Final report

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Synaptic and dendritic mechanisms underlying information coding by hippocampal CA3 pyramidal neurons

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Short summary of the research

The main goal of our research project was to address the question: how does the place- or context-dependent activity of CA3 pyramidal neurons (CA3PNs) depend on their synaptic inputs and their dendritic excitability? We aimed to elucidate the contribution of regenerative dendritic spikes and mossy fibre inputs (from the dentate gyrus) to spatial selectivity of CA3PNs.

Our original work plan was to use *in vivo* somatic patch-clamp recordings from individual CA3PNs in awake head-fixed mice during spatial navigation on a treadmill. This approach turned out to be very challenging, because successful experiments needed to fulfill multiple criteria: they had to be 1) high quality, 2) sufficiently long duration, 3) from identified CA3PNs that 4) have clear spatial activity profile 5) during high-performance behavior. To optimize our methods to achieve this goal, we developed an advanced virtual behavioral setup and made extensive efforts to improve recording stability and quality. This work and the results are detailed below in **Section I** of the report. Yet, after persistent efforts, we had to conclude that the above combined challenges make these experiments so difficult that it is not realistic to collect sufficient amount of data to make statistically supported conclusions.

In parallel we performed *in vitro* dendritic and somatic patch-clamp recordings combined with two-photon Ca^{2+} imaging in acute slices of adult rats and mice, in order to better understand the forms of regenerative dendritic activity and consequential firing modes in CA3PNs. These experiments revealed i) unexpected novel qualities of CA3PN dendrites, and ii) a large degree of heterogeneity among CA3PNs, partly related to anatomical properties. I will describe these results in detail in **Section II**. The findings made it clear that somatic recordings can provide only limited information about dendritic activity and its effects on action potential (AP) output.

Therefore, as an alternative to *in vivo* electrophysiology, we employed *in vivo* two-photon Ca^{2+} imaging in CA3PNs during virtual spatial navigation. This approach allowed us to simultaneously measure Ca^{2+} signals (as a proxy of activity) in both the soma as well as dendritic segments of individual CA3PNs expressing the genetically encoded Ca^{2+} sensors GCaMP6f or GCaMP8s. Our experiments so far demonstrate frequent strong somatic activity events that are accompanied by widespread large Ca2+ signals in dendrites, indicating prominent regenerative dendritic activity. I will summarize the results of these ongoing experiments in **Section III**.

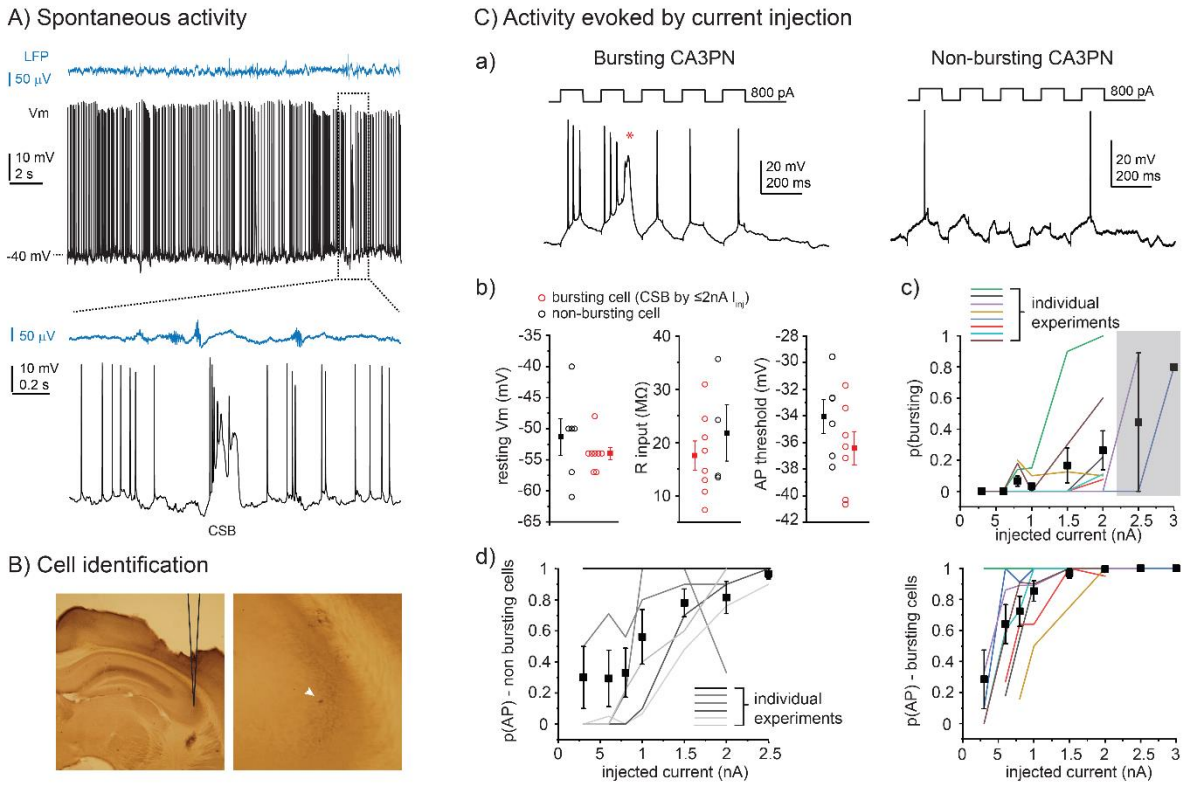
I. *In vivo* electrophysiology experiments

