacuum grease layer in the atmosphere of 5% CO₂.

Neurons were imaged 14-16 days after plating in a Tyrode solution, supplemented with 20 mM D-Glucose and 20 mM HEPES pH 7.2 with 5 sec time laps interval.

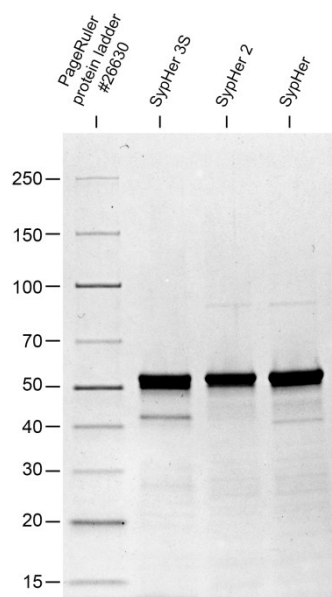
Microscopy of cells was carried out using Leica DMI6000 at 37 °C in HBSS supplemented with 20 mM of HEPES . SypHer3s fluorescence was sequentially excited at 427 and 504 nm using 427/10 and 504/12 band-pass excitation filters; emission of the indicator was collected using a 525/50 band-pass emission filter. GCaMP6s fluorescence were imaged with GFP filter (excitation BP 470/40 with central band 504 nm band, dichromatic mirror 565 and emission filter BP 525/25). For Sph-tdTomato TX2 filter cube was used (excitation: BP 560/40, dichromatic mirror 595, emission: BP 645/75). Recording intervals were 5 s. The final processing of images was performed using the Fiji (ImageJ) software.

For pH measurement calculation, stacks corresponding to 427 nm and 504 nm excitation of SypHer were converted to 32-bit after background subtraction. Stacks were thresholded to remove pixel values from the background. A 504-nm stack was divided by the corresponding 427-nm stack frame by frame. Time course of SypHer3s fluorescence was calculated for cell regions and background location outside the cell. For colocalization analysis custom macros was used. z-stacks for 2 channels of SypHer3s were divided for ratiometric result, then Sph-tdTomato were merged and colocalisation with deconvolution analysis were produced. Cololasion counted as overlay of mitochondrial automatic founded ROIs to Sph-tdTomato automatic founded ROIs. All graphs were processed in OriginPro8.1 (OriginLab).

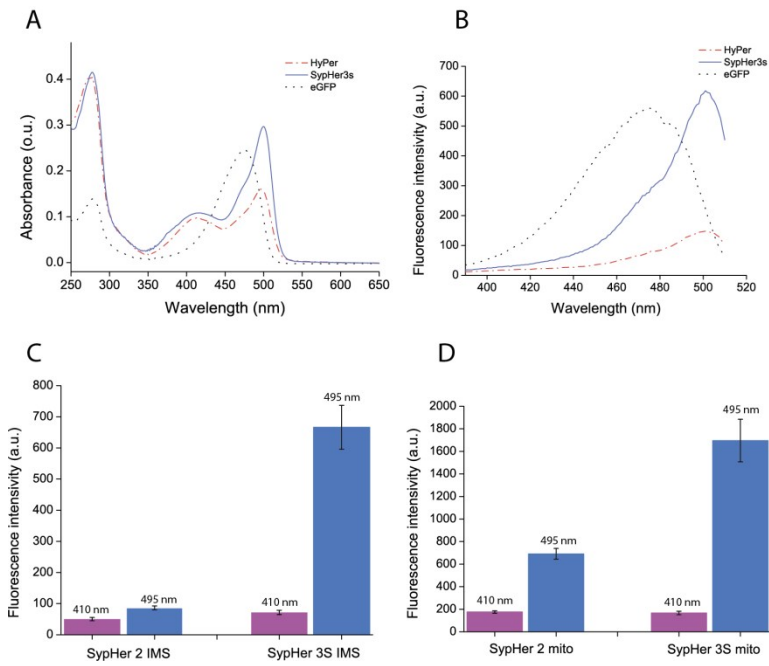
Animal Husbandry

Zebrafish of the wild type were maintained and raised under recommended conditions. mRNA encoding SypHer3s was synthesized using mMessage mMachine SP6 Transcription kit (Ambion). 0.1 µg/µL mRNA was injected into yolk-sacs of zebrafish embryos at the stage of a single cell. Then embryos with fluorescent signal at 48 hours post-fertilization were selected. Injected embryos were raised at 26,5°C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, and 0.1% Methylene Blue, equilibrated to pH 7.0) in Petri dishes in a room with 12/12 day-night maintenance. Before microscopy, embryos were dechorionated, immobilized in a 0.02% tricaine solution (Sigma) and enclosed in 1% low-melting agarose. In order to translate SypHer3s ratio in fish tissues to pH values, we calibrated the biosensor in a culture of HeLa-Kyoto cells at the same microscope parameters.

