Title: Improved spike inference accuracy by estimating the amplitude of unitary [Ca2+] transients in weakly GCaMP6f expressing hippocampal pyramidal cells

Authors: Timea Eltes
Miklos Szoboszlay
Katalin Kerti-Szigeti
Zoltan Nusser

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Improved spike inference accuracy by estimating the peak amplitude of unitary [Ca^{2+}] transients

in weakly GCaMP6f expressing hippocampal pyramidal cells

Tímea Éltes[1,2,3], Miklos Szoboszlay[1,4], Katalin Kerti-Szigeti[1,5] and Zoltan Nusser[1]

[1] Laboratory of Cellular Neurophysiology, Institute of Experimental Medicine, Hungarian

Academy of Sciences, Budapest 1083, HUNGARY

[2] János Szentágothai School of Neurosciences, Semmelweis University, Budapest 1085, Hungary

[3] Present address: National Institute of Clinical Neurosciences, Department of Neurology, 1145

Budapest, Hungary

[4] Present address: Mortimer B. Zuckerman Mind Brain Behavior Institute, Department of

Neuroscience, Columbia University, New York, NY 10027, USA

[5] Present address: Institute of Science and Technology, A-3400 Klosterneuburg, Austria

Correspondence: Zoltan Nusser, Laboratory of Cellular Neurophysiology, Institute of

Experimental Medicine, Szigony str. 43, Budapest 1083, Hungary, E-mail: nusser@koki.hu

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Key Points:

- the amplitude of unitary, single action potential-evoked $[Ca^{2+}]$ transients negatively correlates with GCaMP6f expression, but displays large variability among hippocampal pyramidal cells with similarly low expression levels.

- the summation of fluorescent signals is frequency-dependent, supralinear and also shows remarkable cell-to-cell variability.

- the main source of spike inference error is the variability in the peak amplitude, and not in the decay or supralinearity.

- we developed two procedures to estimate the peak amplitudes of unitary $[Ca^{2+}]$ transients and show that spike inference performed with MLspike (Deneux *et al.*, 2016) using these unitary amplitude estimates in weakly GCaMP6f expressing cells results in error rates of ~5%.

Abstract

Investigating neuronal activity using genetically encoded Ca^{2+} indicators in behaving animals is hampered by inaccuracies in spike inference from fluorescent traces. Here we combine two-photon $[Ca^{2+}]$ imaging with cell-attached recordings, followed by *post hoc* determination of the expression level of GCaMP6f, to explore how it affects the amplitude, kinetics and temporal summation of somatic $[Ca^{2+}]$ transients in mouse hippocampal pyramidal cells (PCs). The amplitude of unitary $[Ca^{2+}]$ transients (evoked by a single action potential) negatively correlates with GCaMP6f expression, but displays large variability even among PCs with similarly low expression levels. The summation of fluorescent signals is frequency-dependent, supralinear and also shows remarkable cell-to-cell variability. We performed experimental data-based simulations and found that spike inference error rates using MLspike (Deneux *et al.*, 2016) strongly depend on unitary peak amplitudes and GCaMP6f expression levels. We provide simple methods for estimating the unitary $[Ca^{2+}]$ transients in individual weakly GCaMP6f expressing PCs, with which we achieve spike inference error rates of ~5%.

Introduction

Monitoring the activity of large populations of neurons in behaving animals is essential for understanding the function of neuronal networks during complex behaviors. The ideal recording method should simultaneously capture the temporal dynamics of spiking activity of thousands of genetically and morphologically identified neurons. *In vivo* electrophysiological recordings have the required temporal resolution, but they allow the monitoring of the activity of a relatively modest number of cells for short periods and provide limited information about the identity of the recorded cells (Buzsaki, 2004). Optical imaging techniques, however, have the advantage of monitoring action potential (AP)-evoked fluorescent changes in a large number of individual neurons. Moreover, genetically encoded fluorescent indicators, in contrast to synthetic dyes, grant long-term recordings (weeks, months) from genetically defined neuronal populations (reviewed in: Broussard *et al.*, 2014; Jercog *et al.*, 2016; Lin & Schnitzer, 2016). The most ideal optical method would be the direct imaging of transmembrane voltage changes using genetically encoded voltage sensors, but their use for *in vivo* imaging at single cell resolution is still sporadic, due to critical shortcomings such as low brightness, imperfect membrane localization, or low signal to noise ratio (SNR; reviewed in Broussard *et al.*, 2014; Knopfel *et al.*, 2015; but see Piatkevich *et al.*, 2018). Neuronal spiking activity can be indirectly monitored by recording AP-evoked $[Ca^{2+}]$ changes with Ca^{2+} indicators. Genetically encoded Ca^{2+} indicators (GECI) underwent successive structure-guided mutagenesis over the past decade to improve sensitivity, dynamic range, SNR and brightness, resulting in the GCaMP6 family with sensitivities comparable to those of synthetic Ca^{2+} indicators (Nakai *et al.*, 2001; Tian *et al.*, 2009; Akerboom *et al.*, 2012; Chen *et al.*, 2013). GCaMP6f became the most widely used GECI in behaving animals because it combines high sensitivity and large dynamic range with relatively fast kinetics (Jercog *et al.*, 2016). Despite these properties, APs cannot be directly inferred from the fluorescent transients with millisecond temporal resolution (Jercog *et al.*, 2016; Lin & Schnitzer, 2016). To overcome this limitation, several approaches have been developed to determine AP firing underlying the fluorescent traces: template-matching (Greenberg *et al.*, 2008; Grewe *et al.*, 2010; Onativia *et al.*, 2013), deconvolution (Yaksi & Friedrich, 2006; Park *et al.*, 2013; Friedrich *et al.*, 2017), approximate Bayesian inference (Vogelstein *et al.*, 2009; Pnevmatikakis *et al.*, 2016) and supervised learning techniques (Sasaki *et al.*, 2008; Theis *et al.*, 2016), but the accuracy of estimation for high frequency events is still only 40-60% (Lin & Schnitzer, 2016;

Theis *et al.*, 2016). Deneux et al. (2016) improved spike estimation performance by introducing baseline drift (accounting for low frequency, large amplitude baseline fluctuations) and nonlinearity of the indicator (p_{nonlin}). Most of these algorithms are based on the estimation of the model parameters from a limited number of available simultaneous optical and electrical recordings, which might not capture the full range of the key parameters (Deneux *et al.*, 2016; Theis *et al.*, 2016). The spike inferring accuracy is known to depend on peak amplitude and decay kinetics of single AP-evoked (unitary) [Ca^{2+}] transients, SNR, baseline fluctuations and the nonlinear nature of GECIs (Wallace *et al.*, 2008; Lutcke *et al.*, 2013; Wilt *et al.*, 2013; Rose *et al.*, 2014; Deneux *et al.*, 2016). These parameters do not only vary among different cell types, preparations and optical recording conditions, but also among individual cells of the same type (Mao *et al.*, 2008; Tian *et al.*, 2009; Akerboom *et al.*, 2012; Ohkura *et al.*, 2012b; Zheng *et al.*, 2015). A key factor that determines many of these parameters is the concentration of the Ca^{2+} indicator: low indicator concentrations provide signals with higher peak amplitudes, lower SNR and faster kinetics (Hires *et al.*, 2008; Broussard *et al.*, 2014; Dana *et al.*, 2014; Rose *et al.*, 2014). Unfortunately, the concentration/expression of the GECIs cannot be controlled and made uniform among the transfected cells when they are expressed with viral vectors, leaving an inherent source of error for transforming the fluorescent traces to spike trains.

Here we sparsely express GCaMP6f in mouse dorsal hippocampal CA1 pyramidal cells (PCs) by mixing highly diluted Cre-recombinase expressing adeno-associated viral (AAV) vectors with high concentrations of flexed-GCaMP6f-containing AAVs and explore how the expression level of GCaMP6f affects peak amplitude of unitary [Ca^{2+}] transients, their kinetics and temporal summation. We prepared acute hippocampal slices for simultaneous optical and electrical recordings and recorded cells in the cell-attached configuration to preserve their intracellular milieu, while single APs or bursts of APs were evoked with extracellular axonal stimulation. The recorded cells were then *post hoc* identified and the expression levels of GCaMP6f were determined using confocal microscopy after chemical fixation. We then applied the spike inference method MLspike (Deneux *et al.*, 2016) on synthetic fluorescence data of different frequencies to test how the variability in the amplitude, kinetics and supralinearity affects spike estimates. Finally, we estimated the peak amplitudes of unitary [Ca^{2+}] transients by a model-independent ('*presumed unitary*') and a model-dependent ('*detected unitary*') method in

individual, weakly GCaMP6f expressing cells, with which we could reliably estimate the spiking activity of weakly GCaMP6f expressing PCs.

Materials and Methods

Animal Care:

All experiments were conducted in accordance with the Hungarian Act of Animal Care and Experimentation (1998, XXVIII, section 243/1998) and with the ethical guidelines of the Institute of Experimental Medicine Protection of Research Subjects Committee. All experimental protocols were approved by the Protection of Research Subjects Committee of the Institute of Experimental Medicine. Animals were housed within the vivarium of the Institute in a normal 12 h light 12 h dark cycle with food and water available ad libitum. Animals from the same litter were kept together in the same cage. The environment of the animals was enriched with Sizzle-Pet and cardboard tubes as play tunnels.

Stereotaxic virus injection:

Male FVB/Ant mice (n = 57, 30 - 52 days old) were anesthetized with 100 µl / 10 g body weight of the mixture of Ketamine (625 µl in 10 ml), Xylazin (625 µl in 10 ml) and Pypolphen (250 µl in 10 ml). Mice were not treated with analgesics after operations. The head was fixed in a Kopf stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) and a small craniotomy (0.5 - 1 mm) was made bilaterally, above the CA1 region of dorsal hippocampus, at the following coordinates: anterior - posterior: 2.46 - 2.0 mm, medial - lateral: -1.5 mm, dorsal - ventral 1.3 - 1.5 mm. To obtain a sparse expression of the genetically encoded Ca^{2+} indicator GCaMP6f, we used a mixture of AAV9.Syn.Flex.GCaMP6f.WPRE.SV40 (qTiter 7.744e13 GC/ ml, Penn Vector Core, Pennsylvania, PA, USA) and AAV9.CMV.PI.CRE.rBG (qTiter 5.86e13 GC/ ml, Penn Vector Core) in 1:100 dilution. A volume of 100 nl was injected (2.3 nl steps at a speed of 11 bpm) bilaterally using thin glass pipettes (World Precision Instruments, Sarasota, FL, USA) and a Nanoject II Injector (Drummond Scientific Company, Broomall, PA, USA). Mice were returned to their cage for at least two weeks before performing *in vivo* imaging or preparing acute slices.

Imaging window implant for *in vivo* Ca^{2+} imaging:

The surgery was performed according to the method of Dombeck et al. (Dombeck *et al.*, 2010) 21 days after virus injection. Briefly, a 73 days old mouse was anesthetized with isoflurane and a craniotomy (~3 mm) centered above the left dorsal hippocampus was made. After removing the dura, the cortex was slowly removed by aspiration. A cannula (3 mm diameter, 1.5 mm long, filled with optical adhesive; Norland Products, Cranbury, NJ, USA) with glass coverslips on both ends was introduced and sealed to the skull with a mix of RelyX Luting cement (3M, St. Paul, MN, USA) and superglue. Once the cement has set, a custom-made head plate (Supertech Instruments, Pecs, Hungary) was also cemented to the skull.