I.1. Recordings from anaesthetized mice

We began our work with the task to optimize the *in vivo* patch-clamp recording technique in hippocampal CA3PNs in anaesthetized mice. We have successfully achieved good quality intracellular current-clamp recordings from >30 neurons. These cells had resting membrane potential below -40 mV (typically ~-50-55 mV) and fired overshooting action potentials. As anticipated, the main challenges proved to be 1) obtaining acceptably low access resistance (on average ~40 MOhm in good recordings, typically 30-80 MOhm), and 2) extending the recording duration (on average ~10 minutes, range: 1.5-25 minutes). In order to better interpret the single cell intracellular signals in the context of network activity, we also implemented the insertion of a second recording pipette to the CA3 region for LFP recordings concurrent with patch-clamp recordings; as expected, we have observed both theta oscillation and sharp wave ripples (Figure 1A).

Several factors indicated that *post hoc* localization and identification of the recorded neuron is particularly important for interpreting our results. First, our *in vitro* experiments revealed that the propensity of CA3PNs to fire dendritic Ca^{2+} spikes and consequential complex spike bursts (CSBs) depends strongly on the topographic position of the neuron within CA3 (see Section II). Second, a few of our *in vivo* recordings, where we could find the biocytin-loaded neuron, proved to be located outside of CA3 (in CA2 or in adjacent tissue). After testing different immunolabeling protocols, we were able to optimize a pipeline where we could identify ~40% of the recorded cells (Figure 1B). However, survival of the cell can only be expected when detachment of the pipette does not lead to membrane destruction, and therefore cells that were suddenly lost during recording could typically not be recovered in *post hoc* histology.

In several of the recorded neurons we observed spontaneous occurrence of large, long duration depolarizations following action potentials, either in isolation or driving a complex spike burst of progressively smaller and wider spikes (Figure 1A). Our *in vitro* results strongly suggested that these voltage responses are generated by dendritic Ca^{2+} spikes. In 14 histologically identified CA3PNs that were recorded in anaesthetized mice, we tested whether CSB propensity in mice *in vivo* shows similar cell-to-cell heterogeneity as we found in acute slices from rats (see Section II). Applying the same depolarizing current injection protocol as *in vitro*, we found (Figure 1C) that a subset (57%) of CA3 pyramidal cells expressed CSBs upon somatic depolarization, whereas 43% fired only simple APs even to ≥2 nA current injection (regular spiking cells). In CSB cells, the CSB probability increased with stronger current injection. Basic electrophysiological properties (resting membrane potential, resting input resistance and action potential threshold) were not different between CSB cells and regular spiking cells. However, the regular spiking cells appeared to have lower excitability, as the probability of action potential firing increased slower with increasing current injections than that in CSB cells. Thus, our results indicated that the heterogeneity of CSB propensity among CA3 pyramidal cells is robust across different rodent species and experimental conditions.



