Investigation of Glutamatergic Synaptic Transmission in Hippocampal Pyramidal Cells

Ph.D. thesis outline

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Budapest,
2019

1. INTRODUCTION

Regulated control of synaptic transmission is essential for gating of information flow through neuronal circuits. Assessing the efficacy and reliability of synaptic transmission in a dynamic system -in which a plethora of connection types respond differently to diverse patterns of presynaptic firing activity- are crucial for understanding the ongoing information transfer, that will result in formation of memories.

Synapses formed by heterogeneous, or even apparently homogeneous pre- and postsynaptic cells demonstrate remarkable diversity regarding their synaptic properties. A special form of this diversity is the so-called target cell type-dependent difference in release probability (P_r), when the axon of a single pyramidal cell (PC) can transmit different aspects of information coded in a complex spike train to distinct postsynaptic cell types. There is little data available regarding the mechanisms through which this process is regulated. In 2001 Rozov et al. put forward an elegant hypothesis based on their experiments involving fast and slow Ca^{2+} buffers. They postulated that the low initial P_r of facilitating cortical PC synapses can be explained by a larger coupling distance between voltage-gated Ca^{2+} channels (VGCC) and Ca^{2+} sensors on the docked vesicles compared with the high P_r PC synapses on fast-spiking (FS) interneurons (INs). Assuming similar Ca^{2+} sensors and docked vesicle distributions, this would suggest a lower average Ca^{2+} channel density within the active zones (AZs) of low P_r synapses.

Understanding the rules of formation of synapses with such diverse properties is crucial for elucidating how individual neurons build up neuronal circuits, which are then responsible for the emergence of complex behaviors and memory formation. Recent results provide evidence that the heterogeneity of principle cells in concert with the heterogeneity of synaptic properties can constitute several distinct, nonuniform parallel circuit modules, underlying the diverse downstream effects of circuit activity. In order to observe the extent of the influence of synaptic diversity on information processing, storage and retrieval, it is necessary to simultaneously and chronically record the activity of large ensembles of neurons that are anatomically and biochemically identifiable. The introduction of two-photon (2P) microscopy in concert with improvement of the signal to noise ratio (SNR) of the fluorescent probes revolutionized the field of optical imaging promoting the real-time investigation of biological processes (Helmchen and Denk, 2005; Svoboda and Yasuda, 2006). Optical imaging techniques have the advantage of monitoring action potential (AP)-evoked fluorescent changes simultaneously in a large number of individual neurons and in a chronic manner, allowing the examination of activity changes during the course of learning, life experience, brain development, or disease progression. Moreover, the genetically encoded activity indicators enable selective interrogation of genetically defined neuronal subtypes, or of cells with specific patterns of anatomical connectivity permitting the sophisticated analyses of ensemble neural activity. The GCaMP family of genetically encoded Ca^{2+} indicators (GenEncCaInds) 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. Therefore, GCaMP6f became the most widely used GenEncCaInd in behaving animals. Despite the improved properties of indicators, APs cannot be directly inferred from the fluorescent transients with millisecond temporal resolution. 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 GenEncCaInds. A key parameter that determines many of these parameters is the concentration of the Ca^{2+} indicator. Unfortunately, the concentration/expression of the GenEncCaInds 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. Understanding the exact relationship between the expression level of GenEncCaInds and the variability of unitary [Ca^{2+}] transients, their SNR and nonlinearity, and how these parameters influence the performance of spike inferring algorithms is crucial to achieve higher accuracy in deciphering the neuronal activity during behavior.

2. OBJECTIVES

The general aim of my PhD work is to investigate aspects of glutamatergic synaptic transmission. In the first part of my PhD thesis I performed experiments to investigate the mechanism which underlies the target cell type-dependent differences in release probability. First, I examined the short-term plasticity of CA3 PC-to-parvalbumin positive (PV^+) or -metabotropic glutamate receptor 1α (mGluR1α$^+$) IN synapses. I also tested whether the postsynaptic presence of extracellular leucine-rich repeat fibronectin-containing protein 1 (Elfn1/2) determines the short-term plasticity at PC-to-mGluR1α$^+$ INs. Next, I investigated potential target cell type-dependent differences in the [Ca^{2+}] transients in axon terminals synapsing onto these two populations of INs. Having found significantly larger [Ca^{2+}] influx in PV^+-IN targeting boutons I performed experiments to search for potential causes. For this, I tested the contribution of P/Q, N type VGCCs to the [Ca^{2+}] influx and the degree of [Ca^{2+}] buffering in these two populations of axon terminals (Éltes et al., 2017).

