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Supporting Material

Nuclear Calcium Sensors Reveal that Repetition of Trains of Synaptic Stimuli Boosts Nuclear Calcium Signaling in CA1 Pyramidal Neurons

C. Peter Bengtson, H. Eckehard Freitag, Jan-Marek Weislogel, and Hilmar Bading

Supplemental File

MATERIALS AND METHODS

Plamids and recombinant adeno associated viruses

The vectors used to construct and package recombinant adeno associated viruses (rAAVs) have been described previously (21). The rAAV expression vector was modified by deletion of the original 5'NheI side and insertion of a multiple cloning site (MCS) containing 5'NheI/ 3'BamHI sides and at the 3'end a myc-tag. The coding sequence of *pEYFP-Nuc* (Clontech) was inserted into 5'NheI and 3'BamHI sides to yield rAAV-*EYFP-Nuc*. 5'NheI and 3'BglII sides were added by PCR to the entire coding sequence of Inverse Pericam (22) and GCaMP1.6 (23). The PCR products were inserted into 5'NheI and 3'BglII sides of rAAV-*EYFP-Nuc* to yield rAAV-*Inverse Pericam-NLS* (rAAV-*IP-NLS*) and rAAV-*GCaMP1.6-NLS*. The following primers were used: 5' TTT TGC TAG CGC CAC CAT GAA GAG G 3' (for IP) and 5' GAT GAC AGC AAA GAG ATC TTC T 3' (for IP rev). 5' TTA GTG AAC CGT CAG ATC CGC TAG 3' (for GCaMP1.6) and 5' AGG CAA GAT CTC TTC GCT GTC ATC 3' (for GCaMP1.6 rev). 5'AgeI and 3'BglII sides were added by PCR to the entire coding sequence of *GCaMP2.0* (24). The PCR product was inserted into 5'AgeI and 3'BglII sides of rAAV-*EYFP-Nuc* to yield rAAV-*GCaMP2.0-NLS*. The following primers were used: 5' TTA ACC GGT GGA TCC CGC CAC CAT GCG GGG 3' (for *GCaMP2.0*) and 5' AGC CAG ATC TCT TCG CTG TCA TCA 3' (for *GCaMP2.0* rev). rAAV-*GCaMP2.0*-NLS was further subcloned in the CaMKII-driven rAAV expression vector (rAAV-*eGFP*) using 5`AgeI and 3`EcoRI (CaMKII-driven rAAV-*eGFP* was kindly provided by Peter Seeburg). The vectors used to construct and package rAAVs have been described previously (21, 25).

Stereotaxic delivery of rAAVs to the rat brain

Viral vectors carrying nuclear calcium indicators were delivered by stereotaxic injection (Kopf Instruments, Tujunga, CA) into the hippocampus of Sprague Dawley rats (Charles River Breeding Laboratories) at P22 weighing ~44 g. Animals were anesthetized with an intraperitoneal injection of sleep-mix (Fentanyl (5 µg/kg body weight), Medetomidine (150 µg/kg bw), Midazolam (2 mg/kg bw). A total volume of 2µl containing about $2x10^9$ genomic virus particles were injected unilaterally at flow rate of 200 nl/min using a microprocessor-controlled mini-pump (World Precision Instruments, Sarasota, FA). The following coordinates relative to Bregma were used: anteroposterior, -4.3 mm; mediolateral, -4.1 mm; dorsoventral; -3.9and -3.6 mm from the skull surface (1 µl plus 1 µl after retracting the needle 0.3 mm). After the operation, animals were injected subcutaneously with wake up mix (Atipamezol (750 µg/kg bw), Flumazenil (200 µg/kg bw), Naloxone (120 µg/kg bw)).

Animals had free access to food and water and were housed under diurnal lighting conditions. The experiments were carried out according to ethical guidelines for the care and use of laboratory animals for experiments, and were approved by the local animal care committee (Karlsruhe, Germany).