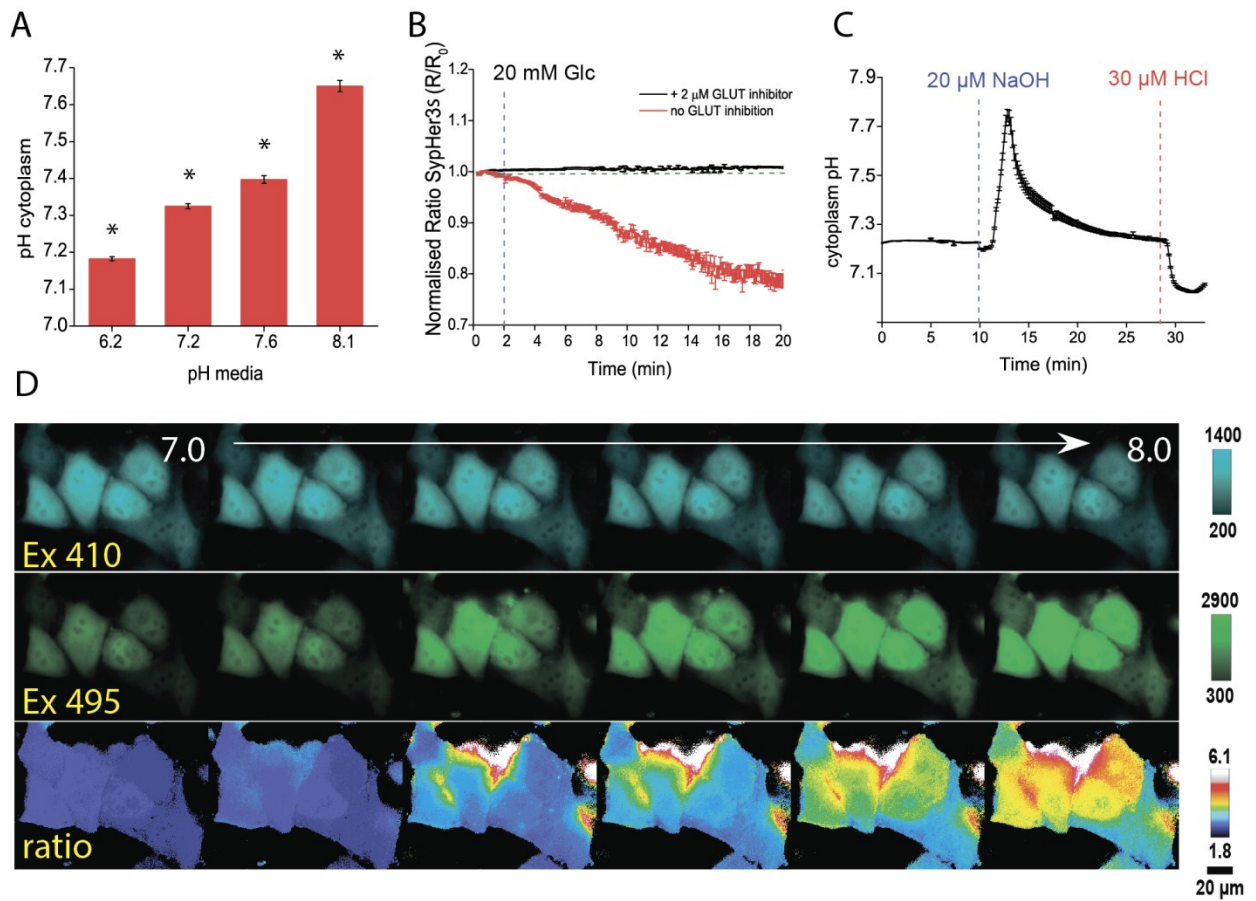
Supplementary figures



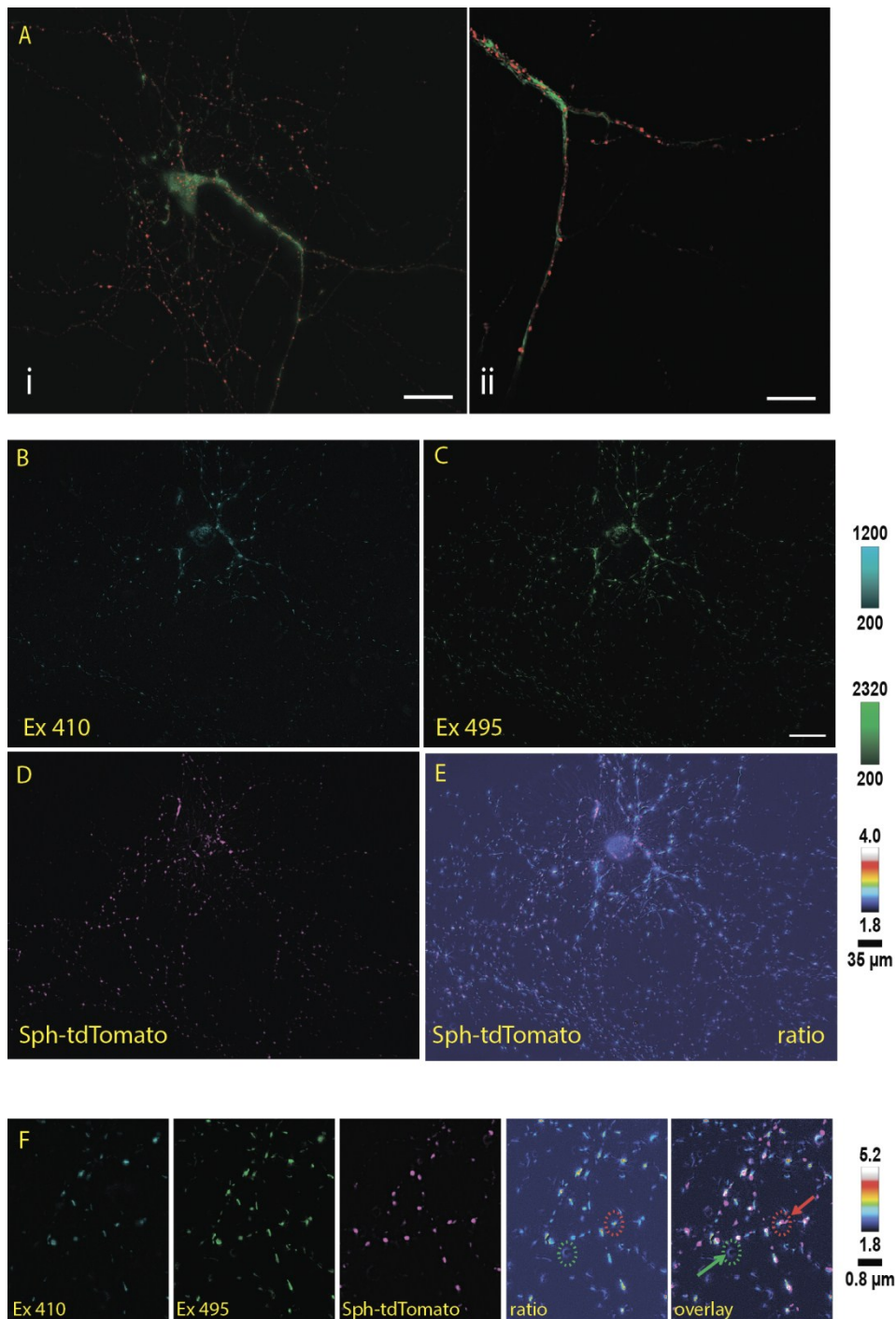
Suppl. Fig 1. SDS-PAGE of the purified SypHer3s, SypHer and SypHer2.