Investigation of diverse synaptic properties and their functional consequences on network activity during behavior is hampered by the inaccurate spike inference from the optically recorded fluorescent transients. Therefore, in the second part of my PhD work I aimed to understand the relationship between the expression level of the most widely used genetically encoded Ca^{2+} indicator, GCaMP6f and the variability in [Ca^{2+}] transients, their signal to noise ratio and nonlinearity. I also aimed to elucidate which of these parameters is a key source of error in spike inference, and to develop a procedure that improves the spike estimation (Éltes et al., 2019).

Contributions: In the first part of my thesis, the short-term plasticity measurements of CA3 PC and PV^+/mGluR1α$^+$ INs, and the Ca^{2+} imaging experiments from boutons of CA3 PCs

(with 300μM Fluo5F) are pooled from my recordings and that of my collaborator, Noémi Holderith's. In the second part of my thesis the simulations were conducted together with my collaborator, Miklós Szoboszlay. The anti-GFP immunoreactions and quantification of nativeGCaMP6f and anti-GFP intensities were performed by Katalin Szigeti. She also performed 3dimensional (3D) reconstructions of the somata of a subselected population of the recorded cells and measured their surface-to-volume ratios.

3. MATERIALS AND METHODS

3.1. 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.

3.2. PART I: Investigation of the mechanism of target cell-type dependent differences in neurotransmitter release probability

3.2.1. Slice preparation and electrophysiological recordings of CA3 PCs and INs

Male Wistar rats (n = 97, 14 - 17 days old) were killed by decapitation and acute hippocampal slices were prepared as described previously (Holderith et al., 2012; Éltes et al., 2017). Cells were visualized using a Femto2D microscope equipped with oblique illumination and a water-immersion lens (25X, numerical aperture (NA) = 1.05, Olympus, or 25X, NA = 1.1, Nikon). Whole-cell voltage- or current-clamp recordings were performed from CA3 PCs or INs located in the strata oriens and pyramidale. For voltage-clamp recordings of evoked excitatory postsynaptic currents (EPSCs) in INs, the intracellular solution contained picrotoxin (0.6 - 0.8 mM). For current-clamp recordings and Ca^{2+} imaging in PC axons, 100 or 300 μM Fluo5F and 20 μM Alexa Fluor 594 were added to the intracellular solution. The firing pattern of the INs was determined with a series of 500-ms long hyperpolarizing and depolarizing current pulses with amplitudes of 125 - 500 pA. For extracellular stimulation, a unipolar stimulating electrode was placed in the stratum oriens at least 100 μm away from the soma. INs were held at -70 mV and 5 stimuli (0.2 - 0.3 ms duration, 20 - 200 pA) at 40 or 50 Hz were applied. Pyramidal cells were held at -70 mV (with a maximum of -100 pA DC current) and single action potentials (APs) were evoked with 2- to 4-ms-long depolarizing current pulses (1 - 1.2 nA).

3.2.2. *In vitro* two-photon [Ca^{2+}] imaging of CA3 PC axon terminals

Experiments were performed with a Femto2D (Femtonics) laser scanning microscope equipped with a MaiTai femtosecond pulsing laser tuned to 810 nm. Cells were filled for 2 h with a Ca^{2+}-insensitive (20 μM Alexa Fluor 594) and a Ca^{2+}-sensitive fluorophore (100 or 300 μM Fluo5F). Boutons were imaged in line scan mode (at 1 kHz, 2 -

3 scans averaged for each bouton) with a laser intensity of 2 - 6 mW at the back aperture of the objective lens. Single AP-evoked changes in fluorescence were quantified during the recording as $G/R(t) = (F_{green(t)} - F_{rest, green})/(F_{red} - I_{dark, red})$. To normalize data across batches of dyes, G_{max}/R values were measured by imaging a sealed pipette filled with intracellular solution containing 10 mM $CaCl_2$ for each cell. G/R measurements from boutons were divided by G_{max}/R, yielding the reported values of G/G_{max}. The effects of 1 μM ω-CTX MVIIC was tested by comparing the peak amplitudes of presynaptic [Ca^{2+}] transients in individual boutons, averaged from two to three consecutive scans, in control conditions and after 30 min of wash-in of the drug.

3.2.3. Identification of the postsynaptic target of the imaged boutons

At the end of the recordings a high-resolution image stack of the measured boutons was acquired; after which the slices were chemically and double-immunolabeling for PV and mGluR1α or mGluR1α and Elfn1/2 was performed. Biocytin was visualized with Alexa Fluor 488- or Cy5-conjugated streptavidin. The imaged boutons were then identified based on superposition of the 2P and *post hoc* acquired zstacks.