Organotypic slice culture

Brain slices were prepared from 6-days-old Sprague–Dawley rats. The pups were decapitated and the brains were removed and placed into ice-cold dissection medium (EBSS containing $CaCl_2$ and $MgCl_2$, 22 mM glucose) for blocking. Brains were then transferred to the Teflon stage of a tissue chopper (H. Saur Laborbedarf, Reutlingen, Germany) and 400 µm thick

slices were cut. Hippocampal slices were micro-dissected from each brain slice in ice-cold dissection medium and transferred onto PTFE-cell culture inserts (Millipore, Billerica, MA, USA). The slices were kept in a humidified atmosphere (5% CO_2/95 % air) at 37° C for 2 weeks as described previously (26), except that the culturing medium had the following composition: (50% v/v Eagle's basal medium, 25 % v/v Earle's balanced salt solution, 25 % v/v horse serum (Invitrogen, Karlsruhe, Germany), 22 mM glucose, 1 mM L-glutamine, Phenol Red (Sigma, Taufkirchen, Germany) and 0.5 % penicillin/streptomycin (Sigma, Taufkirchen, Germany). Half of the medium was replaced every three days. Organotypic slice cultures were infected at DIV5 or DIV6 by adding 1-2 µl of rAAV onto the surface of the slice. Recordings were made at DIV14. Solutions for all culture recordings consisted of (in mM) NaCl 140, KCl 5.3, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, glycine 0.01, glucose 30, Na-pyruvate 0.5. GCaMP1.6-NLS infected cultures were transferred to a 4% CO_2 incubator at 28°C two to six hours before recordings.

Acute brain slice preparation

Brain slices were prepared from P35-P42 rats. Rats were anaesthetized with 2-bromo-2-chloro-1,1,1-trifluoroethane (Sigma) by inhalation and killed by decapitation. The brain was rapidly removed and submerged in ice cold slicing solution (in mM: Sucrose, 150; NaCl, 40; KCl, 4; $MgCl_2$, 7; NaH_2PO_4, 1.25; $CaCl_2$, 0.5; glucose, 25; $NaHCO_3$, 26; gassed with 95% O_2 and 5% CO_2). A vibratome was used to cut 300µm thick slices at an angle of approximately 30° above the horizontal in slicing solution maintained at 0°C (CU65 Cooling Unit & HM650V Vibratome, Microm, Walldorf, Germany). Hippocampii were dissected out of each slice and transferred to a holding chamber containing artificial cerebrospinal fluid (ACSF, in mM: NaCl, 125; KCl, 3.5; $MgCl_2$, 1.3; NaH_2PO_4, 1.2; $CaCl_2$, 2.4; glucose, 25; $NaHCO_3$, 26; gassed with 95% O_2 and 5% CO_2). Slices were maintained at 32°C for the first 30 min and then returned to room temperature until used for recordings over the subsequent four hours.

Patch-clamp recordings

Single slices were transferred to a recording chamber (PM-1, Warner Instruments, Hamden, CT, USA or PC-R, Siskiyou, OR, USA), secured with a platinum harp and completely submerged with continuously flowing (3 ml/min) ACSF. Whole-cell patch-clamp recordings were made from CA1 hippocampal pyramidal neurons. Patch electrodes (3-4 MΩ) were made from borosilicate glass (1.5 mm, WPI, Sarasota, FL, USA) and filled with a potassium methylsulphate based solution (containing in mM: KCH_3SO_4, 145; NaCl, 8; HEPES, 10; K_2-phosphocreatine, 10; Mg_2-ATP, 4; Na_3-GTP, 0.3; 0.125; pH 7.35 with KOH). Recordings were made with a Multiclamp 700A or 700B amplifier, digitized through a Digidata 1322A A/D converter and acquired and analyzed using pClamp 9 software (Axon Instruments and Molecular Devices, CA, USA). Access resistance (range: 10 – 28 MΩ) was monitored regularly during voltage clamp recordings and data was rejected if changes greater than 20% occurred. All membrane potentials have been corrected for the calculated junction potential of -11 mV (JPCalc program by Dr. Peter H. Barry).