In vivo two-photon Ca^{2+} imaging and data analysis:

Imaging was performed 9 days after window implant surgery with a Femto2D (Femtonics Ltd., Budapest, Hungary) resonant scanning microscope equipped with a Chameleon Vision (Coherent) femtosecond pulsing laser tuned to 925 nm and a water-immersion lens (16X, NA = 0.8, Nikon). The animal was anaesthetized with isoflurane (4 – 5% for induction, 1 – 1.5% during imaging). A field of view (FOV) of 300 µm x 300 µm was imaged at a frame rate of 32 Hz and a resolution of 0.65 µm/ pixel for 10 minutes. After performing rigid motion correction (NonRigid4Reso Toolbox, Femtonics Ltd.) we drew regions of interest (ROIs) by hand around somata and exported the fluorescent traces. A Gaussian filtering (8.7 ± 1.4 Hz) was applied to all fluorescent traces and the peak fluorescence was measured as the maximum value of the fluorescence. To correct for drifts in baseline we subtracted the mean baseline measured before each [Ca^{2+}] transient individually. Changes in fluorescence with a SNR >2 (2*SD of the baseline) were considered [Ca^{2+}] transients. To estimate the variability in the ΔF/F caused by variations of the background fluorescence (F$_{bk}$), we measured F$_{bk}$ in ROIs of similar areas (n = 9) in the vicinity of each GCaMP6f expressing cell. We then subtracted its values from each cell's baseline fluorescence. We recalculated the ΔF/F as ΔF/F = (F$_{peak}$ - F$_{baseline}$) / (F$_{baseline}$- F$_{bk}$) with the 9 different F$_{bk}$ values and their mean, and found a mean coefficient of variation (CV) of 0.29 (n = 14 cells, 1 transient for each cell with 10 F$_{bk}$ values). This variance accounts for only 7.3% of the total variability observed in our *in vivo* recordings (CV = 1.07).

Slice preparation and electrophysiological recordings:

Adult male FVB/Ant mice (n = 53, 43 - 81 days old for GCaMP6f cell-attached recordings; n = 7, 42 - 59 days old mice without virus injection for Flou5F whole-cell recordings) were anaesthetized with 100 µl / 10 g body weight of the mixture of Ketamine (625 µl in 10 ml), Xylazin (625 µl in 10 ml) and Pypolphen (250 µl in 10 ml). Mice were transcardially perfused with an ice-cold cutting solution containing the following (in mM): sucrose, 205.2; KCl, 2.5; $NaHCO_3$, 26; $CaCl_2$, 0.5; $MgCl_2$, 5; NaH_2PO_4, 1.25; and glucose, 10; saturated with 95% O_2 and 5% CO_2. After decapitation, the brain was quickly removed and placed into the ice-cold cutting solution. 300 µm thick coronal slices were cut from the dorsal part of the hippocampus with a vibratome (Leica VT1200S). Slices were incubated in an interface-type holding chamber in ACSF containing the following (in mM): NaCl, 126; KCl, 2.5; $NaHCO_3$, 26; $CaCl_2$, 2; $MgCl_2$, 2; NaH_2PO_4, 1.25; and glucose, 10; saturated with 95% O_2 and 5% CO_2, pH = 7.2 – 7.4, at 36°C that was gradually (~1 hour) cooled down to room temperature. Experiments were performed at 22 - 24°C up to 6 h after slicing. Cells were visualized using a Femto2D microscope equipped with an oblique illumination and a water-immersion lens (25X, NA = 1.1, Nikon). A MultiClamp 700A amplifier (Molecular Devices) was used to obtain cell-attached recordings from CA1 PCs expressing GCaMP6f, and whole-cell recordings in experiments using the synthetic Ca^{2+} indicator Fluo5F (300 µM). Recorded traces were filtered at 3 – 4 kHz and digitized online at 20 kHz. Patch pipettes (resistance 4 – 7 MΩ) were pulled from thick-walled borosilicate glass capillaries (Sutter Instruments) with an inner filament. Recording pipettes for cell-attached recordings were filled with ACSF. For whole-cell experiments the intracellular solution contained the following (in mM): Fluo5F (Invitrogen), 0.3; Alexa Fluor 594 (Invitrogen), 0.02; K-gluconate, 110; KCl, 5; creatine phosphate, 10; HEPES, 10; ATP, 2; GTP, 0.4; and biocytin, 5, pH = 7.3; 290 – 300 mOsm. APs were evoked antidromically by extracellular stimulation (0.2 – 0.3 ms duration, ~500 µA) using a monopolar stimulating electrode, placed at least 100 µm away from the soma in the stratum oriens/alveus. Several stimulating protocols were used: single stimulus, 5 stimuli at 10 Hz or 50 Hz, 3 stimuli at 100 Hz, 12 stimuli at 0.25 Hz, place cell firing pattern stimuli (timing of spikes from (Losonczy *et al.*, 2002) and a burst recovery protocol. For whole-cell recordings, PCs were held at -70mV. APs were evoked with 2 to 4 ms-long depolarizing current pulses (1 – 1.2 nA).

***In vitro* two-photon Ca^{2+} imaging and data analysis:**

Experiments were performed using a Femto2D (Femtonics Ltd.) laser scanning microscope equipped with a Chameleon femtosecond pulsing laser (Coherent) tuned to 925 nm (as described in (Holderith *et al.*, 2012). Somata of CA1 PCs expressing GCaMP6f were imaged in line scan mode (0.5 - 1 kHz sampling rate) with 10 – 15 mW laser intensity at the back aperture of the objective lens. To measure background fluorescence (F_{bk}), each line scan protocol included a short (~10 μm) background line over the neuropil in the vicinity of the cell (~10 μm; F_{bk}: 20 ± 20 AU n = 53 cells), which was scanned for 45 ms. After that, the cell was scanned for 400 ms before delivering the stimulus ($F_{baseline}$). The resting fluorescence of the cell was calculated as $F_{rest} = F_{baseline} - F_{bk}$ (F_{rest}: 169 ± 362 AU n = 53 cells) and the peak fluorescence was measured as the maximum value of the fluorescence within a 300 ms window following the stimulus. The $\Delta F/F$ value was calculated as: $\Delta F/F = (F_{peak} - F_{baseline}) / F_{rest}$. Raw fluorescence values (F_{rest} and ΔF) and SNR (peak amplitude divided by standard deviation of $F_{baseline}$) are reported only for cells imaged with the same laser intensity (10 mW, ~90% of data). We acquired images of the cells at the start of the recordings (either before or immediately after the cell was patched) and after the withdrawal of the recording pipette. We excluded cells from our analysis if the mean intensity of fluorescence changed >25% from the beginning to the end of the cell-attached recordings. The remaining cells had a mean intensity at the start of the recording of 364 ± 367 AU and 377 ± 355 AU (n = 55) at the end of the recordings. Image stacks (2 μm step size) were also taken to allow *post hoc* identification of the recorded cells. ROIs around the somata were drawn in Icy BioImage Analysis software (http://icy.bioimageanalysis.org/) and mean GCaMP6f intensities were measured in the optimal focal plane. The laser intensity was the same (10 mW) for all images used for quantification. We reconstructed the soma of a subset of cells in 3D and calculated their surface and volume as follows: (1) volume: we multiplied the ROI areas measured from each frame with the z-step size; (2) surface: we multiplied the ROI circumference measured from each frame with the z-step size, and added the areas of top and bottom ROIs. In some experiments, $[Ca^{2+}]$ transients were measured with the synthetic dye Fluo5F (300 μM) in CA1 PCs from control animals without virus injection. The intracellular solution also contained a Ca^{2+}-insensitive fluorophore (20 μM Alexa Fluor 594). After 7 to 10 minutes in whole-cell configuration, the stabilization of peak amplitude and decay time constant of $[Ca^{2+}]$ transients indicated that the dye reached an equilibrium in the soma. For the measurement of variability in unitary peak $[Ca^{2+}]$ transient amplitudes, data was collected from the following 5

minutes. AP-evoked changes in fluorescence were quantified as $G/R(t) = (F_{green(t)} - F_{rest, green})$ / $(F_{red} - I_{dark, red})$ where $F_{green(t)}$ represents the green fluorescence signal as a function of time, $F_{rest, green}$ is the green fluorescence before stimulation, and $I_{dark, red}$ is the dark current in the red channel (Holderith *et al.*, 2012; Eltes *et al.*, 2017). To normalize data across batches of dyes, G_{max}/R values were measured by imaging a sealed (tip melted and closed by heating) pipette filled with intracellular solution containing 10 mM $CaCl_2$ for each cell. G/R measurements were divided by G_{max}/R, yielding the reported values of G/G_{max}. A uniform Gaussian filtering was applied to all fluorescent traces.

Quantification of native GCaMP6f intensities in cell-attached recorded PCs
After recordings, the slices were immersion fixed in a solution containing 4% paraformaldehyde (PFA), 0.2% picric acid in 0.1 M phosphate buffer (PB), pH = 7.4, at 4°C overnight. Image stacks were acquired with an Olympus FV1000 confocal microscope with a 20X (NA = 0.75, Olympus) objective. For quantification of native GCaMP6f intensities we used the same laser intensities and PMT settings for all cells. The recorded cells were identified based on two-photon image stacks. Mean native GCaMP6f intensities were quantified as described above. The background was measured in each slice over five ROIs placed close to each cell, and their mean value was subtracted from the fluorescence values measured over the cells. We tested the accuracy of hand drawing five ROIs around the cells for 10 cells, and found that the variance in the intensity measurements from these ROIs was negligible (CV = 0.02 ± 0.02). We also measured the GCaMP6f intensity for 15 cells from ROIs including or excluding the nuclei and found almost perfect correlation between these values ($\rho = 0.96$; Spearman correlation), indicating that both measurements reflect equally well the intensity of GCaMP6f fluorescence.

Immunohistochemistry and quantification of native GCaMP6f and anti-GFP intensities:
One month after virus injection, three male mice (55 – 76 days old) were deeply anesthetized with isoflurane and i.p. injection of Ketamine (0.2 ml / 20 g body weight). The animals were transcardially perfused with 0.9% saline (for 1 min) followed by 2% PFA and 0.2% picric acid in 0.1M PB for 16 min. The brain was quickly removed from the skull and postfixed for another hour after which it was placed in PB. Blocks were cut out from the forebrain and 50 μm coronal sections were cut with a vibratome (Leica VT1000s). Sections from all three mice were used to

determine the intensity distribution of native GCaMP6f in perfusion fixed tissue (Figure 3b). Sections from one mouse were washed in 0.1M PB and Tris-buffered saline (TBS; pH 7.4) containing 0.1% TritonX-100 and blocked in normal goat serum (NGS, 10%) for 1 h made up in TBS. Sections were then incubated in mouse anti-GFP (1:5000, catalog number 75-132, NeuroMab) IgG diluted in TBS containing 0.3% TritonX-100 and 2% NGS overnight. Sections were then washed and Cy3 conjugated goat anti-mouse IgG2a (1:500, Jackson Laboratories) secondary antibody was applied for 2.5 h at room temperature. Sections were mounted in Vectashield (Vector Laboratories).

Image stacks were acquired with an Olympus FV1000 confocal microscope with 20X (NA = 0.75, Olympus) and 60X (NA = 1.35, oil-immersion, Olympus) objectives. Laser intensity and PMT settings were the same for all cells. Cells located within the top 6 µm of the sections were selected for quantification. The background fluorescence was determined over 78 ROIs and its mean value was subtracted from each cell.