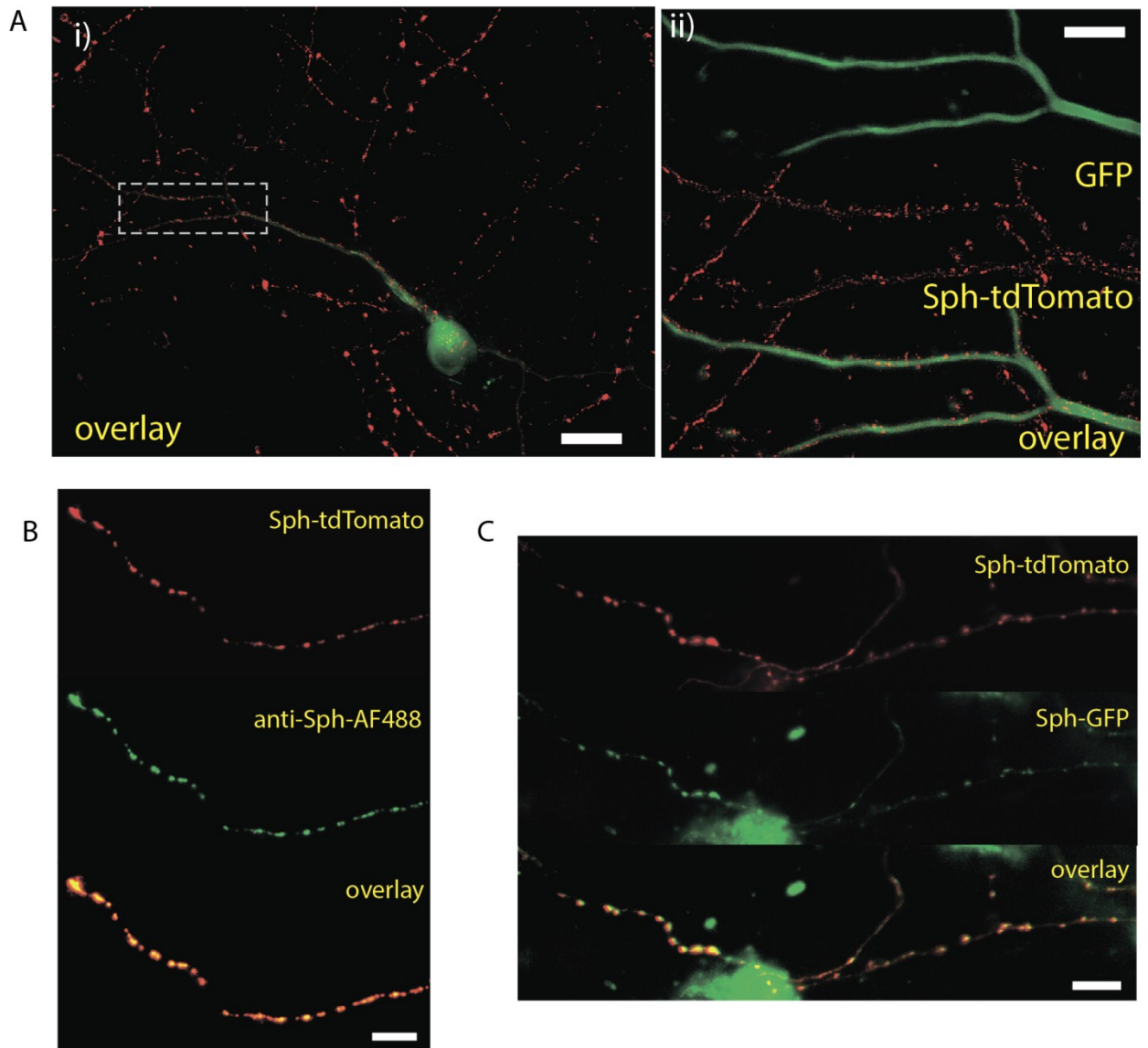
Suppl. Fig 2. Brightness of SypHer3s. (A) Absorbance spectra of HyPer, SypHer3s and EGFP in PBS. (B) Excitation spectra of HyPer, SypHer3s and EGFP in PBS. (C) Fluorescence intensity of HeLa-Kyoto cells expressing IMS-targeted version of SypHer2 or SypHer3s. (D) Fluorescence intensity of HeLa-Kyoto cells expressing SypHer2 or SypHer3s in the mitochondrial matrix.