<u>Figure 1.</u> In vivo patch-clamp recordings in anaesthetized mice. A) Spontaneous activity in an example recorded CA3PN. Bottom panel shows a spontaneous CSB magnified. B) Post hoc identification of CA3PNs. C) Testing bursting by somatic I_{inj} in histologically identified CA3PNs. a) Example bursting and non-bursting cells. b) Basic electrophysiological properties of the two groups. c) Summary of burst (top) and AP (bottom) probability in bursting cells. d) AP probability in non-bursting cells.

I.2. Spatial navigation in virtual environments

Because of the limitations of recording duration, and in order to potentially test place coding in multiple environments with different levels of novelty in an individual cell, we set up a virtual reality (VR) navigation system instead of a simple treadmill, and developed a new LabView-based custom control software for the VR system (Figure 2A). We designed and optimized a training protocol for mice for a spatial context discrimination task using two different virtual environments with different wall patterns and water reward locations (Figure 2B). After optimizing the training, mice can learn the task in ~ one week, as assessed by change in running speed and anticipatory lick responses at the specific reward locations in the two environments (Figure 2C).

We also developed the custom analysis pipelines necessary for processing and evaluating the recorded electrophysiology data, including statistical evaluation of behavioral parameters and neuronal selectivity (i.e. place cells) using bootstrapping statistics.

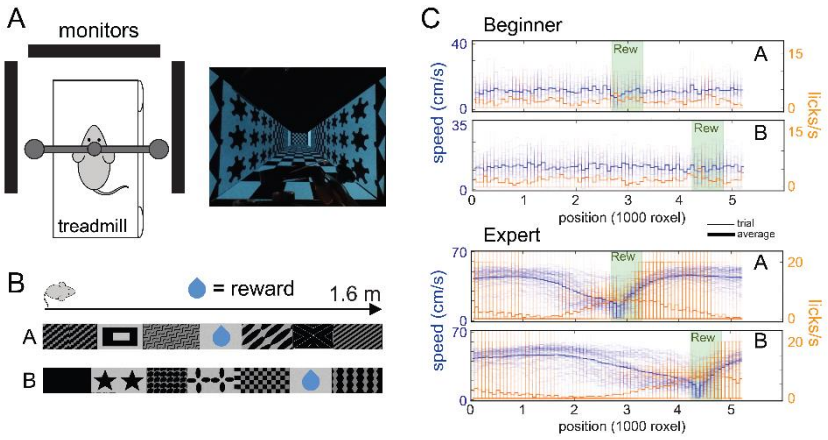


Figure 2. Spatial navigation in virtual environments (VEs). A) Design of the setup. B) Task: navigation to different reward zones in two visually different VEs. C) Behavioral parameters (running speed and lick rate) indicate improvement of performance with training (example mouse).

I.3. Recordings in awake behaving mice

Obtaining high-quality patch-clamp recordings of sufficient duration in awake behaving mice performing the task correctly proved to be very difficult despite all our persistent and hard efforts. We took several measures to overcome the technical challenges. 1) Since recordings were often lost early due to hunching or sudden movement of the animals, we optimized the behavioral training protocol so that mice were not stressed and moved smoothly during the experiment. 2) We developed an optimized head-post that provided much better head fixation stability than our previous design. 3) We also tried several approaches to increase the mechanical stability of the pipette attached to the cell. However, even when technical conditions seemed to be optimal, the recording duration we achieved during spatially guided behavior in VR-trained mice remained short (typically only a few tens of seconds, sometimes a few minutes). In addition, the animals remained engaged to the task for ~20-25 minutes, so the recordings had to be made during this time frame. In the few experiments of sufficient quality and duration, spatially tuned activity of the recorded CA3PCs in the virtual environments could not be confirmed by statistical comparison to shuffled data (Figure 3).