3.3. PART II. Improving spike inference accuracy from GCaMP6f fluorescent transients in hippocampal CA1 PCs

3.3.1. Stereotaxic viral delivery of GCaMP6f to the CA1 region of the hippocampus

Male FvB/Ant mice (n = 57, 30 - 52 days old) were anesthetized and a small craniotomy (0.5 - 1 mm) was made bilaterally, above the CA1 region of dorsal hippocampus. To obtain a sparse expression of the genetically encoded Ca^{2+} indicator GCaMP6f, I used a mixture of AAV9.Syn.Flex.GCaMP6f.WPRE.SV40 (Penn Vector Core) and AAV9.CMV.PI.CRE.rBG (Penn Vector Core) in 1:100 dilution.

3.3.2. 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 (n = 1).

3.3.3. *In vivo* two-photon [Ca^{2+}] imaging and data analysis

Imaging was performed 9 days after window implant surgery with a Femto2D 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. 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.) the changes in fluorescence were quantified as ΔF/F.

3.3.4. Slice preparation for electrophysiological recordings in CA1 hippocampal region from adult mice

Adult male mice (n = 53, 42 - 81 days old) were anaesthetized and were transcardially perfused with an ice-cold, standard cutting solution. After decapitation, the slices were cut with the same procedure as in the previous paragraph. I performed cell-attached recordings from CA1 PCs expressing GCaMP6f, and whole-cell current-clamp recordings in control experiments using the synthetic Ca^{2+} indicator Fluo5F (300 μM). Recording pipettes for cell-attached recordings were filled with ACSF; for whole-cell experiments I used standard intracellular solution. APs were evoked antidromically by extracellular stimulation (0.2 - 0.3 ms duration, ~500 μA) using a monopolar stimulating electrode, placed in the stratum oriens/alveus. For whole-cell recordings, PC were held at -70mV and APs were evoked with 2- to 4 ms-long depolarizing current pulses (1 - 1.2 nA).

3.3.5. *In vitro* two-photon [Ca^{2+}] imaging of somatic GCaMP6f signals and data analysis

Experiments were performed using a Femto2D (Femtonics) laser scanning microscope equipped with a Chameleon femtosecond pulsing laser (Coherent) tuned to 925 nm. Somata of CA1 PCs expressing GCaMP6f were imaged in line scan mode (0.5-1 kHz sampling rate). AP-evoked changes in fluorescence were quantified as ΔF/F. To tetst for any potential damage during the recordings I acquired images of the cells at the start of the recordings and after the withdrawal of the recording pipette and comapared the mean GCaMP6f intensities. Image stacks were also taken to allow *post hoc* identification of the recorded cells. In another set of experiments, cells were filled with a Ca^{2+}-insensitive (20 μM Alexa Fluor 594) and a Ca^{2+}-sensitive fluorophore (300 μM Fluo5F).

3.3.7. Immunohistochemistry and quantification of nativeGCaMP6f and anti-GFP intensities

After recordings, the slices were chemically fixed and the recorded cells were identified based on two-photon image stacks and their mean native GCaMP6f intensity was quantified. In another experiment 3 animals were transcardially perfused (55 - 76 days old), and anti-GFP immunolabelling was performed. The mean native GCaMP6f intensity and anti-GFP intensity was quantified.

3.3.8. Simulations

The simulations were performed together with my colleague Miklos Szoboszlay. To infer APs from fluorescent Ca^{2+} traces, we used the MLspike software. First, we estimated the algorithm's built-in parameters of nonlinearity (p_{nonlin}) and the '*baseline drift*' by fitting in vitro recorded fluorescent [Ca^{2+}] transients evoked by bursts consisting of 5 APs at 50 Hz. 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 single spike-evoked unitary fluorescent [Ca^{2+}] transients, their measured noise

and the abovementioned p_{nonlin} and *baseline drift* values. 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). I estimated the peak amplitude of putative unitary [Ca^{2+}] transients with two methods. (1) First, I 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, I calculated that two third of the temporally isolated events are single APs, the rests are bursts. I detected temporally 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, I 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). Then, I 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. Then, I performed spike inference using the '*putative unitary'* or '*detected unitary'* [Ca^{2+}] transient amplitudesS and mean decay, p_{nonlin} and *baseline drift* parameters of each cell.