Simulations

To infer APs from fluorescent Ca^{2+} traces, we used the MLspike software (Deneux *et al.*, 2016), which detects the most likely underlying spike train. The spike matching tolerance window, i.e. the maximal time difference between an inferred and the original AP, within which the inferred AP is registered as 'correctly detected', was set to 60 ms for all simulations, almost an order of magnitude shorter than that used by Deneux et al. (0.5s). First, we fitted *in vitro* recorded fluorescent $[Ca^{2+}]$ transients evoked by either a single AP or bursts consisting of 5 APs at 50 Hz with MLspike. From the burst protocol (n = 37 cells), we first estimated the p_{nonlin} (reflects the nonlinearity of the Ca^{2+} sensor) and '*baseline drift*' (accounts for low frequency, large amplitude baseline fluctuations) parameters. The accuracy of the fitting is quantified in error rates (ERs) as the harmonic mean of sensitivity (% of missed spikes, i.e.: that were not detected within the 60 ms time window from the real spike) and precision (% of false detections). The ERs were calculated for p_{nonlin} values ranging from 0 to 2.1 with an increment of 0.1 and *baseline drift* values from 0.001 to 0.101 with an increment of 0.005 for determining those parameters that produce the smallest ERs (see Figure 9b). If there were multiple parameter combinations resulting in minimal ER values, the smallest *baseline drift* value was selected with its corresponding maximal p_{nonlin} value.

We then generated synthetic fluorescent transients with MLspike at three different frequencies (0.1 Hz, 1 Hz, 10 Hz) of Poisson spike trains (n = 10 repetitions for each cell). Fluorescent transients were generated with 37 cells' experimentally determined amplitude and decay of unitary fluorescent $[Ca^{2+}]$ transients, their measured noise and the abovementioned p_{nonlin} and *baseline drift* values. For spike inference, we implemented the following four scenarios: 1) all of the parameters were the cells' own (i.e. with which the synthetic fluorescent data were generated); 2) own amplitude, p_{nonlin} and *baseline drift* parameters, average of n = 37 cells' decay; 3) own decay, p_{nonlin} and *baseline drift* parameters and average of n = 37 cells' amplitude; and 4) all four parameters were the averages of the 37 cells (see Figure 9d-i).

To test the efficacy of our approach under more realistic conditions, we generated fluorescent Ca^{2+} traces using *in vivo* recorded spike times of hippocampal CA1 PCs (Grosmark, Long and Buzsaki; CRCNS.org, http://dx.doi.org/10.6080/K0862DC5; (Grosmark & Buzsaki, 2016). We estimated the peak amplitude of putative unitary $[Ca^{2+}]$ transients with two methods. (1) First, we approximated the percentage of temporally isolated (no spike >4s before and >1s after a given AP), single AP-evoked $[Ca^{2+}]$ transients. From the spike train of the *in vivo* recorded data, we calculated that two third of the temporally isolated events are single APs, the rest are bursts with inter-spike intervals <20 ms. We detected temporally well isolated fluorescent $[Ca^{2+}]$ transients and calculated the '*presumed unitary*' peak amplitudes as the mean of the smallest 66.6% of the events. (2) In a second procedure, we inferred spiking activity with MLspike using the mean amplitude, decay time constant, p_{nonlin} and *baseline drift* parameters of weakly GCaMP6f expressing cells (n = 20). We then selected the temporally isolated (no spike > 4s before and >1s after), potentially single AP-evoked $[Ca^{2+}]$ transients and measured the peak amplitudes of these so called *'detected unitary'* $[Ca^{2+}]$ transients. In few cases, the detected isolated AP followed a missed (i.e. not inferred) AP, and a preceding $[Ca^{2+}]$ transient was clearly distinguishable. These transients were omitted when more than 10 isolated $[Ca^{2+}]$ transients were already detected in the cell (n = 4 cells).

We then performed spike inference using the '*presumed unitary*' or *'detected unitary'* $[Ca^{2+}]$ transient amplitude of each cell (n = 20) with the mean of the decay, p_{nonlin} and *baseline drift* parameters. All simulations were performed on a desktop computer, running Matlab (version: 2017b) under Windows 10.

Statistical analysis:

All statistical tests and plotting of data were performed using OriginPro. Normality of data was tested using Shapiro-Wilk test. Statistical significance was assessed with: Spearman correlation for non-normally distributed data (Figure 1d inset; Figure 2f ; Figure 3f; Figure 4f, g, h; Figure 6a, b, d, e; Figure 7a, c, d; Figure 8b, c, e, f; Figure 9j; Figure 11b, c); Pearson correlation for normally distributed data (Figure 8h); Kruskal-Wallis ANOVA test (multiple independent samples; Figure 7b); Two-Sample Kolmogorov-Smirnov Test (Figure 3b, Figure 7f; Figure 11a); Three-Way ANOVA and Bonferroni *post hoc* test (Figure 9e, g, i, k); Friedman ANOVA, with Paired Sample Wilcoxon Signed Rank *post hoc* test and Bonferroni correction (Figure 11g); Paired Sample Wilcoxon Signed Rank test (Figure 2e). Data are presented as mean ± SD or median and interquartile range one (IQR1) – interquartile range three (IQR3). Results were considered significant when $p < 0.05$.

Results

Variability in the amplitude of the unitary GCaMP6f [Ca^{2+}] transients

We obtained sparse expression of GCaMP6f in dorsal hippocampal CA1 PCs by injecting a mixture of highly diluted Cre-recombinase-expressing AAV vectors and concentrated flexed-GCaMP6f-containing AAVs. Three weeks after the virus injection, we implanted a chronic imaging window above the dorsal hippocampus to provide optical access for recording neuronal activity in a head-fixed, lightly anaesthetized mouse. The peak amplitude of [Ca^{2+}] transients (0.26 ± 0.3 ΔF/F, median = 0.16 ΔF/F, interquartile range 1-3 (IQR): 0.07 – 0.36 ΔF/F, n = 311 transients) showed substantial variability (mean coefficient of variation (CV) = 1.07 ± 0.42, n = 13 cells; Figure 1a-c) in our imaged cells, similar to that published previously (Chen *et al.*, 2013). The variability in the peak [Ca^{2+}] transients could reflect different single AP-evoked, unitary [Ca^{2+}] transient among cells, different ratios of single APs vs. high frequency bursts, different numbers of APs within bursts and different degrees of nonlinearity of the GECI.

To address the contribution of these parameters, we recorded single AP-evoked unitary [Ca^{2+}] transients in acute hippocampal slices. Whole-cell patch-clamp recording perturbs the

intracellular milieu of the cells and dialyzes soluble proteins, including GCaMP6f; therefore, we decided to record extracellularly-evoked APs in cell-attached configuration and the corresponding [Ca^{2+}] transients with two-photon microscopy in a line-scan mode over the somata. APs were evoked by extracellularly stimulating PC axons in stratum oriens/alveus.

The mean peak amplitude of [Ca^{2+}] transients evoked by a single AP (0.2 ± 0.2 ΔF/F, median = 0.14 ΔF/F, IQR: 0.06 – 0.27 ΔF/F, n = 121 cells) was similar to that described earlier (e.g. (Chen *et al.*, 2013), with profound cell-to-cell variability (CV = 0.96; Figure 1d, e). The SNR was also highly variable (11.4 ± 7.4, median = 10.3, IQR: 5.6 – 15, n = 115), and positively correlated with the unitary [Ca^{2+}] signals (ρ = 0.88, p < 0.001; Spearman correlation; Figure 1d inset). The trial-to-trial variability in the amplitude of unitary [Ca^{2+}] transients was assessed by evoking 12 APs at 0.25 Hz. These experiments revealed similar peak amplitudes throughout the train without any significant run-up or run-down (normalized mean of the 12th transient = 1.06 ± 0.16, n = 45 cells) and demonstrated very little within-cell, trial-to-trial variance (CV = 0.13 ± 0.06, median = 0.12, IQR: 0.1 – 0.17, n = 45 cells; Figure 1e). This is large cell to cell variance is not only found for the extracellularly evoked unitary responses, but is also observed for the occasionally occurring (n = 6 cells) spontaneous single AP-evoked responses, which had peak amplitudes and variances very similar to the evoked responses (CV = 0.81, Figure 2).

Variability in GCaMP6f expression level

Virally expressed proteins, including GCaMP6f, are known to demonstrate variable expression levels (Dana *et al.*, 2014). To quantify the variability in the expression level, we first measured GCaMP6f intensities in slices following fixation in 4% paraformaldehyde-containing fixative using confocal microscopy (Figure 3a, b). We argue that by chemically fixing the slices, variability in fluorescence will no longer reflect differences in intracellular [Ca^{2+}], but will purely reflect GCaMP6f expression levels. We found that CA1 PCs show widely differing GCaMP6f intensities (482 ± 542 AU, median = 262 AU, IQR: 109–654 AU; CV = 1.12, n = 297 cells). The distribution of CA1 PCs according to their GCaMP6f expression level in perfusion fixed tissue was also skewed and showed large variability (IQR: 131–979 AU, CV = 1.05, n = 639 cells) with a somewhat higher mean intensity (624 ± 653 AU, median = 345 AU), compared

to that obtained from *in vitro* slices after fixation (p = 0.006; Two-Sample Kolmogorov Smirnoff test, Figure 3b), consistent with preferential death of the most intensive cells following slicing.

Another potential way of determining the expression level of GCaMP6f is measuring the intensity of the cells at the isosbestic wavelength (~810nm) of GCaMP6f. We therefore measured the fluorescent intensity in acute hippocampal slices at 810 nm excitation wavelength and found that it significantly correlates with the intensity determined *post hoc* after fixation (ρ = 0.76, p < 0.001, Spearman correlation; Figure 3c). However, we failed to find a significant correlation for cells that weakly express GCaMP6f (ρ = 0.33, p = 0.12, Spearman correlation, Figure 3d).

To provide unequivocal evidence that the intrinsic fluorescence of GCaMP6f in fixed tissue is indeed proportional to the amount of GCaMP6f protein, we immunolabelled the perfusion fixed tissue with an anti-GFP antibody (Figure 3e) and examined its relationship with the native GCaMP6f intensity. The almost perfect positive correlation (ρ = 0.97, p < 0.001; n = 87; Spearman correlation; Figure 3f) between the anti-GFP immunoreactivity and intrinsic GCaMP6f fluorescence after fixation indicates that the latter is an excellent correlate of GCaMP6f protein level.