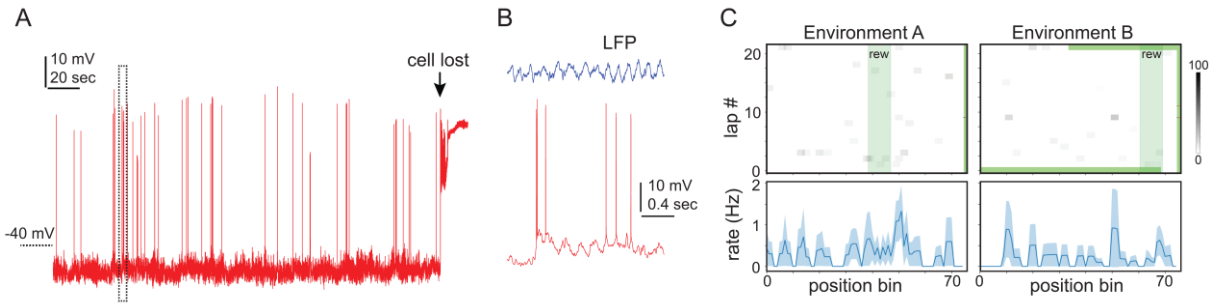


Figure 3. Example experiment in an awake behaving mouse. A) In vivo patch-clamp recording from a putative CA3PN lasting for ~4 minutes. B) The segment in the dashed box in A is shown on higher temporal scale. C) Lap-by-lap activity (top) and average AP rate (bottom, mean ± SD) of the cell in A-B in the two VEs. No significant spatial tuning of this cell was observed in any of the two environments.

As an alternative method to assess the prevalence of spatially tuned CA3 pyramidal neurons, we conducted loose cell-attached recordings and detected action potentials extracellularly. This method yielded longer recordings, however, we still did not observe spatially modulated activity in any of the CA3PNs recorded (n=12).

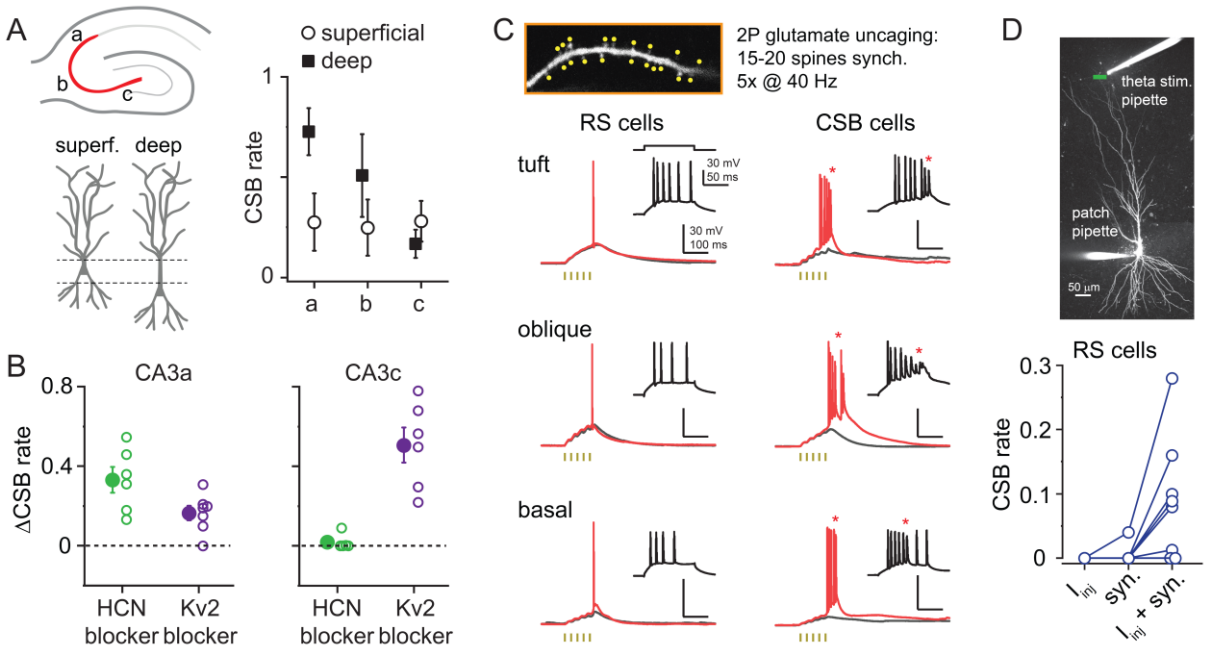
II. *In vitro* experiments in CA3PN in acute slices