4. RESULTS

4.1. PART I. Target cell type-dependent differences in Ca^{2+} channel function underlie distinct release probabilities at hippocampal glutamatergic terminals

4.1.1. Distinct short-term plasticity of EPSCs in different IN types of the hippocampal CA3 region

I chose CA3 PC local axon collaterals as the subject of my study because they are amenable to presynaptic [Ca^{2+}] measurements and establish synaptic contacts onto both FS/PV^+ INs and mGluR1α^+ INs. First, I characterized the short-term plasticity of EPSCs recorded from PV^+, and mGluR1α^+ INs in the CA3 region of young Wistar rats. For this, I performed whole-cell voltage-clamp recordings from the somata of GABAergic INs located in the stratum oriens or stratum pyramidale, with biocytin-containing intracellular solution and I evoked five EPSCs by extracellular stimulation of PC axons.

The cells were characterized based on their firing patterns, *post hoc* determined dendritic and axonal arbors, and their PV or mGluR1α immunoreactivity. EPSCs evoked by a train of stimuli at 40 or 50 Hz in PV^+ INs showed short-term depression ($EPSC_{fifth}/EPSC_{first}$: 0.52 ± 0.19, n=16). In contrast, five stimuli elicited facilitating EPSCs in mGluR1α^+ INs ($EPSC_{fifth}/ EPSC_{first}$ = 3.02 ± 1.9, n = 31). On average the excitatory inputs to mGluR1α^+ cells showed a different short-term plasticity pattern compared with those recorded from PV^+ cells. Next, I tested the potential role of Elfn1/2 in setting the short-term plasticity of EPSCs onto mGluR1α^+ cells. Elfn1/2 and mGluR1α double-labeled INs showed a robust short-term facilitation, the degree of which was significantly larger than that detected in mGluR1α^+, but

Elfn1/2$^-$ cells. These results reveal a previously unseen diversity in the short-term plasticity of PC-to-mGluR1α^+ IN synapses and indicate a potential role of Elfn1/2 in its regulation.

The demonstrated robust differences, in the short-term plasticity of evoked EPSCs in PV$^+$ and mGluR1α^+ INs in the CA3 area, are similar to those observed in the CA1 area and the neocortex, offering the use of these molecules in this brain region as markers to label the postsynaptic compartments of functionally distinct presynaptic axon terminals.

4.1.2. Target cell type-dependent differences in Ca^{2+} inflow in axon terminals

I performed whole cell current-clamp recordings of CA3 PCs and evoked single APs by a depolarizing current pulse. To examine [Ca^{2+}] specifically in axon terminals with identified postsynaptic target cell types, I fixed the slices after the *in vitro* imaging experiments and then I visualized the intracellular biocytin, and immunolabeled the tissue for mGluR1α and PV for confocal microscopic analysis. From the total of 692 imaged boutons, I found 26 and 61 as potential presynaptic elements to PV$^+$ and mGluR1α^+ INs, respectively. My *post hoc* analysis revealed that the peak amplitude of the [Ca^{2+}] transient was 1.25 times larger in PV$^+$ dendrite-targeting boutons compared with their mGluR1α^+ dendrite-targeting counterparts (PV: median: 0.134 G/G_{max}, IQR: 0.105 - 0.168 G/G_{max}, n = 26; mGluR1α: median: 0.107 G/G_{max}, IQR: 0.081 - 0.138 G/G_{max}, n = 61, unidentified target: median: 0.114 G/G_{max}, IQR: 0.084 - 0.145 G/G_{max}, n = 605).

To minimize potential errors caused by slightly different dye concentrations in distinct PCs, I calculated peak amplitude ratios with two other methods. First, I restricted my analyses to cells that contained both PV$^+$ and mGluR1α^+ dendrite-targeting boutons and calculated within-cell ratios and found a similar 1.28 times higher value in boutons targeting PV$^+$ dendrites (n = 10 cells). Second, I normalized the peak amplitude of the [Ca^{2+}] transients in each PV$^+$ and mGluR1α^+ dendrite innervating bouton to the mean of peak amplitudes recorded from all boutons of a given cell and again found a 1.21 times larger value in boutons targeting PV$^+$ dendrites.

Next, I assessed the contribution of the N/P/Q-type Ca^{2+} channels to the [Ca^{2+}] transients. For this, I applied ω-CTX MVIIC (a selective N- and P/Qtype Ca^{2+} channel blocker) in a concentration (1 μM) that almost completely abolished the evoked EPSCs in both INs in the stratum oriens of the CA3 area. The toxin reduced the peak amplitudes of the presynaptic [Ca^{2+}] transients by 47% (n = 296 boutons in n = 13 cells). The extent of the block was similar in PV-innervating boutons (45 ± 10%; n = 12) and in mGluR1α-innervating boutons (46 ±18%; n = 19; p > 0.05, MW U test). These results demonstrate that N/P/Q-type Ca^{2+} channels contribute similarly to the [Ca^{2+}] transients in the two bouton populations.