Variability in the amplitude of unitary [Ca^{2+}] transients among cells with similar GCaMP6f expression level

Having demonstrated large cell-to-cell variability in the amplitude of unitary [Ca^{2+}] transients and GCaMP6f expression levels, we asked whether these parameters show any correlation (Figure 4). To ensure that even cell-attached recordings do not cause any alterations in the fluorescence of the cells, we measured GCaMP6f intensities before establishing the cell-attached configuration and after the withdrawal of the pipette in 83 out of 121 PCs in acute slices. We observed that in 28 out of 83 recorded neurons, the fluorescence intensities increased more than 25% during the 10–30 minutes of cell-attached recordings. We thus, restricted our further analysis to cells that showed changes <25% (Figure 4a, b). The peak amplitudes of unitary [Ca^{2+}] transients (0.20 ± 0.16 ΔF/F, median = 0.15 ΔF/F, IQR: 0.07 - 0.3 ΔF/F, n = 55) and the variability (CV = 0.79) of these subselected cells were very similar to those of the whole

population (CV = 0.96), indicating that the changes in fluorescence during the recordings did not selectively occur in cells showing small or large unitary $[Ca^{2+}]$ transients. This large variance was also apparent among cells recorded from the same slices (CV = 0.64 ± 0.4, n = 10 slices) and from the same animals (CV = 0.65 ± 0.36, n = 18 animals, 2 – 4 cells/animal). At the end of the recordings we acquired Z image stacks of the recorded cells and the surrounding areas to enable their *post hoc* identification after fixation. Despite the lack of intracellular labels (e.g. biocytin or fluorescent dyes), the sparse labelling allowed us to unequivocally identify 43 of the 55 recorded cells in the fixed sections. We then measured their native GCaMP6f intensity levels using confocal microscopy (Figure 4c) and observed a substantial variability in their intensity (277 ± 478 AU, median = 151 AU, IQR: 66–252 AU, CV = 1.73, n = 43 cells; Figure 4e).

We found a significant negative correlation between the peak amplitude of unitary $[Ca^{2+}]$ transients and the GCaMP6f expression level (n = 43, ρ = -0.69, p < 0.001; Spearman correlation, Figure 4f), with highly expressing cells showing very small fluorescence changes upon an AP. The relationship seems to follow a power law, where the peak amplitude is proportional to the expression level^{-1}. However, there was a very pronounced variance around the fit at cells with low expression levels. In cells selected for similarly low native GCaMP6f intensities (the lowest 66 percentile of the population, <180 AU), the peak amplitudes were somewhat higher (0.26 ± 0.14 ΔF/F, median = 0.22 ΔF/F, IQR: 0.14 – 0.39 ΔF/F), but were still highly variable (CV = 0.56, n = 26). When the peak amplitude of unitary $[Ca^{2+}]$ transient was plotted against the GCaMP6f expression level for these subselected cells, no significant correlation was found (ρ = -0.22, p = 0.28; Spearman correlation, Figure 4g). The amount of GCaMP6f also correlates negatively with the SNR (ρ = -0.60, n = 42, p < 0.001; Spearman correlation, Figure 4h), but it is also highly variable (CV = 0.6) among cells with low intensities.

Non-biological sources of variance could contribute to the observed large variability in the peak amplitude of unitary $[Ca^{2+}]$ transients. The background fluorescence (F_{bk}) varies within the slices and because its exact value influences our estimate of ΔF/F, we estimated its contribution to the total variance of unitary $[Ca^{2+}]$ transients. In a subset of slices, we recorded 2 cells each having its 'own' background line (i.e.: scanned quasi simultaneously with the recorded cell). We calculated the ΔF/F for each cell with its 'own' F_{bk} and also with the other cell's F_{bk} and calculated the variance in the peak ΔF/F. The mean CV was 0.17, which accounts for ~10% of

the total variance (CV = 0.56). Furthermore, the positioning of our scanning line over the cell body can result in different nucleus/cytosol ratios, which could be another biologically-irrelevant source of variance. We, therefore, analyzed the nucleus and the cytoplasm parts of the lines over the cells and calculated the peak $\Delta F/F$ values. This analysis revealed a very tight, significant correlation ($r^2 = 0.96$, p < 0.001, Pearson's correlation) between the $\Delta F/F$ values of the cytoplasm and the entire cell, with very similar variances (CV of whole cell: 0.63; CV of cytoplasm only: 0.57, n = 20 cells). To test whether out-of-focus fluorescence has any contribution to the variability of unitary fluorescent transient in sparse labelling conditions, we performed the following analysis and experiments. First, we argue that if a labelled process (axon or dendrite) passed below or above our imaged cell and its activity contributed to the fluorescence, then the chance that it contributed equally to both the 'left' and 'right' halves is very small. Thus, we divided our line scans into two halves and analysed them separately. This analysis resulted in very similar unitary amplitudes with a CV of only 0.13 ± 0.1 (n = 20 cells). In addition, we measured unitary peak amplitudes in three different focal planes (in the so called 'middle' of the cell and 3-4 µm above or below this focal plane) over cell bodies of weakly GCaMP6f expressing cells and calculated the CV in the mean unitary amplitudes between the three focal planes (Figure 5). The mean amplitudes showed very little dependencies on the focal planes, resulting in a CV of only 0.10 ± 0.07 (n = 7 cells). Finally, we also tested how the variance in the peak amplitude of unitary $[Ca^{2+}]$ transients depended on the time between the virus injection and the recordings (i.e. expression time) and found no correlation with the expression time ($\rho= 0.25$, p = 0.32; n = 18 animals, Spearman correlation), ruling out its major contribution to the total variance.

We next analysed the relationship between the decay time constants of the unitary $[Ca^{2+}]$ transients and the GCaMP6f expression level and found a significant positive correlation for all cells ($\rho = 0.6$, p < 0.001; n = 41, Spearman correlation, Figure 6a) and also for the low GCaMP6f intensity-subselected cells ($\rho = 0.51$, p = 0.008; n = 26, Spearman correlation, Figure 6b). When analysing the relationship of unitary peak amplitudes and decay time constants, we found that cells with smaller unitary peak amplitudes (and higher GCaMP6f intensity) displayed mostly longer decay times, resulting in a significant correlation between them ($\rho = -0.27$, p = 0.048; n = 53, Spearman correlation, Figure 6c, d); whereas for the GCaMP6f intensity subselected group of

cells (intensity < 180 AU) there was no significant correlation between these parameters (ρ = -0.001, p = 0.99; n = 26, Spearman correlation, Figure 6e).

Variability in the amplitude of unitary $[Ca^{2+}]$ transients reported by GCaMP6f can be the consequence of different amounts of $[Ca^{2+}]$ entering the soma, different somatic surface to volume ratios or different $[Ca^{2+}]$ buffering. Because the amount of $[Ca^{2+}]$ entering through voltage-gated Ca^{2+} channels during an AP is strongly dependent on the AP waveform (Sabatini & Regehr, 1999; Geiger & Jonas, 2000), we measured AP width (full width at half-maximal amplitude, FWHM) as recorded in the cell-attached configuration. The AP width does not correlate significantly with the peak amplitude of unitary $[Ca^{2+}]$ transients (ρ = 0.20, p = 0.17; Spearman correlation, Figure 7a). To assess the effect of potential differences in endogenous buffers such as calbindin, which is expressed in superficial PCs (Baimbridge *et al.*, 1991), we grouped the recorded cells based on their somatic location in the PC layer as deep, middle and superficial cells. We found no significant difference in the peak amplitude of unitary $[Ca^{2+}]$ transients among the three groups (p = 0.13, Kruskal-Wallis ANOVA test with multiple independent samples; Figure 7b). Next, we fitted the decay of averaged $[Ca^{2+}]$ transients with single exponentials and found very similar decay time constants of these subgroups (superficial: 419 ± 154 ms, n = 19; middle: 376 ± 127 ms, n = 18; deep: 400 ± 210 ms, n = 11; p = 0.35, Kruskal-Wallis ANOVA test with multiple independent samples), arguing against considerable differences in endogenous Ca^{2+} buffering by calbindin. To test for potential differences due to different surface to volume ratios, we reconstructed the soma of a subset of the recorded PCs in 3D from the two-photon Z image stacks and calculated their surface to volume ratios. The peak amplitude of the unitary $[Ca^{2+}]$ transients of the subselected cells did not correlate with the surface to volume ratio (ρ = 0.07, p >0.05; Spearman correlation, Figure 7c). Differences in resting $[Ca^{2+}]$ could also be an important factor in determining cell-to-cell variability in the peak unitary $[Ca^{2+}]$ transients. We could not provide an unequivocal estimate of resting $[Ca^{2+}]$, but obtained a measure that should reflect it. We normalized the resting fluorescence (F_{rest}) of each cell to its native GCaMP6 fluorescence intensity after fixation. The lack of correlation between this measure and the amplitude of its unitary $[Ca^{2+}]$ transients (ρ = -0.25, p = 0.12; Figure 7d) indicates that the large variance in unitary $[Ca^{2+}]$ transient amplitudes is probably not the sole consequence of the variability in resting $[Ca^{2+}]$.

Finally, we tested cell-to-cell variability in the amplitude of unitary $[Ca^{2+}]$ transients in CA1 PCs using a synthetic Ca^{2+} dye (300 µM Fluo5F), which is known to report physiologically relevant $[Ca^{2+}]$ linearly (Figure 7e). We found substantially smaller variability in the peak amplitude (0.02 ± 0.006 G/G_{max}, median = 0.02 G/G_{max}, IQR: $0.019 - 0.023$ G/G_{max}, n = 23) with a CV of only 0.27. The mean-normalized distribution of unitary $[Ca^{2+}]$ signals reported with Fluo5F was significantly narrower than that reported by GCaMP6f (p = 0.014, Two-Sample Kolmogorov Smirnoff test, Figure 7f), suggesting that the observed variability in the amplitude of GCaMP6f $[Ca^{2+}]$ transients is not mainly the consequence of biological variability in peak $[Ca^{2+}]$.

Supralinear temporal summation of GCaMP6f $[Ca^{2+}]$ transients

To examine the relationship between GCaMP6f expression levels and the temporal summation of fluorescent $[Ca^{2+}]$ transients, we evoked trains of APs at different frequencies. $[Ca^{2+}]$ transients showed substantial temporal summation already at 10 Hz, which was more pronounced at 50 Hz (Figure 8). We calculated a linearity index by dividing the peak of $[Ca^{2+}]$ transients evoked by the short trains with the respective mathematical sum of their unitary events. The summation of GCaMP6f $[Ca^{2+}]$ transients was use-dependent (Figure 8g) and supralinear at every tested frequency and showed a slight frequency dependence (linearity index: 5 AP at 10 Hz: 1.67 ± 0.54, median = 1.54, IQR: $1.31 - 1.94$, n = 34; 3 AP at 50 Hz: 1.70 ± 0.51, median = 1.57, IQR: $1.42 - 2.02$, n = 26; 5 AP at 50 Hz: 1.92 ± 0.63, median = 1.89, IQR: $1.48 - 2.27$, n = 40; 3 AP at 100 Hz: 1.83 ± 0.66, median = 1.82, IQR: $1.23 - 2.26$, n = 18). In contrast, the summation of $[Ca^{2+}]$ transients measured with Fluo5F was quasi linear (linearity index: 5 AP at 10 Hz: 0.93 ± 0.07, median = 0.95, IQR: 0.9 - 0.96, n = 10), indicating that the supralinear summation of GCaMP6f fluorescent transients is not the consequence of an increased Ca^{2+} influx during the AP trains. The linearity of 5 AP-evoked GCaMP6f $[Ca^{2+}]$ transients at 10 Hz did not show significant correlation either with the unitary $[Ca^{2+}]$ transient amplitude ($\rho = 0.14$, p = 0.4; Spearman correlation, Figure 8b), or with GCaMP6f expression level ($\rho = -0.33$, p = 0.09; Spearman correlation, Figure 8c). The correlation between the linearity of $[Ca^{2+}]$ transients evoked by 5 APs at 50 Hz and the peak amplitude of unitary $[Ca^{2+}]$ transients was also not significant ($\rho = 0.3$, p = 0.06; Spearman correlation, Figure 8e), but the correlation between the linearity at 50 Hz and the GCaMP6f expression level is significant ($\rho = -0.46$, p = 0.006;

Spearman correlation, Figure 8f). The tight correlation between the linearity index for 3 APs and 5 APs at 50 Hz indicates similar summation for low numbers of APs ($r^2 = 0.86$, Pearson correlation; Figure 8h).