II.1. Diverse dendritic Ca^{2+} spike forms in CA3PNs

At the beginning of our project, still relatively little was known about the active properties and synaptic integration in dendrites of CA3PCs compared to other cortical PN types. To better understand the active integrative properties of CA3PN dendrites and the types of AP outputs they produce, we used patch-clamp electrophysiology combined with two-photon (2P) Ca^{2+} imaging in rat CA3PCs in acute slices. In particular, we were interested in dendritic Ca^{2+} spikes and consequently evoked somatic CSBs in these neurons, because these events have recently been shown to contribute to place coding in CA1PNs, but they have not yet been investigated in detail in CA3PNs.

First, we performed somatic recordings, and observed a remarkable heterogeneity in CA3PCs in their ability to generate CSBs by somatic current injection or synaptic stimulation. A large fraction of CA3PNs produced CSBs even to mild stimuli, whereas another population of CA3PCs did not produce CSBs even by large depolarization (regular spiking or RS cells), and a minority of neurons showed an intermediate phenotype. We demonstrated that CSBs were generated by dendritic Ca^{2+} spikes (preliminary data for these results were included in Fig. 1 of the original proposal).

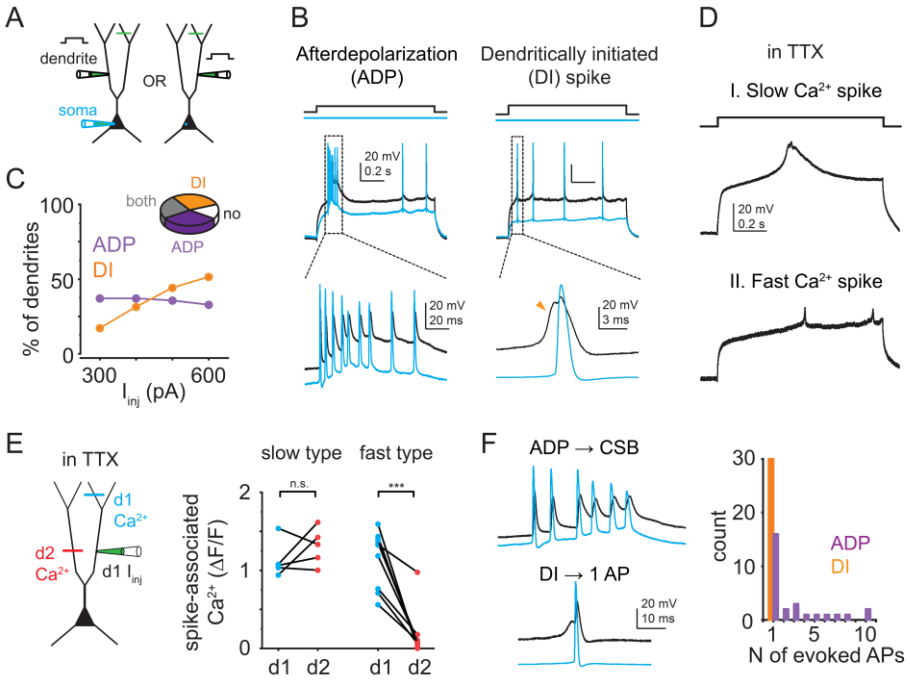
Looking into the reasons behind this heterogeneity, we discovered that the propensity of CA3PNs to produce CSBs depends on the topographical location of the cell within the CA3 region, so that cells in distal CA3a-b and in deeper layers of str. pyramidale had the highest CSB propensity (Figure 4A). Investigating the active dendritic components of this regulation using pharmacological manipulations, we pinpointed two dendritic ion channel types (HCN and Kv2), whose prominent proximodistal subregion-specific activity modulates CSB generation (Figure 4B). Using various synaptic stimulation approaches (2P glutamate uncaging and electrical stimulation) to elucidate the synaptic input-output transformation conveyed by CSBs, we revealed that the I-O transformation rules triggering CSBs are not uniform: in regular spiking cells CSBs (if evoked) require conjunction of proximal and distal apical synaptic inputs, whereas in CSB cells they can be triggered by any input type clustered in any individual branch (Figure 4C). These results, which were **published in Nature Communications** (**Raus Balind et al., 2019**), suggested versatile, location-dependent properties and input-output functions by dendritic Ca^{2+} spikes in CA3PNs.