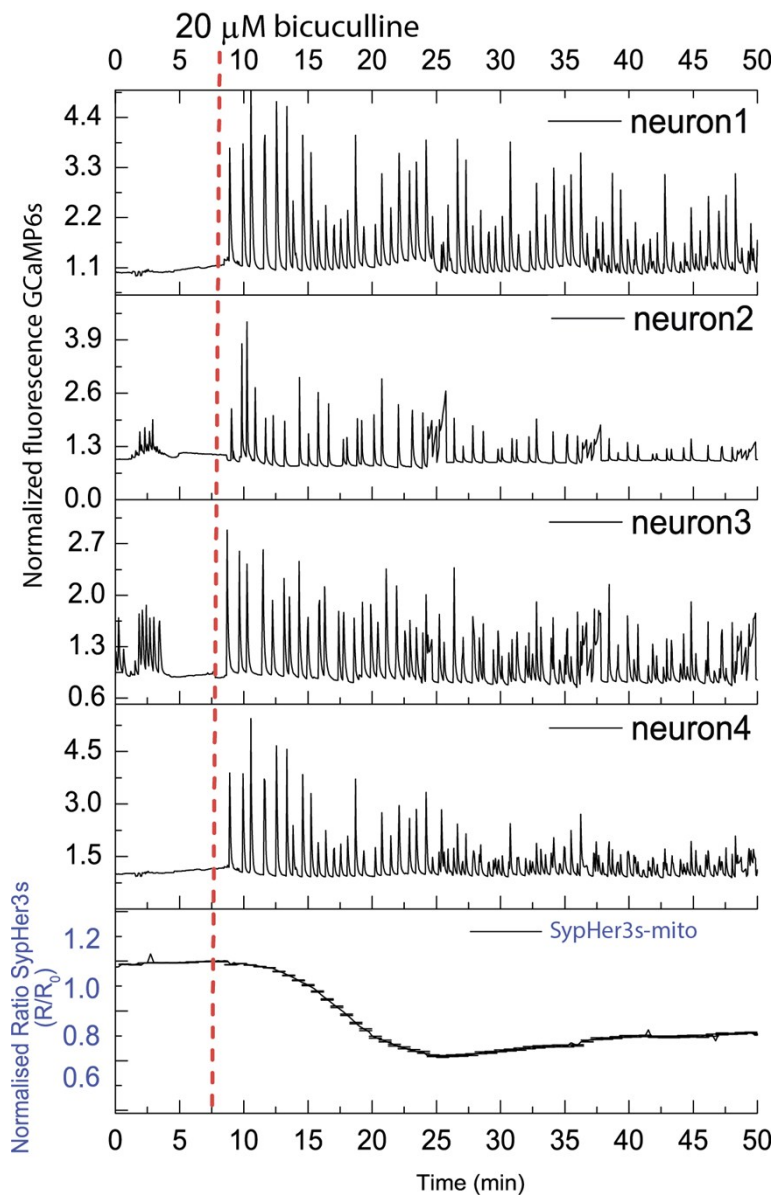
Suppl. Fig 3. Monitoring pH using SypHer3s in HeLa-Kyoto cells. (A) Cytosolic pH of HeLa-Kyoto cells depending on pH of Hank's solution containing 20 mM HEPES. (B) SypHer3s signal dynamics in cytoplasm of HeLa-Kyoto cells after 20 mM glucose addition. (C) Stressing HeLa-Kyoto cells with rapid media pH changes in Hank's solution containing 20 mM HEPES. (D) HeLa-Kyoto cells expressing SypHer3s in HEPES buffer containing nigericin and monensin under buffer pH change from 7.0 to 8.0. * $P < 0.01$, one-way ANOVA, followed by Bonferroni correction and Duncan post hoc test. The error bars represent the s.e.m.



Suppl Fig 4. Synaptic and non-synaptic mitochondrial matrix pH. Neuronal mitochondrial matrix pH depends on co-localisation with Sph-tdTomato. Images representing the colocalization of the SypHer3s-mito with Sph-tdTomato in presynaptic compartment. (A) 488 nm SypHer3s mito excitation, full picture (i, scale bar 60 μm), and one magnification (ii, scale bar 15 μm). (B) 410 nm SypHer3s mito excitation (scale bar 90 μm). (C) 488 nm SypHer3s-mito excitation, (D) 543nm excitation for Sph-tdTomato, (E) Overlay SypHer3s of ratio F495/F410 with Sph-tdTomato. (F) Magnified fragment of the neuron from the panels B-E. Images representing the co-localization of the SypHer3s-mito with Sph-tdTomato in presynaptic compartment. Red line – synaptic mitochondria, green line – non-synaptic mitochondria. Images were captured using the Leica 6000 DM Microscope at separate channels using 60X magnification.



Suppl. Fig 5. Sph-tdTomato characterization in neuronal culture. (A) Neuron (i) cotransfected with non-localised GFP and Sph-tdTomato. Scale bar 50 μm; ii) fragment of the axon, scale bar 16.5 μm. (B) Sph-tdTomato transfected neurons stained with mouse antibodies to Sph (7H12, Cell Signaling) and anti-mouse goat Alexa-Fluor488 (Thermos Fisher Scientific). Scale bar 8.0 μm. (C) Co-transfection of Sph-tdTomato and Sph-GFP. Scale bar 15 μm.



Suppl. Fig 6. pH_{mito} dynamics and calcium oscillations in neurons after 20 μM bicuculline treatment.

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