Interestingly, we observed a dramatic increase in the peak amplitude of the unitary $[Ca^{2+}]$ transients, when they followed high frequency bursts by several seconds (Figure 8i). To quantitatively describe this phenomenon, we applied a burst recovery protocol consisting of a 10 AP at 50 Hz burst and single recovery pulses at different time points (2.5, 4, 6, 8, 10 or 15 s; Figure 8j). We normalized the amplitude of $[Ca^{2+}]$ transients to the amplitude of a unitary $[Ca^{2+}]$ transient evoked >8 seconds before the burst. $[Ca^{2+}]$ transients evoked by 10 AP at 50 Hz were supralinear (linearity index: 2.54 ± 0.94, median = 2.24, IQR: 1.98 – 3.14, n = 8 cells). 2.5 s after the burst the peak amplitude of the unitary $[Ca^{2+}]$ transient was more than twice (2.06 ± 0.46, median = 2.14, IQR: 1.7 – 2.45, n = 6 cells) as that of the control transient and returned to its initial value >10 second later (Figure 8k, l). Fitting a monoexponential to the normalized recovery of $[Ca^{2+}]$ transients yielded a time constant of 3.95 s (Figure 8l). In contrast, when the same protocol was applied to cells which did not express GCaMP6f and in which $[Ca^{2+}]$ transients were recorded using Fluo5F, the unitary peak amplitude did not increase after a burst (4s after burst: 0.84 ± 0.09, median = 0.87, IQR: 0.77 - 0.92, n = 9; normalized to the unitary transient before the burst), indicating that the aforementioned phenomenon is likely due to the nonlinear nature of GCaMP6f and not the short-term facilitation of the Ca^{2+} flux.

Because the supralinear enhancement of peak amplitudes of unitary $[Ca^{2+}]$ transients can be a potential source for the observed large cell-to-cell variance of the unitary $[Ca^{2+}]$ transients of GCaMP6f expressing cells, we subselected cells with traces where the single AP-evoked transients were not preceded by higher frequency events by >10 s. The resulting mean unitary $[Ca^{2+}]$ transient displayed similarly large cell-to-cell variance (n = 35, CV = 0.95), which was also present among cells with low GCaMP6f expression levels (<180 AU, n = 15; CV = 0.73).

Spike inference ERs are strongly affected by the cell-to-cell variability in the peak amplitude of unitary $[Ca^{2+}]$ transients

To infer APs from fluorescent $[Ca^{2+}]$ transients, we adopted the method (MLspike) of Deneux et al. (2016), but we set the tolerance window of spike matching to 60 ms (i.e. an inferred AP should be within a 60 ms time window from the original AP to be registered as 'correctly detected'), a value almost an order of magnitude smaller than that used by Deneux et al. (500 ms). MLspike estimates the most likely spike trains underlying the fluorescent transients by using a model that includes *baseline drift*, the nonlinear feature of the Ca^{2+} sensor (p_{nonlin}), the peak amplitude and the decay of the unitary $[Ca^{2+}]$ transients. First, we constrained the *baseline drift* and p_{nonlin}values by fitting 5 AP-evoked $[Ca^{2+}]$ transients recorded in individual PCs (n = 37 cells; Figure 9a-c). Next, we generated synthetic data with MLspike using experimentally measured peak amplitudes, decay times, SNR of unitary $[Ca^{2+}]$ transients, the corresponding *baseline drift* and p_{nonlin}values with spike timings obtained from Poisson distributions at 0.1 Hz, 1 Hz, and 10 Hz (Figure 9d, f, h). We applied 4 different fitting scenarios to investigate the parameters that critically influence the ER: 1) fitting the transients with the amplitude, decay, *baseline drift* and p_{nonlin} values used to generate the data; 2) mean decay value of the 37 cells with the cell's own amplitude, *baseline drift* and p_{nonlin} values; 3) mean amplitude value of the 37 cells, with the cell's own decay, *baseline drift* and p_{nonlin} values; 4) the mean amplitude, decay, *baseline drift* and p_{nonlin} values of the 37 cells. The first scenario resulted in virtually zero errors for all tested mean firing frequencies (red in Figure 9e, g, i). Similarly, low ERs were obtained when the mean decay time constant was used at frequencies <10 Hz (orange in Figure 9e, g). However, when the mean peak amplitude was used, the ERs were substantially higher at all frequencies (cyan in Figure 9e, g, i), similar to the scenario when all parameters used for fitting were the mean values of the 37 cells (blue in Figure 9e, g, i; Three-Way ANOVA: p < 0.001 for scenarios; p > 0.05 for frequency; p > 0.05 for scenarios–frequency interaction). The ERs in the 4th scenario depended on the difference between the cell's unitary peak amplitude and the mean amplitude; the ER was negligible when the difference was small (Figure 10a-c). This is explained by a larger fraction of missed APs for cells with lower unitary peak amplitudes (Figure 10d-f) and by a larger fraction of incorrectly detected APs for cells with higher unitary peak amplitudes (Figure 10g-i). These data support the key role of the variance in the amplitude of the unitary $[Ca^{2+}]$ transients in determining the precision of spike inference.

Our analysis also revealed that the ER (4th scenario) and GCaMP6f intensity show significant positive correlations at all frequencies (0.1 Hz: ρ = 0.5, p = 0.007; 1 Hz: ρ = 0.59, p = 0.001; 10

Hz: $\rho = 0.46$, $p = 0.013$; $n = 28$; Spearman correlation; Figure 9j). When the cells were grouped based on their native GCaMP6f intensities into weakly (<180 AU, $n = 20$) and strongly (>180 AU, $n = 8$) expressing groups, the ERs were found to be significantly smaller for weakly expressing cells at 0.1 Hz and 1 Hz (Three-Way ANOVA: $p < 0.001$ for intensity; $p < 0.001$ for intensity–scenarios interaction; $p > 0.05$ for intensity–frequency interaction; $p > 0.05$ for intensity–scenarios–frequency interaction; pairwise Bonferroni *post hoc* test: 0.1 Hz: $p < 0.001$, 1 Hz: $p < 0.001$, 10 Hz: $p > 0.05$; Figure 9k). These results demonstrate that the key parameter that critically determines the spike inference ER is the peak amplitude of the unitary $[Ca^{2+}]$ transients. Furthermore, our data also provide clear evidence that the ER for weakly GCaMP6f expressing cells is significantly lower than that for the strongly expressing ones.

Reduction of spike inference ER by fitting with estimated unitary amplitudes of $[Ca^{2+}]$ transients

In the final set of simulations, we generated fluorescent traces using the amplitude and decay of unitary $[Ca^{2+}]$ transients recorded in 20 PCs that weakly express GCaMP6f (< 180 AU), their respective *baseline drift*, p_{nonlin} parameters, noise and the spike timings of 20 CA1 PCs recorded from behaving animals using tetrodes (obtained from Grosmark, A.D., Long J. and Buzsaki, G; CRCNS.org; http://dx.doi.org/10.6080/K0862DC5). The distribution of the mean firing rates of the PCs was positively skewed ($n = 48$ cells) and was very similar for the 20 randomly selected cells compared to the 48 cells of the dataset ($p = 0.27$, Two-Sample Kolmogorov-Smirnov test; Figure 11a).

Because our previous simulations revealed that the amplitude of unitary $[Ca^{2+}]$ transients is the key parameter in determining the ER, we aimed at determining the amplitude of unitary $[Ca^{2+}]$ transients from the fluorescent traces with a model-independent and a model-dependent method. For this, first we analysed the spike times of *in vivo* recorded CA1 PCs. Because the amplitude of a $[Ca^{2+}]$ transient increases substantially when it is preceded by a burst, we selected APs, which were preceded and followed by at least a 4 and 1 second silent period, respectively. In the analysed 20 *in vivo* recorded spike trains, $10.4 \pm 5.5\%$ of the APs fulfilled these criteria. However, the slow kinetics of GCaMP6f $[Ca^{2+}]$ transients does not allow the unequivocal distinction between single AP-evoked and isolated high-frequency (ISI < 20 ms) burst-evoked

[Ca^{2+}] transients; therefore, we also calculated the percentage of such bursts. We found that among such temporally separated events, 77.6 ± 8.4% were single APs with a minimum of 63.6% per spike train, the remaining ones were bursts of APs. Thus we argued that if we detect [Ca^{2+}] transients that are separated by 4 and 1 seconds from other transients (15 ± 8% of the total transients), the smallest two-third of them will be likely single AP-evoked '*presumed unitary*' [Ca^{2+}] transients. Next, we detected [Ca^{2+}] transients in all 20 synthetic fluorescent traces, from which we selected such temporally segregated events and calculated the mean of the amplitudes of the smallest two-third of them. The ratio of the '*presumed unitary*' and the real amplitude of the [Ca^{2+}] transients was 1.01 ± 0.05 (median = 1.0, IQR: 0.98 – 1.02), and even the largest error was only 16% (Figure 11b), indicating that this method provides an excellent estimate of the amplitude of unitary [Ca^{2+}] transients. Due to spike sorting issues, especially in case of bursts of APs (Buzsaki, 2004), the calculations of the fraction of single APs and burst of APs from the isolated events can be erroneous. However, the burst-evoked [Ca^{2+}] transients have much larger peak amplitudes compared to the single AP-evoked responses; therefore, it should be reflected by a larger, separate peak on the amplitude distributions. In such cases, the amplitude of the first peak of the distribution should be applied. In our data, the accuracy of such an estimation of '*presumed unitary*' [Ca^{2+}] transients was almost identical (1.05 ±0.05; largest error: 23%) to that described above.

In a second method, we selected those [Ca^{2+}] transients that were inferred by MLspike as single AP-evoked ones, in a scenario where mean amplitude (0.248 ΔF/F), decay time constant (389 ms), p_{nonlin} (0.56) and *baseline drift* (0.011) parameters of the 20 PCs were used for fitting (blue in Figure 11d). We included only those detected unitary events in our analysis which were temporally segregated from other events (>4s before and >1s after). The accuracy of this estimation method was slightly lower than that of our first method; the ratio of '*detected unitary*' and real peak amplitude was 1.08 ± 0.17 (median = 1, IQR: 0.97 – 1.16, max = 1.51; Figure 11c). The main inaccuracies occurred for cells with small unitary peak amplitudes resulting in a significant negative correlation between the real peak amplitude and the ratio of '*detected unitary*'/real amplitudes (ρ = -0.78, p < 0.001; Spearman correlation, Figure 11c).