<u>Figure 4.</u> A) Topographic heterogeneity of CSB propensity in CA3PNs. B) Subregion specific regulation of CSB propensity by HCN and Kv2 ion channels. C) Local synaptic stimulation (by 2PGU) in different dendritic domains uniformly evokes either regular spiking or CSB at the soma of individual CA3PNs. D) Pairing distal synaptic stimuli with somatic I_{inj} could evoke CSBs in some of the RS cells.

To better elucidate the differences in dendritic spike mechanisms supporting these variable response properties, we next performed dendritic and dual dendrite-soma recordings. Our hypothesis was that the threshold of Ca^{2+} spikes is heterogeneous among PNs, leading to heterogeneity in their capacity to produce CSBs. However, instead we discovered a variety of different Ca^{2+} spike mechanisms in CA3PC dendrites (Figure 5A), which we classified into two major groups: 1) afterdepolarizations (ADPs) following action potentials (APs), and 2) fast spikes generated independently from APs („dendritically initiated (DI) spikes"). Dissecting these components revealed that they are mediated by two types of regenerative mechanisms: a) slow global Ca^{2+} spikes and b) fast compartmentalized Ca^{2+} spikes, which can recruit additional Na^{+} channel activation to create a novel, hybrid form of d-spike. Importantly, the different d-spike types had opposing effect on somatic output: while ADPs evoked CSBs, the novel DI spikes evoked strictly single APs. *Thus, our results disproved the preexisting postulate that dendritic Ca^{2+} spikes generally evoke burst output, and revealed that a specific novel Ca^{2+} spike type can instead directly promote regular spiking phenotype.*

We also uncovered a close relationship between the dominant Ca^{2+} spike form and certain morphological traits of the dendritic tree. Furthermore, the Ca^{2+} spike forms we found in CA3PNs were also distinct from that of CA1PNs. Curiously, the novel fast Ca^{2+} spike we discovered in CA3PC dendrites is similar to a recently described d-spike form in human cortical L2/3 pyramidal neurons (Gidon et al, 2020, Science). The human study proposed that these spikes may endow cortical neurons with human-specific computational abilities, but our results in rat cells refuted this idea. The results on the novel dendritic Ca^{2+} spike forms in CA3PNs have been **published in eLife** (**Magó, Kis et al., 2021**).



<u>Figure 5.</u> A) Recording configuration. B) Two types of regenerative Ca^{2+} spikes evoked by Iinj into the dendrites of CA3PNs. C) Propensity of the two types of Ca^{2+} spikes (n=70 dendrites). D) Two types of Ca^{2+} spikes after blockade of Na^{+} channels by tetrodotoxin (TTX).E) Slow Ca^{2+} spikes propagate globally, whereas fast Ca^{2+} spikes are compartmentalized to individual dendritic families. F) The two types of Ca^{2+} spikes evoke opposing forms of AP output at the soma.

In further experiments we examined the kinetic varieties of compound Ca^{2+} spikes as measured at the soma, resulting from the summation of the various Ca^{2+} d-spikes. We could distinguish three somatic forms of Ca^{2+} spikes (fast, slow and mixed). The somatic Ca^{2+} spike phenotypes again showed a clear topographic organization within CA3, similar to that of CSB propensity. To better understand the mechanisms behind the heterogeneity, we next investigated the contribution of various Ca^{2+} and K^{+} channels to shaping the kinetics of Ca^{2+} spikes. We found that L-type voltage-gated Ca^{2+} channels and I_A and I_M type voltage-gated K^{+} channels play the most prominent roles. Finally, we also discovered that the Ca^{2+} spike and CSB properties are robustly regulated by cholinergic activity that can convert fast spikes into the slow, burst-inducing form, indicating that the dendritically driven output form also depends on neuromodulatory state. **A manuscript is in preparation** for publication of these results.