Stimulation protocols

Evoked excitatory post-synaptic currents (eEPSCs) were recorded in response to 100 µs long constant current pulse stimuli at intensities given in the results text from constant current bipolar stimulus isolator units (A365, World Precision Instruments, Sarasota, Florida, USA). Stimulus isolators were connected to either the two chambers of a theta glass electrode filled with ACSF or to an Ag/AgCl electrode in the recording chamber and a glass patch pipette filled

with ACSF and placed onto the surface of the slice. One stimulating electrode was positioned in stratum radiatum approximately 100 µm from the stratum pyramidale 50 to 100 µm toward the CA3 from the patched cell. Paired pulse recordings were performed in standard solutions using a stimulation intensity adjusted to produce an EPSC around 300 pA in amplitude. For LTP recordings, a second stimulating electrode was placed in the stratum oriens approximately 50 µm from the patched cell and single stimuli were applied to each pathway (radiatum and oriens) at an interval of 30 s. Stimulation intensities were set to the approximate threshold for eliciting an action potential with a single stimulus (30 to 80 µA), which was independently assessed in each slice (see figure 2 and main text). Stable baseline measurements were obtained for at least 8 minutes before LTP was induced. High frequency stimulation (HFS) protocols consisted of 100 Hz stimulations of 1 s (i.e., 100 stimuli). Theta burst stimulations (TBS) consisted of 10 repetitions at 5 Hz of groups of 4 stimuli delivered at 100 Hz. TBS trains were repeated at 60 s intervals. HFS trains were repeated at 30 s, 60 s or 5 min intervals as indicated in the results text.

Calcium imaging

All recordings from cultures and slices were performed at 32-34°C except GCaMP1.6-NLS infected material which was recorded at room temperature. Neurons were viewed with differential interference contrast optics, infrared illumination through a 20x (XLUMPLFL20xW, N.A. 0.95, Olympus, Hamburg, Germany) objective on an wide field upright microscope (BX51WI, Olympus) equipped with a CCD camera (Photometrics Coolsnap HQ, Roper Scientific, Ottobrunn, Germany) connected through a software interface (Metafluor, Universal Imaging Systems and Molecular Devices, Downington PA, USA) to a computer monitor. Fluorescent excitation light was generated by a monochromator coupled to a light source with a 75W xenon arc lamp (Optoscan and Optosource, Cairn, Faversham, UK). All images were corrected for background fluorescence using a measurement from the same image in a region devoid of GECI expression. GCaMP1.6 and 2.0 were excited at 480nm (20 nm bandwidth) through a BA470-490 excitation filter, DM505 dichroic and a BA510-550 emission filter (Olympus). Inverse Pericam was excited at 495 nm (20 nm bandwidth) through a 515DCXR dichroic and a 545/50 emission filter (Chroma, Fürstenfeldbruck, Germany). All indicators showed rapid exponential decay in fluorescence at the start of imaging but recovered fluorescence after several seconds in the dark, indicating that the fluorescence decrease results from photoisomerization (reversible bleaching) but not from bleaching. For this reason, all experiments were performed at a constant exposure (15-30 ms with 4x4 binning) and imaging rate (2 Hz) and stimulations were presented after baseline intensities had stabilized. IP-NLS showed some bleaching which caused a maximal loss of fluorescence of 1% per minute for a typical recording of one 50 ms exposure per second. This imposed a linear baseline drift for inverse pericam recordings which was corrected during analysis. Data for GCaMP1.6 and GCaMP2.0 is presented as: $\Delta F/F_0 = (F - F_0)/F_0$ and data for inverse pericam is presented as: $\Delta F/F = (F_0 - F)/F$ where F represents the average background subtracted emission fluorescence intensity in a region of interest (ROI) and F_0 represents the baseline F measured prior to each stimulation. Fold increase in responses to stimulation was calculated from $\Delta F/F$ and $\Delta F/F_0$ data as the response amplitude of the largest response in the train series divided by the amplitude of the response to the first train in the series. Both fold increase (see Results text) and normalization to the maximum response (see Figure 3B) were calculated on a within-cell basis and thus mean values do not represent the reciprocal of each other. Where possible, the identity of patched cells in fluorescence images was confirmed after L-LTP recordings using suprathreshold