Next, we hypothesised that by using the reasonably accurately estimated peak amplitudes ('*presumed unitary*' and '*detected unitary*') of each cell, the mean decay time constant (389 ms),

p_nonlin (0.56) and *baseline drift* (0.011), inference error should be smaller compared to the scenario when all parameters were the means of the 20 PCs (60 ms tolerance window). Indeed, the ERs were significantly reduced (p = 0.01, Friedman ANOVA test; Figure 11g) from $15.0 \pm 15.2\%$ (median = 10.5%, IQR: $4.7 - 19.5\%$) to $6.3 \pm 4.9\%$ (median = 5.8%, IQR: $2.4 - 9.5\%$) when the '*presumed unitary*' amplitude (green in Figure 11g; Paired Sample Wilcoxon Signed Rank *post hoc* test: p = 0.008) and to $6.4 \pm 4.8\%$ (median = 5.3%, IQR: $3 - 8.3\%$) when the '*detected unitary*' amplitude was used for fitting (purple in Figure 11g; Paired Sample Wilcoxon Signed Rank *post hoc* test: p = 0.003). The ER did not show significant correlation with the estimated unitary peak amplitudes in either scenario (Figure 11e, f). These results further support the key role of the variability in peak amplitudes in spike inference accuracy; whereas, the decay time constant, *p_nonlin* and *baseline drift* contribute to only a minor part of the ER (~6%). Indeed, the ER was reduced to virtually zero when in addition to the estimated amplitudes, the original decay time constant, *p_nonlin* and *baseline drift* values were used ('*presumed unitary*': ER = $0.05 \pm 0.13\%$, median = 0%, IQR: $0 - 0\%$; '*detected unitary*': ER = $0.09 \pm 2.35\%$, median = 0%, IQR: $0 - 0.05\%$).

Finally, we analyzed our synthetic dataset with the algorithm (OASIS) published by Friedrich et al., (2017). We used two different settings of the algorithm and compared the correlation coefficients (CC) between the ground truth spike trains to those inferred with OASIS and MLspike. In the first sets of simulations, we let the OASIS algorithm to fit the rise and decay time constants of 5 isolated events. Then we used the obtained values to infer the spike train underlying the fluorescent data. This procedure resulted in a CC of 0.667 ± 0.177 between the ground truth and the inferred spike trains, which was substantially lower than that obtained with MLspike (0.821 ± 0.112). During a second set of simulations, we used the average decay time constant of unitary responses in 20 cells of our dataset (389 ms) and set the rise time to 10 ms for all cells. With these parameters the CC between the ground truth and the OASIS inferred spike trains was even lower (CC = 0.571 ± 0.198) compared to the first setting. These results demonstrate that in our simulations MLSpike performs better than OASIS.

Discussion

In the present study we demonstrate that the variability in the peak amplitudes of GCaMP6f [Ca^{2+}] transients recorded *in vivo* in a population of cells is not only the consequence of the mixture of single APs and high-frequency bursts, but is also due to a substantial cell-to-cell variability in the peak amplitude of unitary [Ca^{2+}] transients and the variable degree of supralinearity of GCaMP6f. We also show that the expression level of GCaMP6f is highly variable among CA1 PCs, partially underlying the variability in the amplitude, decay and summation of unitary [Ca^{2+}] transients. However, to our surprise, substantial variability is still observed in the peak amplitude and supralinearity when PCs were subselected with similarly low GCaMP6f expression. Our modelling with MLspike revealed that the main source of spike inference error is the variability in the peak amplitude of unitary events and not in the decay or supralinearity. We developed two procedures to estimate the peak amplitudes of unitary [Ca^{2+}] transients and show that spike inference ERs using these unitary amplitude estimates in weakly GCaMP6f expressing cells are as low as ~6%.

In the present study, we determined for the first time the expression level of GCaMP6f in functionally characterized nerve cells, allowing us to correlate the amplitude, kinetics and supralinearity of unitary fluorescent [Ca^{2+}] transients with the amount of GECI. We estimated the expression level of GCaMP6f 1) by measuring its native fluorescence in aldehyde fixed tissue, arguing that this measure should not reflect differences in [Ca^{2+}]; and 2) with anti-GFP immunohistochemistry. Our experiments revealing a very tight correlation between the intrinsic GCaMP6f fluorescence and anti-GFP immunoreactivity provide a strong support for the notion that the intrinsic fluorescence of GCaMP6f in fixed tissue is an excellent measure of the protein level. Determining the expression level of GCaMP6f in every imaged cell after dense population recordings is laborious, therefore we also analysed the relationship between the intrinsic GCaMP6f fluorescent intensities as measured in acute slices with two-photon microscopy and those measured after fixation. The weak correlation, with very low predictive power at low intensities, indicates that the resting fluorescence of GCaMP6f in living tissue is not a reliable indicator of the amount of GCaMP6f protein irrespective whether 810 nm or 925 nm excitation laser was used. Therefore, we conclude that reliable measurement of the expression level of GCaMP6f requires analysis of fluorescent intensities after chemical fixation. We also suggest that applying a cut off value of ~5% intensity of the most intensely labelled cells or ~30% intensity value of the mean intensity of the population should be applied to subselect cells in

which the unitary [Ca^{2+}] transients should have large amplitudes with favourable SNR. Such intensity-subselection resulted in the exclusion of approximately one third of the cells.

We simultaneously measured electrically the APs and optically GCaMP6f [Ca^{2+}] transients in acute hippocampal slices and not *in situ* in the brain, but we argue that our approach of using cell-attached recordings with antidromic stimulation has the advantage of permitting the *post hoc* identification of the unlabeled cells and of performing such experiments in hundreds of PCs. The size of our dataset is substantially larger than that of *in vivo* recordings and our data is not confined to monitoring naturally occurring spike trains, but we were able to perform the same, standardized protocols (e.g. single AP, bursts with different frequencies and spike number, recovery protocol) for all recorded cells. Our results demonstrate large variance in the peak amplitude of single AP-evoked unitary GCaMP6f [Ca^{2+}] transients in hippocampal CA1 PCs (CV= 0.96 for all recorded cells; CV = 0.95 for temporally isolated unitary events in 35 cells; CV = 0.73 for temporally isolated unitary events in cells with low GCaMP6f expression, n = 15). The cell-to-cell variability in [Ca^{2+}] transients in visual cortical *in vivo* recorded cells using GCaMP6f (Chen *et al.*, 2013) or GCaMP6s (Greenberg et al., 2019) was somewhat smaller (CV = 0.5 and 0.6, respectively), which might be the consequence of the differences between hippocampal and visual cortical PCs or differences between *in vitro* and *in vivo* conditions, including the temperature (room vs. body temperature). Indeed, the peak amplitude of single AP-evoked [Ca^{2+}] transients and its variance is known to depend on the preparations (hippocampal slice cultures, acute cortical slices, *in vivo*) and recording conditions (Mao *et al.*, 2008; Tian *et al.*, 2009; Akerboom *et al.*, 2012; Ohkura *et al.*, 2012a; Chen *et al.*, 2013). Biological variation in the behavior of GCaMP6f among distinct nerve cell types is also plausible, because the ionic strength and the composition of intracellular milieu has been shown to affect the affinity, cooperativity, and dynamic range of GCaMPs (Hires *et al.*, 2008). However, the parameters responsible for the variability among individual cells of the same types are unknown. Biophysical and biochemical mechanisms can potentially account for such complexities. Greenberg et al. (2019) developed a kinetic model and proposed that the cooperativity across the four Ca^{2+} binding sites of GCaMP, the slow and multiphasic kinetics of the indicator, and the total GCaMP concentration can account for variability and nonlinearity of GCaMP-reported fluorescent transients. Moreover, GCaMP molecules display two states with different fluorescent properties. The dimeric form, though less prevalent, contributes to background fluorescence of

the GCaMP expressing cells, however it is [Ca^{2+}] insensitive (Akerboom *et al.*, 2009) and can complicate the interpretation of fluorescent transients. Some mutations have been shown to result in photoisomerization (Akerboom *et al.*, 2009), or in the accumulation of non-responsive proteins in lysosomes (jRGECO1a, Dana *et al.*, 2016). It has also been shown that GCaMP-associated calmodulin disrupt Ca^{2+} influx and dynamics, and gene expression by affecting the gating of L-type Ca^{2+} channels in an expression-dependent manner (Yang *et al.*, 2018). We also demonstrate that peak amplitude of unitary [Ca^{2+}] transients is not only different among cell types and among cells of the same type, but also varies according to the cell's firing history. The use-dependent nonlinearity of $\Delta F/F$ has been reported previously (Mao *et al.*, 2008; Tian *et al.*, 2009; Akerboom *et al.*, 2012; Ohkura *et al.*, 2012a; Chen *et al.*, 2013) and is probably a result of the cooperativity of calcium binding by calmodulin. We provide a first evidence for a >2-fold increase of the unitary peak amplitude after a high frequency burst (10 AP at 50 Hz), and for the extremely slow recovery time course (~4 s) from this process. The mechanism underlying this long time-constant of the burst-evoked potentiation of unitary responses is probably different from that which underlies the supralinearity of the GCaMP6f responses within bursts (e.g. [Ca^{2+}]-dependent association constants (Nakai *et al.*, 2001), or different Ca^{2+} binding sites with different on and off rates). We also provide first evidence for variable degrees of supralinearity of GCaMP6f among CA1 PCs and its weak dependency on the expression level of the sensor.

The expression level of the [Ca^{2+}] indicator is thought to play a key role in defining [Ca^{2+}] transients. Lower concentration of Ca^{2+} indicators should report the same [Ca^{2+}] with higher peak amplitude, faster kinetics and lower SNR (Hires *et al.*, 2008; Broussard *et al.*, 2014; Dana *et al.*, 2014). Unexpectedly, our results show, that even though there is a significant negative correlation between the peak amplitude and GCaMP6f expression, in case of cells with similarly low intensities the variance remains substantial (CV = 0.56 – 0.75); therefore, homogenisation of the expression level by using transgenic mice (e.g. Dana *et al.*, 2014) might not provide a sufficient solution for the problem. One explanation of these results is a cell-to-cell variability in Ca^{2+} influx upon single APs, resulting in a different intracellular [Ca^{2+}] that will be reported as a highly differing fluorescence change upon the same stimulation. However, the lack of correlation of the unitary [Ca^{2+}] transients with the AP width or with the surface to volume ratio, and the lack of difference between peak amplitudes and decay time constants in cells with potentially different endogenous buffers, provide evidence that the substantial variability in unitary

GCaMP6f [Ca^{2+}] transients and its summation are likely due to differences in the aforementioned intrinsic properties of GCaMP6f instead of a different [Ca^{2+}]. This hypothesis is supported by the results with a synthetic dye (Fluo5F) in CA1 PCs not expressing GCaMP6f, showing much more uniform amplitudes of unitary [Ca^{2+}] transients among CA1 PCs.

We demonstrate that the cell-to-cell variability in the peak amplitude of unitary [Ca^{2+}] transients is a key source of spike inference ER on simulated data (with experimentally derived parameters) using the MLspike algorithm (Deneux *et al.*, 2016). Fitting with mean peak amplitude, but own decay time constants, *p_{nonlin}* and *baseline drift* parameters results in a similarly high ER compared to fitting when the mean is used for all parameters. Our ER with the mean parameters is similar to that obtained by (Deneux *et al.*, 2016) and also by a recent study using simple non-negative deconvolution (Pachitariu *et al.*, 2018). During *in vivo* optical recordings, the unitary peak amplitude of the several hundreds of recorded cells is not available; nonetheless, our results indicate that a decrease in spike inference ER may already be achieved by subselecting cells based on their GCaMP6f expression level. In the weakly expressing cell population, when simulations were performed using real spike timings of *in vivo* recorded CA1 PCs, we were able to accurately estimate the unitary peak amplitude with a model-independent ('*presumed unitary*') and a model-dependent ('*detected unitary*') procedure. Providing either of these estimated peak amplitudes to MLspike resulted in a significant reduction of the spike inference ER. To our knowledge there is currently no other spike inference algorithm or model that can estimate spiking activity with such low ERs (~6%). Importantly, this low ER is achieved in our simulations with a detection window (60 ms) that is an order of magnitude briefer than that used by others. The use of spiking statistics for the estimation of unitary [Ca^{2+}] transient amplitudes should be a generalizable method provided the firing properties of the cells of interest are known from *in vivo* silicon probe or tetrode recordings, based on which one can calculate the ratio of temporally isolated single APs and bursts of APs. The key issue with both of our methods is to achieve a SNR in the *in vivo* recordings, which permits detection of single AP-evoked fluorescent transients. One solution might be to reduce the number simultaneously recorded nerve cells, resulting in a better SNR. Our results support the necessity to obtain *in vivo* the full range of the critical parameters and to consider the variability of these when developing spike inferring algorithms.