Altogether our *in vitro* results revealed that the active dendritic properties of CA3PNs are unique and more diverse than initially thought, and that distinct forms of regenerative dendritic Ca^{2+} spikes can lead to opposite forms of action potential output, such as single spikes versus long bursts. The results underscored that, without measuring local activity in the dendrites, we cannot get a complete understanding of the impact of dendritic spikes on somatic activity of CA3PNs *in vivo*.

III. *In vivo* Ca^{2+} imaging

The difficulties experienced with in vivo patch-clamp and the new insights provided by our in vitro results prompted us to change strategy and explore alternative techniques to address the main questions of the research program. We have set up an in vivo two-photon imaging system to investigate the somatic and dendritic activity in CA3PNs, employing the same behavioral task. There are several advantages provided by switching to this method: 1) we can record activity (via Ca^{2+} signals reported by genetically encoded Ca^{2+} indicators such as GCaMP) not only from the soma but also locally in multiple dendrites of the same neuron; 2) we can unambiguously identify CA3PNs as well as visualize their morphology; and 3) we can record from multiple cells at the same time for long durations and even across days. On the down-side, Ca^{2+} imaging has poorer temporal resolution, is less informative about subthreshold signals, and can be primarily used in the more superficial dorsal and distal subregions of CA3.

Mice were injected in the dorsal CA3 area with AAVs to express Cre-recombinase and Cre-dependent GCaMP (6f or 8s) Ca^{2+} sensor in sparse sets of CA3PNs. The animals were then implanted with a glass-bottomed stainless steel cannula placed above the left dorsal hippocampal CA3 area with the overlying cortex removed. We obtained adequate optical access to somata and dendrites of labelled CA3PCs at ~300-500 micrometer depth (Figure 6A-D).