depolarization to elicit VOCC-mediated calcium responses (see Figure 4C and Supplementary Figure 2D). At the end of all recordings, depolarizing solution containing 45 mM K^+ and 4 mM calcium was applied to the slice to elicit a near maximal calcium response. Depolarizing solution evoked larger fluorescence increases than depolarisation to a similar membrane potential with a voltage step (see Figure 4) largely due to the contribution of photons emitted by nearby cells especially above and below the focal plane which cannot be excluded with the wide-field microscopy. For this reason single cell and multiple cell responses are only compared in Figure 4 where a normalization procedure was necessary to correct the data (see Results text and Figure legend). Dynamic range was calculated as the ratio of fluorescence at saturating (Fmax) and minimum (Fmin) calcium concentrations. Fmin was estimated with EGTA. Fmax was estimated with depolarizing solution in slices and ionomycin in dissociated cell cultures. Ionomycin seemed to penetrate poorly in slices. Despite the steep Hill coefficient of GCaMP (27-29), HFS and TBS responses were always smaller than Fmax and thus did not saturate the indicator. Regions of interest for analysis were assigned using images recorded during depolarization to more accurately identify and localize infected neurons with weak basal fluorescence. For calcium uncaging experiments, 50 μM D-myo-inositol 1,4,5-trisphosphate, $P_{4(5)}$-1-(2-nitrophenyl)ethyl-ester (NPE-IP_3) and 100 μM Fluo-4 were included in the patch pipette. Uncaging of IP_3 was achieved with whole field illumination with a 40 ms pulse of 360 nm light from the monochromator. Fluo-4 was imaged using the same filter and dichroic as for GCaMP without the excitation filter. All data are expressed as mean ± standard error of the mean. Statistical comparisons were made using t tests for independent or paired samples.

Confocal imaging

Confocal images of GCaMP-NLS were made using a laser scanning confocal microscope (TCS SP2, Leica, Mannheim, Germany) on an inverted fluorescent microscope (DM IRE2, Leica) using Leica confocal scan (LCS) software. Images represented in the results section were scaled to enhance contrast and artificially colored by adapting the look-up table to the color of the corresponding fluorescent tag for better representation.