A larger [Ca^{2+}] transient might be the consequence of lower Ca^{2+} buffering, a smaller bouton volume, or a larger amount of Ca^{2+} entering the bouton. The high fluorescent dye concentration (300 μM) used in the previous experiments might dominate the decay, masking potential differences in the fixed buffer concentration. To circumvent this problem,

I recorded [Ca^{2+}] transients with 100 μM Fluo5F and analyzed their decay times. The [Ca^{2+}] transients recorded with lower dye concentrations displayed a substantially faster decay (300 μM: 463 ms, n = 691 boutons; 100 μM: 210 ms, n = 439 boutons; fitted to the first 260 ms of the averaged traces). When I compared the decay time constants of [Ca^{2+}] transients in boutons innervating PV^+ or mGluR1α^+ dendrites, I found no significant difference (PV: median: 277 ms, IQR: 212 - 403 ms, n = 18; mGluR1α: median: 259 ms, IQR: 207 - 322 ms, n = 35; $p < 0.61$,MW U test), arguing against a robust difference in Ca^{2+} buffering capacity.

To test for potential differences in bouton volumes, Noémi Holderith performed 3D EM reconstructions of PV^+ or mGluR1α^+ dendritic segments and their presynaptic axon terminals. Her experiments revealed no significant difference in the volume of the boutons innervating these IN types, demonstrating that distinct bouton volumes are not the main cause of the observed differences in peak [Ca^{2+}]. She also measured the total AZ areas in the 3D reconstructed terminals and found significantly smaller AZs in PV^+ dendrite-innervating ones. Her bouton volume and active zone measurements, and our [Ca^{2+}] imaging experiments allowed the calculation of the total Ca^{2+} that enters upon an AP (peak [Ca^{2+}] * volume). Assuming that this Ca^{2+} enters through Ca^{2+} channels located in the AZs, we could calculate the functional Ca^{2+}channel density (i.e. total amount of Ca^{2+} that enters the boutons through a unit AZ area). These two combined functional- structural approaches indicated a 1.7 - 1.9 times larger functional Ca^{2+} channel density for boutons innervating PV^+ INs compared to the mGluR1α^+ dendrite-targeting ones.

4.2. PART II. Improvement of spike inference accuracy of GCaMP6f fluorescent transients in hippocampal pyramidal cells

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

I obtained sparse expression of GCaMP6f in dorsal hippocampal CA1 PCs by injecting the mixture of highly diluted Cre-recombinase-expressing AAV vectors and concentrated flexed GCaMP6f-containing AAVs. Three weeks after the virus injection, I implanted a chronic imaging window above the dorsal hippocampus to provide optical access for recording neuronal activity in a head-fixed, lightly anaesthetized mice. 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) in my imaged cells, similar to that published previously. 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 and bursts of APs, different numbers of APs within bursts, and the different degrees of nonlinearity of the GenEncCaInds.

To address the contribution of these parameters, I recorded single AP-evoked unitary [Ca^{2+}] transients in acute hippocampal slices. The mean peak amplitude of [Ca^{2+}] transients evoked by a single AP (0.2 ± 0.2 ΔF/F, median = 0.14, IQR: 0.06 - 0.27, n = 121 cells) was similar to that described earlier, with profound cell-to-cell variability (CV = 0.96).

The signal to noise ratio 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 ($\rho = 0.88$, $p < 0.01$; Spearman correlation).

4.2.2. Variability in the GCaMP6f expression level

Virally expressed proteins, including GCaMP6f are known to demonstrate variable expression levels. I 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. I 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). To provide unequivocal evidence that the intrinsic fluorescence of GCaMP6f in fixed tissue is indeed proportional to the amount of GCaMP6f protein, Katalin Szigeti immunolabeled perfusion fixed tissue with an anti-GFP antibody and examined its relationship with the native GCaMP6f intensity. The almost perfect positive correlation ($\rho = 0.97$, $p < 0.01$; Spearman correlation) between the anti-GFP immunoreactivity and intrinsic GCaMP6f fluorescence after fixation indicates that the latter is an excellent measure of GCaMP6f protein level.