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Author Contributions: TE, ZN designed the experiments, TE performed all *in vivo* and *in vitro* imaging experiments and analyzed the data; KK-Sz performed immunohistochemistry experiments; MS performed the modelling, MS and TE analyzed the synthetic data; TE and ZN wrote the manuscript.

Data Accessibility: The recorded fluorescent traces and the corresponding GCaMP6f intensities are available upon request.

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Figure legends

FIGURE 1. Large cell-to-cell variability in the amplitude of unitary $[Ca^{2+}]$ transients.

(a) A representative maximum intensity projection image (20 image frames) of the hippocampal CA1 area *in vivo* in an anaesthetised, head-fixed mouse. The ROIs for some of the analysed cells are overlaid. (b) Fluorescence traces recorded from the cells shown in (a). (c) Histogram of peak amplitudes of *in vivo* recorded $[Ca^{2+}]$ transients (n = 311 transients from 13 GCaMP6f-expressing PCs, 10 minutes recordings). (d) Representative single AP-evoked unitary $[Ca^{2+}]$ transients recorded from the somata of CA1 PCs *in vitro*. Black trace corresponds to average of all recorded PCs (n = 121). Inset: peak amplitude shows significant (p < 0.001, Spearman correlation) positive correlation with signal to noise ratio (SNR). (e) Cumulative probability plot of mean unitary $[Ca^{2+}]$ transients (black, n = 121 cells) demonstrates large cell-to-cell variability, with a much smaller trial-to-trial variance of individual cells (5 individual cells colour-coded, each with multiple trials).

FIGURE 2. Spontaneous and evoked unitary $[Ca^{2+}]$ transients show similar amplitudes and cell-to-cell variability.

(a – b) A fluorescence trace shows two spontaneous (cyan and orange) and one stimulus-evoked (grey) $[Ca^{2+}]$ transients (a) and the corresponding APs recorded in the cell-attached configuration (b). (c – d) The spontaneous and evoked $[Ca^{2+}]$ transients (c) and APs (d) have similar shapes and amplitudes. (e – f) The amplitude of spontaneous single AP-evoked $[Ca^{2+}]$ transients is on average 27% larger compared to that of the stimulus-evoked $[Ca^{2+}]$ transients (e; n = 6 cells, p = 0.036, Paired Sample Wilcoxon Signed Rank test), but their amplitudes show perfect correlation (f, Spearman correlation).

FIGURE 3. Large variability in the amount of GCaMP6f in CA1 PCs.

(a) Maximum intensity projection image of a confocal image stack showing variability in native GCaMP6f intensity of transfected neurons in perfusion fixed tissue. (b) Histograms of native GCaMP6f intensities in perfusion fixed tissues and in immersion fixed acute slices. (c) GCaMP6f intensity after fixation (measured with confocal microscopy) correlates with GCaMP6f intensity as measured in acute slices using 810 nm excitation light with two-photon microscopy for the whole population ($p < 0.001$, Spearman correlation). (d) For the intensity sub-selected cells (lower 65 percentiles of all cells) no significant correlation is found between these parameters ($p = 0.12$, Spearman correlation). (e) High magnification confocal images of perfusion fixed tissue showing native GCAMP6f expression (green) and its anti-GFP immunoreactivity (orange). * and # indicate weakly and moderately expressing cells, respectively. (f) The intensity of the native GCaMP6f signal and that of the anti-GFP immunosignal shows a significantly positive correlation ($n = 87$, $p < 0.001$, Spearman correlation).

FIGURE 4. Single AP-evoked unitary $[Ca^{2+}]$ transients show large variability among cells with similar GCaMP6f expression levels.

(a) Two-photon images of CA1 PCs before cell-attached recordings (white lines: scanning lines). (b) Two-photon images of the same cells after recording pipette was withdrawn at the end of the recordings. Note the lack of detectable changes in the native GCaMP6f intensity in these cells. (c) Confocal images of the same cells following immersion fixation (outline of ROIs in white) (d) Single AP-evoked unitary $[Ca^{2+}]$ transients in the same 6 cells as shown above (individual transients: semi-transparent, mean: bold) show large variability in amplitude. (e) Histogram of native GCaMP6f intensities of PCs for which the intensity between the start of the recording and withdrawal of the pipette did not change >25% ($n = 43$). (f) The amplitude of unitary $[Ca^{2+}]$ transients negatively correlates with the native GCaMP6f intensity of the cells ($p < 0.001$, Spearman correlation; coloured symbols represent the 6 cells shown in a-d). (g) Single AP-evoked $[Ca^{2+}]$ fluorescence signals show large variability among cells with similar intensities (same data as in f, but subselected for intensities <180 AU; $p = 0.28$, Spearman correlation). (h) Native GCaMP6f intensity levels negatively correlate with the SNR of unitary $[Ca^{2+}]$ transients, but show large variability among cells with similar intensities ($p < 0.001$, Spearman correlation).

FIGURE 5. Negligible contribution of out-of-focus fluorescence to the peak amplitude of unitary responses.

(a) Images of the same weakly GCaMP6f expressing cell in three different focal planes separated by 3-4 µm. (b) Averaged unitary fluorescent transients recorded in the three focal planes shown in (a). (c) The peak amplitudes recorded in the different focal planes show little variability (CV = 0.10, n =7 cells; open circles: individual cells; green triangles: mean).

FIGURE 6. Correlation of the decay time constant of the unitary $[Ca^{2+}]$ transients with the GCaMP6f expression level and with the peak amplitude of the unitary responses.

(a, b) The decay time constant of single AP-evoked $[Ca^{2+}]$ transients correlates positively with the GCaMP6f intensity of the recorded PCs for the whole population (a; n = 41; p < 0.001, Spearman correlation) and for the sub-selected cells (b, same data as in (a), but sub-selected for cells with intensities < 180 AU, corresponding to the bottom 65% of the cells; n = 26; p = 0.008, Spearman correlation). (c) Heatmap plot showing the relationship between decay time constants, the peak amplitudes of unitary $[Ca^{2+}]$ transients and GCaMP6f expression level of the recorded cells. (d) The decay time constants correlate negatively with the peak amplitudes of the unitary responses (ρ = -0.27, p = 0.048; n = 53, Spearman correlation) when all cells are examined. (e) However, when only the weakly GCaMP6f expressing cells are subselected (< 180 AU), there is no significant correlation between these parameters (ρ = -0.001, p = 0.99; n = 26, Spearman correlation).

FIGURE 7. The amplitude of unitary GCaMP6f $[Ca^{2+}]$ fluorescent transients does not correlate with the AP width, soma location or surface to volume ratio.

(a) Peak amplitude of unitary $[Ca^{2+}]$ transients does not correlate with AP width recorded in cell-attached configuration (p = 0.17, Spearman correlation; n = 46 cells). (b) The peak amplitudes of unitary $[Ca^{2+}]$ transients are not significantly different among cells situated in superficial, middle or deep layers of the stratum pyramidale (p = 0.13, Kruskal Wallis test; n = 20, 18, and 12 cells in superficial, middle and deep layers, respectively). (c) Surface to volume ratio does not

correlate with the amplitude of unitary $[Ca^{2+}]$ transients (p = 0.78, Spearman correlation; n = 21 cells). (d) Peak amplitude of unitary $[Ca^{2+}]$ transients does not correlate with the GCaMP6f intensity-normalized resting fluorescence (p = 0.12, Spearman correlation). (e) Representative $[Ca^{2+}]$ transients (grey: three individual traces; pink: averaged trace) recorded with 300 μM Fluo5F. Inset shows the scanning line over the PC somata. (f) The distribution of single AP-evoked GCaMP6f $[Ca^{2+}]$ transients (normalized to mean) is wider (n = 55) and significantly different (p = 0.014, two-sample Kolmogorov-Smirnoff test), compared to that of unitary $[Ca^{2+}]$ transients measured with Fluo5F (n = 23 cells).

FIGURE 8. Highly supralinear nature of GCaMP6f fluorescence signals.

(a, d) Examples of $[Ca^{2+}]$ transients normalized to their unitary peak amplitude evoked by 5 APs at 10 or 50 Hz (blue) and the mathematical sum of its corresponding five, spike time-adjusted, unitary $[Ca^{2+}]$ transients (cyan; black vertical line: unitary amplitude). (b, c) Fluorescent signals evoked by 5 APs at 10 Hz are supralinear (mean linearity = 1.67 ± 0.54, n = 34). The linearity index does not correlate with either the peak amplitude of unitary $[Ca^{2+}]$ transients (b, p = 0.4; Spearman correlation) or with their native GCaMP6f expression level (c, p = 0.09; Spearman correlation). (e, f) $[Ca^{2+}]$ transients evoked by 5 APs at 50 Hz sum supralinearly (mean linearity = 1.92 ± 0.63, n = 40). The linearity index does not correlate with the peak amplitude of unitary $[Ca^{2+}]$ signals (e, p = 0.06, Spearman correlation), but shows a negative correlation with the expression of GCaMP6f (f, p = 0.006, Spearman correlation). (g) Normalized peak amplitudes of 3 or 5 AP-evoked $[Ca^{2+}]$ transients are highly variable and show different degrees of summation. (h) The linearity index for 3 and 5 APs at 50 Hz shows a significant positive correlation (r^2 = 0.86, p < 0.001, Pearson correlation). (i) A large $[Ca^{2+}]$ transient is followed by 12 APs evoked at 0.25 Hz. Note the gradual decrease of the unitary $[Ca^{2+}]$ signal during the train. (j) Example of several $[Ca^{2+}]$ transients evoked by burst recovery protocols (magenta: 2.5 s; red: 4 s; cyan: 10 s; blue: 15 s after a 10 AP at 50 Hz burst). Bottom: lines illustrate the timing of the recovery stimuli for the different protocols. Inset: Same traces zoomed in Y scale and compressed in time for better visualization of the transients evoked by the recovery pulses. (k) The increase in peak amplitude of unitary $[Ca^{2+}]$ transients after a burst of 10 APs at 50 Hz persists for several seconds (open circles: mean of individual cells, closed circles: average ± SD). Data are normalized to unitary $[Ca^{2+}]$ transients measured before the burst. (l) Mono-exponential fit (τ =

3.95 s) to the normalized unitary [Ca^{2+}] transients evoked by the recovery pulses (color code same as above).

FIGURE 9. Cell-to-cell variability in the peak amplitude of unitary GCaMP6f [Ca^{2+}] transients leads to inaccurate spike inference.