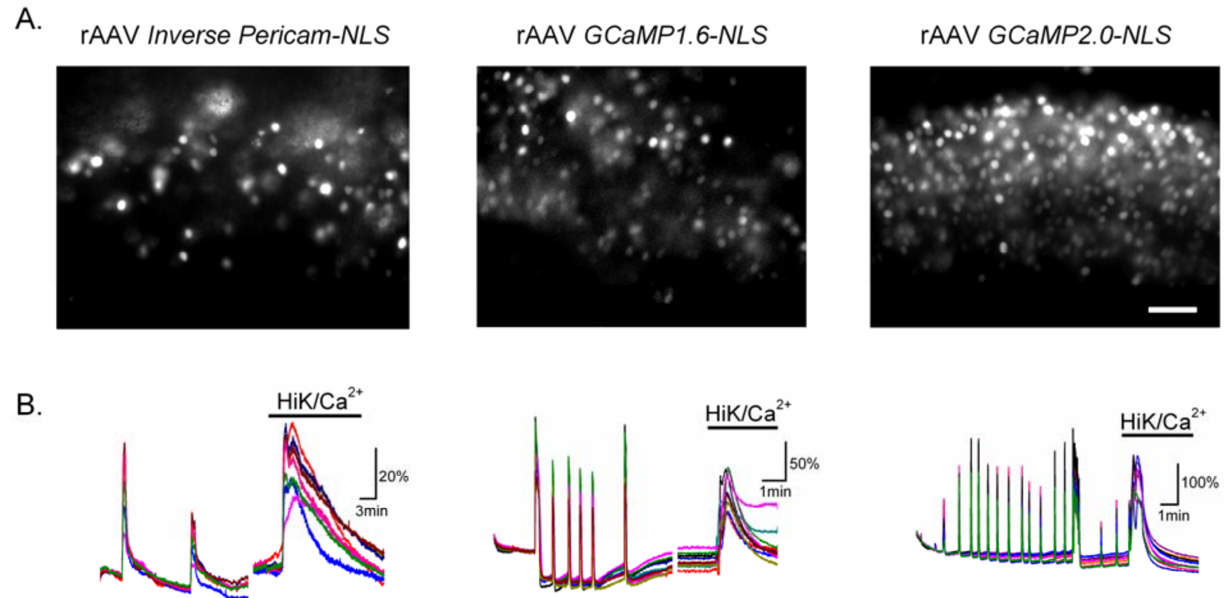
SUPPLEMENTARY TABLE

Supplementary Table 1: Passive membrane properties. Values represent the indicated passive membrane properties measured at -70 mV soon after establishing whole cell mode in CA1 pyramidal neurons in control slices or from neurons infected with the indicated GECI or their uninfected neighbours (GCaMP2.0-NLS negative).

	n	Vrest (mV)	Cm (pF)	Rm (MΩ)	threshold (μA)
Control	48	-72.9± 0.9	197 ± 8	94 ± 4	56 ± 5
IP-NLS	5	-75.1 ± 1.6	200 ± 18	118 ± 22	56 ± 2
GCaMP1.6-NLS	4	-75 ± 2.2	168 ± 19	112 ± 5	47 ± 3
GCaMP2.0-NLS	25	-72.2 ± 1.0	160 ± 8	113 ± 6	51 ± 3
GCaMP2.0-NLS negative	15	-71.9 ± 1.5	167 ± 8	116 ± 5	53 ± 5