4.2.3. Variability 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, I asked whether these parameters show any correlation. Despite the lack of intracellular labels (e.g. biocytin or fluorescent dyes), I could unequivocally identify 43 of the cells. I found a significant negative correlation between the peak amplitude of unitary [Ca^{2+}] transients and the GCaMP6f expression level (n = 43, $\rho = -0.69$, $p < 0.01$; Spearman correlation), with highly expressing cells showing very small fluorescence changes upon an AP. When selecting cells with similarly low native GCaMP6f intensities (the lowest 65% of the cells) the peak amplitudes were still highly variable (CV = 0.56, n = 26) and no significant correlation was found ($\rho = -0.22$, $p = 0.28$; Spearman correlation. The amount of GCaMP6f also correlates negatively with the signal to noise ratio ($\rho = -0.58$, $p < 0.01$; Spearman correlation), but it is also highly variable (IQR: 10.5 - 19.3, CV = 0.6, n = 25) for cells with low intensities.

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 (VGCC) during an AP is strongly dependent on the AP waveform(Sabatini and Regehr, 1999; Geiger and Jonas, 2000)(Sabatini and Regehr, 1999; Geiger and Jonas, 2000)(Sabatini and Regehr, 1999; Geiger and Jonas, 2000)(Sabatini and Regehr, 1999; Geiger and Jonas, 2000), I measured AP width. The AP width does not correlate significantly with the peak amplitude of unitary [Ca^{2+}] transients ($\rho = 0.20$, $p = 0.17$; Spearman correlation). To assess the effect

of potential differences in endogenous buffers such as calbindin, which is expressed in superficial PCs, I grouped the recorded cells based on their somatic location in the PC layer as deep, middle and superficial cells. I 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). The decay time constants of these subgroups was also very similar ($p = 0.35$, Kruskal-Wallis) arguing against considerable differences in endogenous Ca^{2+} buffering. The peak amplitude of the unitary [Ca^{2+}] transients of the subselected cells also did not correlate with the surface to volume ratio ($\rho = 0.07$, $p < 0.78$; Spearman correlation).

Finally, I tested cell-to-cell variability in the amplitude of unitary [Ca^{2+}] transients in CA1 PCs using a synthetic Ca^{2+} dye, which is known to report physiologically relevant [Ca^{2+}] linearly. I 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 ($p = 0.013$, Two-Sample Kolmogorov Smirnoff test), suggesting that the variability in the amplitude of GCaMP6f [Ca^{2+}] transients is not the consequence of biological variability in peak [Ca^{2+}].

4.2.4. 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, I evoked trains of APs at different frequencies. I 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 supralinear at every tested frequency and showed frequency dependence. In contrast, the summation of [Ca^{2+}] transients measured with Fluo5F was quasi linear, 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), or with GCaMP6f expression level ($\rho = -0.33$, $p = 0.09$; Spearman correlation). The correlation between the linearity of [Ca^{2+}] transients evoked by 5APs at 50 Hz and the peak amplitude of unitary [Ca^{2+}] transients was also not significant ($\rho = 0.3$, $p = 0.06$; Spearman correlation), but the correlation between the linearity at 50 Hz and the GCaMP6f expression level reached significance ($\rho = -0.46$, $p = 0.006$; Spearman correlation).

Interestingly, I observed a dramatic increase in the peak amplitude of the unitary [Ca^{2+}] transients, when they followed high frequency bursts by several seconds. To quantitatively describe this phenomenon, I applied a burst recovery protocol consisting of a 10 AP 50 Hz burst and single recovery pulses at different time points (2.5, 4, 6, 8, 10 or 15 s). 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) that of the control transient (before

each burst) and returned to its initial value >10 second later. Fitting a monoexponential to the normalized recovery of $[Ca^{2+}]$ transients yielded a time constant of 3.95 s. 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 burst, indicating that the aforementioned phenomena is likely due to the nonlinear nature of GCaMP6f.

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, I 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 (CV = 0.95, n = 35). The large variance (CV = 0.73) was also present among cells with low GCaMP6f expression levels (200 - 350 AU, n = 15).

4.2.5. Cell-to-cell variability of the peak amplitude of unitary $[Ca^{2+}]$ transients underlies spike inference error rate

To infer APs from fluorescent $[Ca^{2+}]$ transients, we adopted the method (MLspike) of Deneux et al. with my collaborator Miklós Szoboszlay. We set the tolerance window of spike matching to 60 ms (i.e. an inferred spike should be within a 60 ms time window of an original 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 (low frequency, large amplitude baseline fluctuations), nonlinear feature of the Ca^{2+} sensor (pnonlin; saturation γ, Hill exponent n or polynomial coefficient), the peak amplitude and the decay of the unitary $[Ca^{2+}]$ transients. 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).

