Diversity and cholinergic regulation of dendritic Ca^{2+} spikes in hippocampal CA3 pyramidal neurons

PhD thesis

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1. Introduction

The hippocampus is a key brain region involved in memory, learning, and spatial navigation. Among its subfields, the CA3 region plays a critical role in memory encoding and retrieval, particularly in pattern completion and autoassociative network functions. However, CA3 is not a homogeneous structure: recent studies have revealed significant heterogeneity along its proximodistal and radial axes, reflected in differences in dendritic architecture, input patterns, and intrinsic firing behaviors. Pyramidal cells (PCs) in CA3 integrate synaptic inputs through their dendritic trees, where voltage-gated ion channels, especially Na^+, K^+, and Ca^{2+} channels play key roles in shaping electrical responses. Among these, dendritic Ca^{2+} spikes are particularly important for triggering plateau potentials and complex spike bursts (CSBs), a hallmark firing pattern in hippocampal neurons. CSBs are associated with learning, spatial navigation, and sharp-wave ripple events. Furthermore, cholinergic modulation from the medial septum fine-tunes excitability and plasticity in CA3, supporting a dynamic balance between memory encoding and retrieval. Understanding how dendritic integration, intrinsic excitability, and neuromodulation interact in CA3 pyramidal cells is essential for revealing the

cellular basis of hippocampal computation and memory processing.

2. Objectives

The aim of this dissertation is to unravel the properties, biophysical mechanisms and functional significance of dendritic Ca^{2+} spikes and CSBs in CA3PCs by addressing the following questions: What are the intrinsic mechanisms of CSB generation in CA3PCs? What are the biophysical properties and roles of dendritic Ca^{2+} spikes in CA3PCs? How heterogeneous are these properties and functions across CA3PCs? How are they regulated by cholinergic neuromodulation?

3. Methods

3.1. Electrophysiology

Acute hippocampal slices were prepared from adult Wistar rats, FVB/AntJ mice and ChAT-Cre/Ai32 transgenic mice according to approved institutional and EU guidelines. Slices (400 μm in rats, 300 μm in mice) were cut from the dorsal hippocampus and maintained in oxygenated ACSF.

Somatic and dendritic whole-cell patch-clamp recordings were performed in CA3 pyramidal cells (CA3PCs)

using standard K-gluconate-based internal solution with fluorescent Ca^{2+} indicators (OGB-1 or OGB-6). Recordings were obtained in submerged chambers at 32–34 °C. Somatic and dendritic Ca^{2+} spikes were induced by current injection (I_{inj}), and their properties were measured under control conditions and during pharmacological manipulations.

3.2. Two-photon imaging and uncaging

Dual galvanometer based two-photon (2P) scanning systems were used for imaging and MNI-glutamate uncaging. Alexa Fluor 594 and OGB dyes were excited at 860–920 nm; uncaging was performed at 720 nm on 20 clustered spines using 0.5 ms pulses, repeated five times at 40 Hz. Uncaging power was adjusted to evoke near-threshold responses for backpropagating action potentials (bAPs) or dendritic spikes.

3.3. Optogenetic stimulation of cholinergic axons

In ChAT-Cre/Ai32 slices, cholinergic fibers were activated by 447 nm laser light (100 ms pulses at 100 ms intervals for 10 s) through a 60X objective. Current injections were applied with or without preceding light stimulation to compare CSB rate and duration at threshold I_{inj}.

3.4. Chemicals

TTX (1 μM) was used to block voltage-gated Na^+ channels (VGNCs). Selective voltage-gated Ca^{2+} channel (VGCC) blockers included TTA-P2 (T-type), SNX-482 (R-type), CTX (N/P/Q-type), and nifedipine/nimodipine (L-type) and nonselective VGCC blocker Ni^{2+}. Selective K^+ channel blockers were also used: iberiotoxin, apamin dendrotoxin-I (DTX), AmmTx3, XE991. Peptide toxins were prepared with 0.1% BSA to prevent tube binding. Carbachol (2 μM) was applied to mimic physiologically relevant cholinergic modulation. Stock solutions were stored at –20 °C and diluted to final concentrations (≥1:1000) in oxygenated ACSF immediately before use.

3.5. Data analysis

CSBs were identified by bursts of ≥2 APs riding on an afterdepolarization (ADP) and accompanied by distal dendritic Ca^{2+} signals. Rate and duration were quantified at threshold I_{inj}. Ca^{2+} spikes in TTX were analyzed for threshold, amplitude, halfwidth, and dV/dt parameters from multiple sweeps.

Ca^{2+} transients were expressed as $\Delta F/F_0$, aligned to spike onset, and measured as the maximal 5-point average after spike initiation relative to baseline.

TTX-isolated Ca^{2+} spikes were clustered using Ward's method on z-score normalized dV/dt range, $\log_{10}$ (halfwidth), and number of peaks.

3.6. Morphological analysis

Dye-filled cells were imaged in 2P z-stacks for measurement of apical trunk length, radial soma position, and proximodistal location. Sholl analysis quantified dendritic complexity. In some experiments, full reconstructions were made with Vaa3D for detailed morphometry.