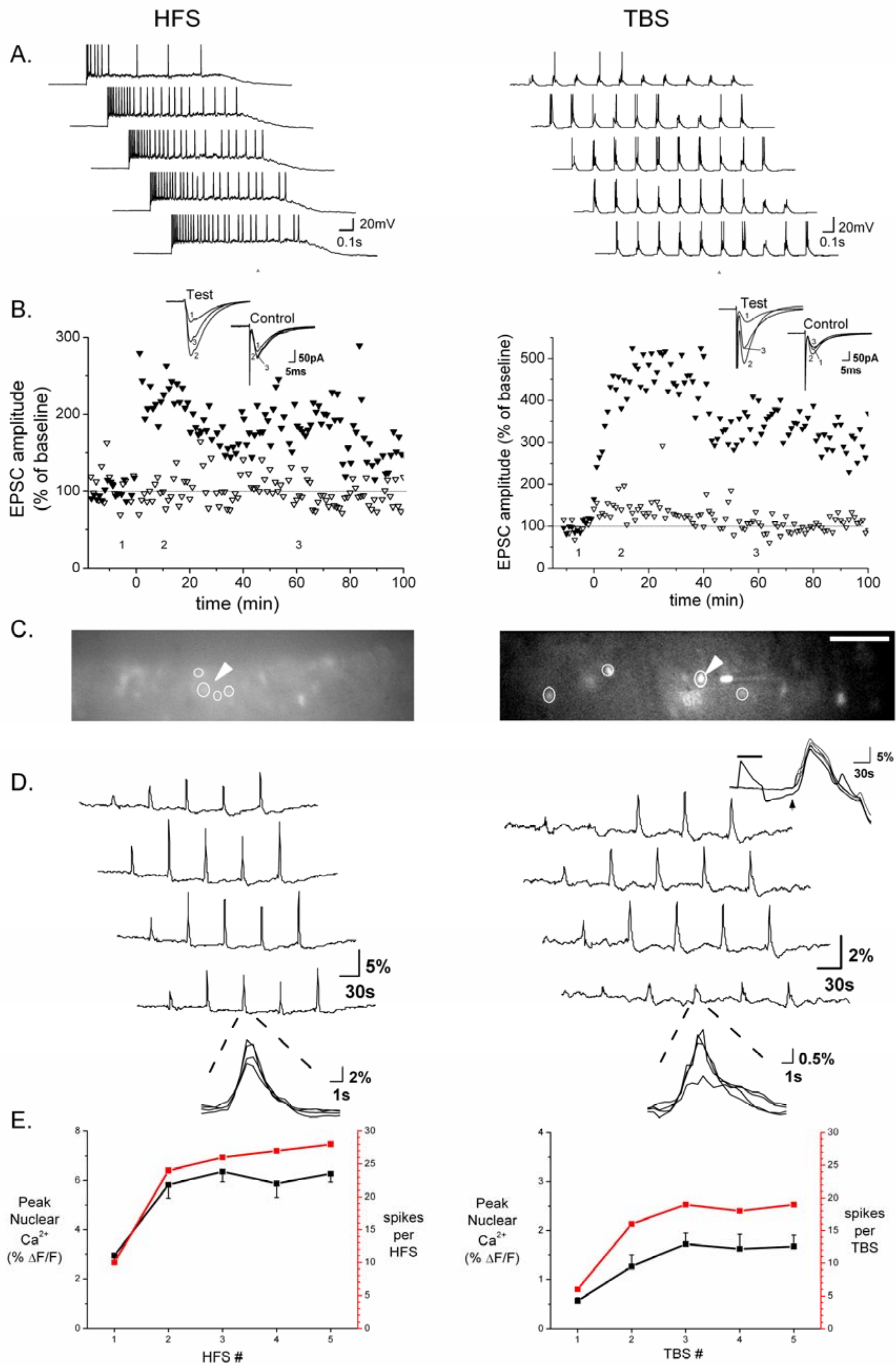
SUPPLEMENTARY FIGURES

Supplementary Figure 1.



Recombinant nuclear calcium indicators used to image activity in slice cultures. **(A.)** Images show 14 day-old organotypic slice cultures of the hippocampus infected 8 or 9 days previously with rAAV containing IP-NLS (left) or GCaMP1.6-NLS (centre) or GCaMP2.0-NLS (right). Scale bar is 50 µm. **(B.)** The results of a live imaging experiment from each culture shown in A. Each trace represents the nuclear calcium signal from one cell. All cells show intermittent nuclear calcium transients due to recurrent and synchronised action potential bursting across the neuronal network following application of the $GABA_A$ receptor antagonist, bicuculline (50 µM) (30). Subsequent application of solution containing 45 mM K^+ and 4 mM calcium evokes a slower rise in nuclear calcium whose fluorescent signal generally returns within a few minutes to baseline or below.

Supplementary Figure 2



Examples of responses to repeated TBS and HFS. Shown are representative recordings from the CA1 region of slices infected with GCaMP1.6-NLS. **(A.)** Bursting in these cells was evoked with five successive trains of HFS (left) or TBS (right) at an interval of 60 s. Trains 1 to 5 are depicted from top to bottom and spikes have been clipped for display purposes. Intensities of 40 μA (left cell) and 35 μA (cell on right) were used for burst induction. These stimulation intensities correspond to threshold for inducing an action potential with a single stimulus (see figure 2 and main text). Stimulation intensity was set to 25 μA for both cells for baseline and post induction EPSC recordings. **(B.)** HFS and TBS stimulation in the test pathway (stimulating electrode in stratum radiatum) induced LTP lasting at least 1.5 hours in the test but not the control (stimulating electrode in stratum oriens) pathway. The inset shows the average of five EPSCs in the control and test pathways at each of the time points indicated in the graph. **(C.)** GCaMP1.6-NLS fluorescence in cells near the neuron recorded in A and B (arrowhead). **(D.)** These traces show the nuclear calcium signal, quantified as ΔF/F, evoked by the five HFS and TBS stimulation trains for each of the four nuclei circled in C. Insets below show the overlayed responses of all cells to the second stimulation train on an expanded timescale. Inset at top right shows responses to depolarisation to -20 mV of the patched cell (bar) and to bath application of ACSF containing 45 mM K^+ and 4 mM calcium (arrow). **(E.)** The histograms show the number of spikes evoked in the patched cell (grey) and the amplitude of the nuclear calcium responses (black) averaged from 16 cells for the HFS and 9 cells for the TBS experiment.

Supplementary Movies. Confocal Z-stack of three GCaMP2.0-NLS expressing neurons. Shown are the Hoechst (**A.**) and GCaMP2.0-NLS (**B.**) channels of a confocal stack through 3 neurons. Note the exclusive nuclear expression of GCaMP2.0 which remains within the confines of the Hoechst staining.