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. I applied 4 different fitting scenarios to investigate the parameters that critically influence the error rate (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. Similarly, low ERs were obtained when the mean decay time constant was used at frequencies <10 Hz. However, when the mean peak amplitude was used, the ER was substantially higher at all frequencies, similar to the scenario when all parameters used for fitting were the mean 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%; Three-Way ANOVA: $p < 0.01$ for scenarios; $p > 0.05$ for frequency; $p > 0.05$ for scenarios*frequency interaction).

My analysis also revealed that the ER (4th scenario) and GCaMP6f intensity show significant positive correlations at all frequencies. The ERs were found to be significantly smaller for weakly expressing cells at 0.1 Hz and 1 Hz (Three-Way ANOVA: $p < 0.01$ for intensity; $p < 0.01$ 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: $p < 0.01$, 1 Hz: $p < 0.01$, 10 Hz: $p > 0.05$). 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, my data also provides clear evidence that the ER for weakly GCaMP6f expressing cells is significantly lower than that for the strongly expressing ones.

4.2.6. Reduction of spike inference error by fitting with the estimated peak amplitudes of unitary [Ca^{2+}] transients

In the final set of simulations, I generated [Ca^{2+}] fluorescent traces using the amplitude and decay of unitary [Ca^{2+}] transients recorded in 20 PCs that weakly express GCaMP6f, their respective *baseline drift*, p_{nonlin} parameters, noise and the spike timings of randomly selected 20 CA1 PCs recorded from behaving animals using tetrodes (obtained from Grosmark, A.D., Long J. and Buzsáki, G; CRCNS.org; http://dx.doi.org/10.6080/K0862DC5).

Because our previous simulations revealed that the amplitude of unitary [Ca^{2+}] transients is the key parameter in determining the ER, I 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 I 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, I selected APs, which were proceeded by at least a 4 second silent period. 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, I also calculated the percentage of such bursts. I found that among such temporally separated events, 77.6 ± 8.4 % were single APs with a minimum of 63.6%, and the remaining ones were bursts of APs. Thus, I argued that if I 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. Then, I detected [Ca^{2+}] transients in all 20 synthetic fluorescent traces, from which I selected such temporally segregated events and calculated the mean of the amplitudes of the smallest 2/3 of them. The ratio of the '*presumed unitary*' and the real amplitude of the [Ca^{2+}] transients was 1.01 ± 0.05 (median = 1, IQR: 0.98 - 1.02).

In a second method, I selected those [Ca^{2+}] transients that were inferred by MLspike as single AP-evoked ones in a scenario where mean amplitude, decay time constant, p_{nonlin} and *baseline drift* parameters of the 20 PCs (only weakly GCaMP6f expressing cells) were used for fitting. I only included such *detected unitary* events in our analysis if they were temporally segregated from other events (>4s before and >1s after). The ratio of '*detected unitary*' and real peak amplitude was 1.08 ± 0.17 (median = 1, IQR: 0.97 - 1.16, max = 1.51).

Next, I hypothesised that by using the reasonably accurately estimated peak amplitudes ('*presumed unitary*' and '*detected unitary*') of each cell, the mean decay time constant (388 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. Indeed, the ERs was significantly reduced ($p = 0.01$, Friedman ANOVA test) from 15.0 ± 15.2 % to 6.3 ± 4.9 % when the '*presumed unitary*' amplitude (Paired Sample Wilcoxon Signed Rank *post hoc* test: $p < 0.01$) and to 6.4 ± 4.8 % when the '*detected unitary*' amplitude was used for fitting (Paired Sample Wilcoxon Signed Rank *post hoc* test: $p < 0.01$). The ER did not show significant correlation with the estimated unitary peak amplitudes in either of the scenarios. These results further support the key role in the variability of 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.