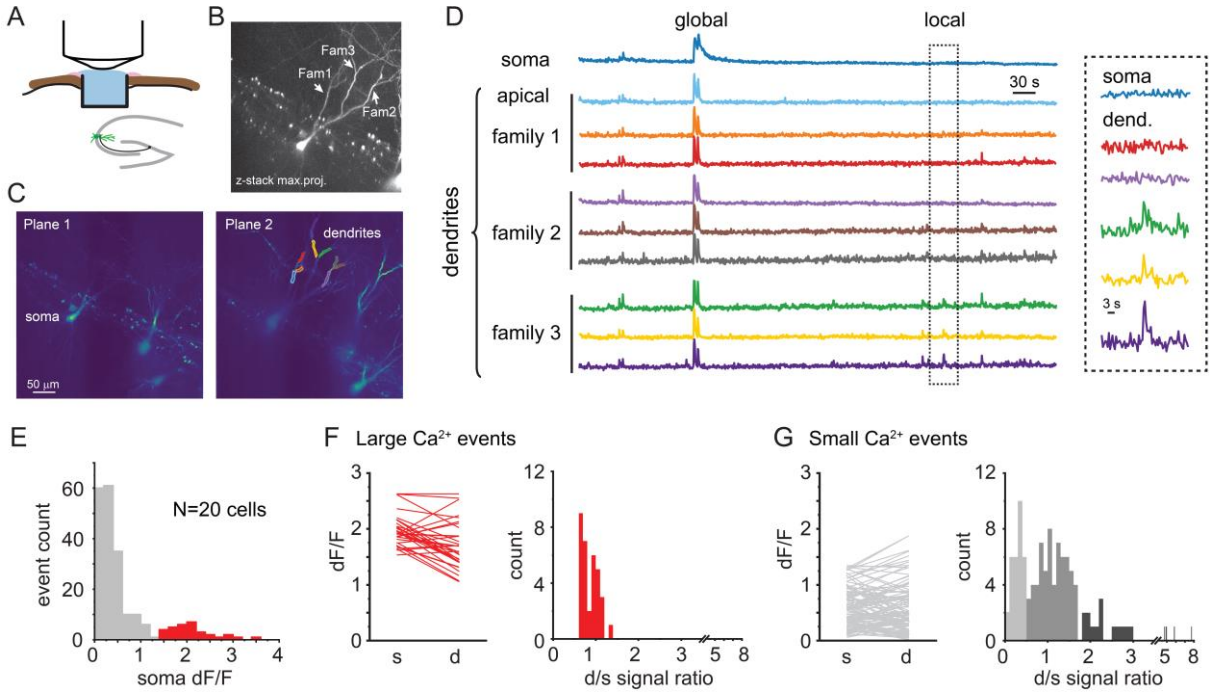


Figure 6. In vivo imaging of CA3PNs. A) Optical window design. B) 2P z-stack of an example GCaMP8s-expressing CA3PN with three apical dendritic families. C) Imaging of the cell in B in two planes (soma and dendrites). D) Ca^{2+} signals from the cell in B-C. Note global and local events (a local event is enlarged in dashed box). E) Histogram of somatic Ca^{2+} signal amplitudes from 20 CA3PNs reveals two groups of somatic events. F) Large somatic Ca^{2+} events (s) are accompanied by similarly large Ca^{2+} signals in dendrites (d, 150-250 µm from soma). G) Small Ca^{2+} events are heterogeneous and can be larger in the dendritic or somatic compartment.

Our preliminary analysis of somatic Ca^{2+} activity in GCaMP8s-labelled CA3PNs revealed that Ca^{2+} signals can be grouped into large and small events based on their amplitude (Figure 6E). Large somatic Ca^{2+} events are always accompanied by widespread large dendritic Ca^{2+} signals (Figure 6F), suggesting slow dendritic Ca^{2+} spikes and CSBs (consistent with *in vitro* measured Ca^{2+} profiles). Small events are characterized by variable dendrite/soma amplitude ratio (Figure 6G) and more spatial heterogeneity, suggesting that some of these events are initiated at the soma and backpropagate to dendrites, while others may originate from the dendrites and forward propagate to the soma. Further experiments and analysis are in progress to collect more data and compare the strength and tuning of dendritic activity in place cells vs. non-place cells. In addition, we confirmed that we can also image Ca^{2+} activity in mossy fibres and recurrent axons. Thus, this new methodical approach - which we are among the firsts to use in the CA3 hippocampal area - allows us unprecedented access to investigate the activity dynamics of diverse subcellular compartments (axons, dendrites and somata) involved in spatial and contextual information coding in this subregion.

IV. Role of dendritic activity in synaptic plasticity

Two other research lines on the relevance of dendritic activity in neuronal coding were closely linked with and inspired by the results of the current project. We elucidated how the fine-scale spatial organization of synaptic input in dendrites and their capacity to evoke dendritic spikes relates to their long-term potentiation (**Magó et al., J. Neurosci. 2020**). Developing a novel computational approach, we also showed that plasticity of functional synapse clusters needs local dendritic rather than global activity mechanisms (**Ujfalussy & Makara, Nature Communication, 2020**).

V. Difficulties

As detailed above, during the course of the project we switched our *in vivo* experimental approach from electrophysiology to imaging. The findings in Section II made it imperative to modify the original strategy: even if *in vivo* patch clamping were practical, because of the newly observed properties of the CA3PNs and their dendrites the original research plan with somatic measurements would have not been sufficiently effective to address our question.

In addition to the scientific challenges, the Covid-19 pandemic complicated the progress of our research program in 2020-21. Because of the virus situation we often had to pause experiments or implement working restrictions in the lab to reduce the risk of infection among lab members, slowing the experiments and compromising the workflow. Since the *in vivo* experimental protocols - including surgery and training of animals - take few weeks for each mouse, the experiments were particularly vulnerable even to shorter Covid-related absence of involved lab members. During the down times we progressed with the development of analysis pipelines for the *in vivo* experiments.

VI. Dissemination of results

The results obtained in the project have been presented at several international conferences (GRC Dendrites, EMBO Dendrites, FENS, MITT). Two papers have been already published in prestigious journals (Raus Balind et al., Nature Communications 2019, Magó, Kis et al., eLife 2021). We are currently preparing a manuscript based on the *in vitro* experiments (to be submitted within ~3 months), and we expect that the first results of the *in vivo* Ca^{2+} imaging experiments that are currently in progress will produce a manuscript in ~1-1.5 years.