(a) A single AP-evoked [Ca^{2+}] transient (black trace) and the best fit using MLspike with the following four scenarios: 1) own amplitude and own decay time constant (red, own A and own τ); 2) own amplitude and mean decay time constant (orange, own A, $\bar{\tau}$); 3) mean amplitude and own decay time constant (cyan, $\bar{A}$, own τ); 4) mean amplitude and mean decay time constant (blue, $\bar{A}$ and $\bar{\tau}$). (b) The inference error rate (ER) of a [Ca^{2+}] transient evoked by 5 APs at 50 Hz is plotted as a function of *pnonlin* and *baseline drift* parameters. (c) Fitting a [Ca^{2+}] transient evoked by 5APs at 50Hz using those maximal *p$_{nonlin}$* and minimal *baseline drift* values that resulted in minimal ER. (d, f, h) Representative synthetic [Ca^{2+}] traces and inferred spike trains calculated with own peak amplitude and decay time constant (red) or with mean peak amplitude and mean decay time constant (blue) values. miss: missed APs; fd: false detection (60 ms tolerance window). Traces were generated with Poisson spike trains at 0.1 Hz (d), 1 Hz (f) and 10 Hz (h). (e, g, i) ERs for the different inference scenarios (mean amplitude value of the 37 cells, with the cell's own decay, *baseline drift* and *p$_{nonlin}$* values: 0.1 Hz: ER = 25.3 ± 33.7%, median = 1.8%, IQR: 0 – 33.1%; 1 Hz: ER = 26.2 ± 30.8%, median = 9.3%, IQR: 0.3 – 46.2%; 10 Hz: ER = 17.4 ± 20.2%, median = 8.9%, IQR: 0.3 – 29.5%; the mean amplitude, decay, *baseline drift* and *p$_{nonlin}$* values of the 37 cells: 0.1 Hz: ER = 22.9 ± 34.2%, median = 1.9%, IQR: 0 - 33.2%; 1 Hz: ER = 27.4 ± 32.9%, median = 9.5%, IQR: 0 - 51.9%; 10 Hz: ER = 26.5 ± 24.5, median = 16%, IQR: 9.6 - 47.3%). (j) Correlations of ERs and GCaMP6f intensity values (0.1 Hz: p = 0.007; 1 Hz: p = 0.001; 10 Hz: p = 0.013; n = 28; Spearman correlation). (k) ERs for low intensity cells (<180 AU, n = 20) are significantly smaller compared to high intensity cells (>180 AU, n = 8). Statistics for panels e, g, i, k: Three-Way ANOVA: p < 0.001 for intensity; p < 0.001 for scenarios; p > 0.05 for frequency; p < 0.001 for intensity–scenarios interaction; p > 0.05 for

intensity–frequency interaction; $p > 0.05$ for scenarios–frequency interaction; $p > 0.05$ for intensity–scenarios–frequency interaction; * <0.05 pairwise Bonferroni *post hoc* test.

FIGURE 10. Relationships of spike inference error rates (ERs), missed APs (miss) and false detections (fd) with the peak amplitude of unitary $[Ca^{2+}]$ transients.

(a – c) ER is low for cells with peak amplitudes similar to the mean unitary peak amplitude (indicated by the vertical dashed line) at each tested firing frequency. (d – f) The fraction of missed APs is higher for cells with small unitary peak amplitudes. (g – i) The fraction of falsely detected APs is higher for cells with large unitary peak amplitudes. (a, d, g: 0.1 Hz, b, e, h: 1 Hz, c, f, i: 10 Hz; n = 33 cells). The ER is the harmonic mean of the false detections and the fraction of missed spikes.

FIGURE 11. The error rate in spike inference can be reduced by estimating the amplitudes of unitary $[Ca^{2+}]$ transients.

(a) The distribution of mean firing rates of CA1 PCs obtained from Grosmark, Long and Buzsaki (CRCNS.org, http://dx.doi.org/10.6080/K0862DC5; n = 48 cells), and that of the subselected cells (n = 20) used for generating synthetic $[Ca^{2+}]$ traces (p = 0.27, Two-Sample Kolmogorov Smirnov test). (b) Accuracy of estimating the amplitude of '*presumed unitary*' $[Ca^{2+}]$ transients shows no significant correlation with original unitary $[Ca^{2+}]$ transients (n = 20 cells; p > 0.05, Spearman correlation). (c) Accuracy of estimating peak amplitudes from $[Ca^{2+}]$ transients detected by MLspike as single AP-evoked ('*detected unitary*') correlates negatively with the real peak amplitudes (n = 20 cells; p < 0.001, Spearman correlation). (d) Synthetic $[Ca^{2+}]$ traces generated based on an *in vivo* recorded spike train (black, $[Ca^{2+}]$ trace; blue, green, and purple $[Ca^{2+}]$ fits; dashed blue, green, and purple, baseline drift). Inferring spikes using MLspike with the mean unitary $[Ca^{2+}]$ transient amplitude and decay (blue) and with the '*presumed unitary*' amplitude and mean decay (green) and the '*detected unitary*' $[Ca^{2+}]$ transient amplitude and

mean decay (purple). (e, f) Peak amplitude of '*presumed unitary*' (puA in e) or '*detected unitary*' (duA in f) $[Ca^{2+}]$ transients does not correlate with the ER (n = 20, p > 0.05, Spearman correlation). (g) Summary plot showing significant reduction in ERs (n = 20; p = 0.01, Friedman ANOVA test) when spike inference is performed using peak amplitude of '*presumed unitary*' $[Ca^{2+}]$ transients (green; Paired Sample Wilcoxon Signed Rank *post hoc* test: p = 0.008) or using the amplitude of '*detected unitary*' $[Ca^{2+}]$ transients (purple; Paired Sample Wilcoxon Signed Rank *post hoc* test: p = 0.003).

(a) *in vivo*

10 μm

(b) *in vivo*

0.5 ΔF/F

10 s

(c) *in vivo*

(d) *in vitro*

ρ = 0.88

peak amplitude (ΔF/F)

SNR

0.1 ΔF/F

200 ms

(e) *in vitro*

○ individual PC
○ cell No. 109
○ cell No. 68
○ cell No. 94
○ cell No. 86
○ cell No. 56

cumulative probability

peak amplitude (ΔF/F)

count

peak amplitude (ΔF/F)

(a) evoked, spontaneous nr. 1, spontanoues nr. 2

0.05 ΔF/F

200 ms

(b) * stim.

100 pA

200 ms

(c) 0.05 ΔF/F

100 ms

(d) 50 pA

1 ms

(e) peak amplitude (ΔF/F)

spontaneous   evoked

*

(f) peak amplitude of evoked (ΔF/F)

ρ = 1

peak amplitude of spontaneous (ΔF/F)

(a) CA1 50 μm

(b) perfusion fixed / immersion fixed
count
150
100
50
0
0   1000   2000   3000
native GCAMP6f intensity (AU)

(c) ρ = 0.76
GCaMP6f intensity after fixation (AU)
3000
2000
1000
0
0   2500   5000   7500
GCaMP6f intensity in slice at 810 nm (AU)

(d) ρ = 0.33
GCaMP6f intensity after fixation (AU)
200
150
100
50
0
0   100   200
GCaMP6f intensity in slice at 810 nm (AU)

(e) native GCaMP6F   anti-GFP   overlay   10 μm

(f) ρ = 0.97
anti-GFP intensity (AU)
800
600
400
200
0
0   500   1000   1500   2000
native GCaMP6f intensity (AU)

(a)

5 μm

(b)

5 μm

(c)

10 μm

(d)

0.2 ΔF/F

500 ms

(e)

count

GCaMP6f intensity after fixation (AU)

(f)

peak amplitude (ΔF/F)

ρ = −0.69

GCaMP6f intensity after fixation (AU)

(g)

peak amplitude (ΔF/F)

ρ = −0.22

GCaMP6f intensity after fixation (AU)

(h)

SNR

ρ = −0.6

GCaMP6f intensity after fixation (AU)

(a) above   middle   below   10 μm

(b) below (n=12)   middle (n=24)   above (n=12)   0.1 ΔF/F   200 ms

(c) normalized ΔF/F   above middle below

(a) ρ = 0.20

peak amplitude (ΔF/F) vs FWHM of AP (ms)

(b) superficial / middle / deep

peak amplitude (ΔF/F)

(c) ρ = 0.07

peak amplitude (ΔF/F) vs surface/volume

(d) ρ = -0.25

peak amplitude (ΔF/F) vs normalized F_{rest}

(e) 0.005 G/G_{max}

1 s

Alexa 594
Fluo5F
5 μm

(f) GCaMP6f CV = 0.79
Fluo5F CV = 0.27

count vs normalized peak amplitude

(a) 5 AP at 10 Hz

(d) 5 AP at 50 Hz

(i)

(b) ρ = 0.14

(e) ρ = 0.3

(j)

(c) ρ = -0.33

(f) ρ = -0.46

(g)

(h) r² = 0.86

(k)

(l) τ = 3.95 s

(a) 0.1 ΔF/F / 0.1 s

Ca²⁺ trace
Ca²⁺ fit with own A, own τ
Ca²⁺ fit with own A, $\bar{\tau}$
Ca²⁺ fit with $\bar{A}$, own τ
Ca²⁺ fit with $\bar{A}$, $\bar{\tau}$

(b) ER (%)
baseline drift
pnonlin

(c) 1 ΔF/F / 0.5 s
Ca²⁺ trace
Ca²⁺ fit
baseline drift
original spikes
inferred spikes

(d) 0.1 HZ
0.5 ΔF/F / 20 s
ER = 0%
(miss: 0%
fd: 0%)
ER = 33.2%
(miss: 0%
fd: 49.9%)

(e) 0.1 HZ
own A, own τ   own A, $\bar{\tau}$
$\bar{A}$, own τ $\bar{A}$, $\bar{\tau}$
ER (%)

(f) 1 Hz
1 ΔF/F / 2 s
ER = 0%
(miss: 0%
fd: 0%)
ER = 33.3%
(miss: 0%
fd: 49.9%)

(g) 1 Hz
ER (%)

(h) 10 Hz
10 ΔF/F / 0.2 s
ER = 0.5%
(miss: 0.6%
fd: 0.5%)
ER = 19.2%
(miss: 0%
fd: 32.1%)

(i) 10 Hz
ER (%)

(j) 0.1 Hz   1 Hz   10 Hz
ER (%)
ρ = 0.5   ρ = 0.59   ρ = 0.46
GCaMP6f intensity after fixation (AU)

(k) □ intensity <180 AU
△ intensity >180 AU
ER (%)
frequency (Hz)
0.1   1   10

(a) Histogram: count vs mean firing rate (Hz), with dataset (gray), selected cells (blue), cell no. 16 (green).

(b) ρ = -0.4; ratio of presumed unitary/real peak amplitude vs real amplitude (ΔF/F).

(c) ρ = -0.78; ratio of detected unitary/real peak amplitude vs real amplitude (ΔF/F).

(d)
$\overline{A}, \overline{\tau}$ — 0.2 ΔF/F, 2 s — ER = 17.4% (miss: 29% fd: 1.4%); original spikes (black), inferred spikes (blue)

presumed unitary A, $\overline{\tau}$ — 0.2 ΔF/F, 2 s — ER = 8.9% (miss: 10.5% fd: 7.25%); original spikes (black), inferred spikes (green)

detected unitary A, $\overline{\tau}$ — 0.2 ΔF/F, 2 s — ER = 8.0% (miss: 10.5% fd: 5.3%); original spikes (black), inferred spikes (purple)

(e) ER puA, $\overline{\tau}$ (%) vs puA (ΔF/F); ρ = -0.27

(f) ER duA, $\overline{\tau}$ (%) vs duA (ΔF/F); ρ = -0.29

(g) ER (%) for $\overline{A}, \overline{\tau}$; puA, $\overline{\tau}$; duA, $\overline{\tau}$; * *