5. CONCLUSIONS

In my dissertation I investigated the mechanism underlying distinct target cell-type dependent P_r and short-term plasticity. First, I determined the short-term plasticity of CA3 PC-to-PV^+ or -mGluR1α^+ INs. My measurements of EPSCs, evoked by extracellular stimulation of CA3 PC axon collaterals demonstrated different (CA3 PC-to PV IN: depressing, CA3 PC-to mGlur1α IN: facilitating) short-term synaptic properties at these synapses, indicating a different initial P_r. I also found that the coexpression of Elfn1 in mGlur1α^+ INs will likely impose facilitating short-term plasticity of CA3 PC-to- mGlur1α^+ IN synapses. My results provided me the use of these molecules (PV and mGlur1α) in this brain region to label postsynaptic compartments of high, respectively low Pr synapses. Next, my [Ca^{2+}] imaging experiments of CA3 PC local axon terminals showed that the [Ca^{2+}] influx upon a single AP is significantly larger in case of high P_r synapses. With the use of ω-CTX MVIIC, a selective N- and P/Q- type Ca^{2+} channel blocker I also provide evidence for a similar contribution of P/Q, N type Ca^{2+} channels to the [Ca^{2+}] influx in these two populations of synapses. Finally, by repeating my [Ca^{2+}] imaging experiments with a lower concentration of the Ca^{2+} sensitive dye, I managed to demonstrate the lack of difference between decay time constants in the high, respectively low P_r bouton populations, indicating similar [Ca^{2+}] buffering in these two bouton populations. These results, in concert with the parallel

experiments of my colleagues showing a ~twofold higher functional Ca^{2+} channel density in the AZs innervating PV^+ INs compared with those innervating $mGluR1\alpha^+$ ones, but only ~15% difference in the Ca^{2+} channel subunit density; provided evidence that a target cell-type different modulation of $[Ca^{2+}]$ function or different subunit composition are the underlying causes of the different P_r at these synapses (Éltes et al., 2017).

Distinct physiological properties and molecular compositions underlie the capability of the neuronal network to form diverse synaptic connections with distinct efficacy of information transfer. Investigating the role of synaptic diversity in a behavioral context-dependent manner is hampered by the limitations of currently available large-scale recording techniques. Therefore, in the second part of my dissertation, I searched for the potential causes of these limitations in case of the most widespread, large-scale optical recording technique, Ca^{2+} imaging using GCaMP6f.

I demonstrate that the variability of peak amplitudes of GCaMP6f $[Ca^{2+}]$ transients recorded *in vivo* is not only the consequence of variable activity but is also due to a substantial cell-to-cell variability in the peak amplitude of unitary $[Ca^{2+}]$ transients. Next, I offer evidence that the expression level of GCaMP6f is highly variable among CA1 PCs, and only partially underlies the variability in the amplitude of single AP-evoked Ca^{2+} transients recorded *in vitro*. Furthermore, I describe the phenomena of unitary Ca^{2+} transients amplitudes' dependence on the preceding firing history of the cell. I also show that GCaMP6f Ca^{2+} transients sum supralinearly and the supralinearity is frequency dependent and varies substantially form cell-to-cell. My parallel control experiments with a synthetic Ca^{2+} dye, that is known to report physiologically relevant $[Ca^{2+}]$ linearly indicate that the observed variability and supralinearity are due to the intrinsic characteristics of GCaMP6f, or the nonlinear characteristic of GCaMP6f amplifies the variance. Our modelling study, performed with the help of my colleague, Miklós Szoboszlay, revealed that the main source of spike inference error is the variability in the peak amplitude, and not in the decay or supralinearity. Finally, I developed a model dependent and an independent procedure to estimate the peak amplitudes of unitary $[Ca^{2+}]$ transients and I demonstrate reliable (mean ER ~5%) spike inference with MLspike using these unitary amplitude estimates in weakly GCaMP6f expressing cells (Éltes et al., 2019)

6. LIST OF PUBLICATIONS:

6.1. Publications that formed the basis of the dissertation:

1. **Éltes T***, Kirizs T*, Nusser Z, Holderith N. (2017) Target cell type-dependent differences in Ca2+ channel function underlie distinct release probabilities at hippocampal glutamatergic terminals. Journal of Neuroscience, 37: 1910–19242. IF: 5.971.

2. **Éltes T**, Szoboszlay M, Kerti-Szigeti K, Nusser Z. (2019): Improved spike inference accuracy by estimating the peak amplitude of unitary [Ca2+] transients in weakly GCaMP6f expressing hippocampal CA1 pyramidal cells. The Journal of Physiology, JP277681 in press, doi.org/10.1113/JP277681, IF:4.540.

Other publications:

1. Unal G, Crump MG, Viney TJ, **Éltes T**, Katona L, Klausberger T, Somogyi P. (2018) Spatio-temporal specialization of GABAergic septo-hippocampal neurons for rhythmic network activity, Brain Structure and Function, doi: 10.1007/s00429-018-1626-0; IF: 4.231.

2. Orbán-Kis K, **Szabadi T**, Szilágyi T. (2015) The loss of Ivy cells and the hippocampal input modulatory O-LM cells contribute to the emergence of hyperexcitability in the hippocampus, Rom J Morphol Embryol, 56(1):155–161; IF: 0.811.

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