4. Results

4.1. Diverse CSB generation in hippocampal CA3PCs

To investigate the role of apical dendrites in CSB generation in CA3PCs, we performed somatic patch-clamp recordings combined with Ca^{2+} imaging using OGB-1 or OGB-6 and Alexa Fluor 594. Somatic depolarizing current injections (5 × 100 ms pulses, 300–600 pA) evoked CSBs, a bursts of ≥2 APs on a prolonged ADP (~20–70 ms), accompanied by all-or-none Ca^{2+} responses in the distal apical dendrites of stratum lacunosum-moleculare.

We quantified CSB propensity as the ratio of CSB-evoking pulses and found it significantly higher in CA3PCs than

CA1PCs. Interestingly, heterogeneity was evident among individual CA3PCs, which we categorized into three groups based on their CSB propensity: 1) high bursting cells (CSB_H), which exhibited CSBs at lower I_{inj} thresholds (300 pA); 2) low bursting cells (CSB_L), which required stronger I_{inj} (400–600 pA) for CSB generation, and 3) regular spiking cells (RS), which did not generate CSBs, even at higher I_{inj} levels (no CSB at 600 pA).

To explore anatomical correlates of this heterogeneity, we analyzed CSB rate across CA3 subregions using soma position along the proximodistal (CA3a, b and c) and radial (deep or superficial) axes. Two-way ANOVA revealed a significant increase in CSB rate from CA3c to CA3a ($p = 0.027$) and a trend toward higher CSB rates in deep CA3a cells (interaction $p = 0.025$). The proportion of CSB-expressing cells was also higher in deep CA3a ($p = 0.034$, χ^2 test). These patterns were not fully explained by passive properties like input resistance or membrane potential.

We next examined whether dendritic morphology contributes to CSB propensity. 3D reconstructions and Sholl analysis revealed that CSB_H cells had significantly longer primary apical dendrites than RS cells, particularly in CA3a ($p < 0.001$), consistent with deeper somatic positions. However, other dendritic parameters, such as total length, branching, or

oblique dendrite positioning, showed no clear correlation with CSB phenotype across regions.

To test if bursting cells in deep CA3a-b were “athorny,” as suggested by a previous study, we examined their proximal apical dendrites. All examined CSB_H cells (n = 11) had at least one, and often several, large thorny excrescences in str. lucidum, indicating they were classical thorny CA3PCs. Thus, our findings indicate that the majority of classical thorny CA3PCs in deep distal CA3 exhibit a CSB phenotype.

4.2. Distinct dendritic Ca^{2+} spike forms

To investigate the dendritic electrophysiological mechanisms underlying the diverse firing patterns in CA3PCs, we focused on dendritic Ca^{2+} spikes, which play a central role in the generation of CSBs described earlier. We performed dendritic and dual patch-clamp recordings combined with 2P Ca^{2+} imaging, targeting higher-order apical trunks 165–400 μm from the soma. 1-s-long dendritic depolarization elicited two distinct dendritic Ca^{2+} spike types (beside other regenerative events including bAPs and Na^+ spikes): ADP-type spikes, which followed bAPs and promoted somatic bursts, and dendritically initiated (DI) spikes, which were fast, locally generated, and bAP-independent.

In a broader dataset (n = 69), 85% of dendrites displayed either or both spike types upon dendritic Iinj. Both forms were abolished by 200 μM Ni^{2+}, confirming their dependence on VGCCs.

To test whether these events could be elicited under more physiological conditions, by synaptic stimulation, we used 2PGU to activate clustered spines (n = 10), in the presence of 50 μM D-AP5 to isolate Ca^{2+} spikes. ADP and DI spikes were successfully evoked, closely resembling current injection-evoked events.

To isolate dendritic Ca^{2+} spikes from other regenerative events, we applied 1 μM TTX to block Na^{+} channels. While bAPs and dendritic Na^{+} spikes were abolished, Ca^{2+} spikes persisted and showed distinct kinetics: slow (166.6 ± 26.8 ms) or fast (9.3 ± 0.7 ms), corresponding to ADP or DI types, respectively.

CA3PC spikes were compared to those in CA1PCs (n = 12). Only slow ADP-type spikes were observed in CA1, with higher current thresholds and no DI spikes, further indicating CA3-specific Ca^{2+} spike diversity. TTX-resistant Ca^{2+} spikes in CA1 had intermediate kinetics (~39 ms) and were similarly Ni^{2+}-sensitive.

We next asked whether fast and slow spikes differ in propagation. Using TTX and high-affinity OGB-1, we imaged Ca^{2+} signals at distal and across-branch dendritic sites. Slow spikes (>60 ms) spread efficiently across dendrites and into basal branches, whereas fast spikes (<20 ms) remained compartmentalized ($p < 0.001$), indicating distinct computational roles.

Importantly, at the somatic level, ADP and DI spikes evoked in the patched dendrites evoked distinct output patterns. ADP spikes promoted bursts of APs, whereas DI spikes produced only single somatic action potentials. These findings suggest that the two spike types serve different computational roles within CA3PCs, potentially contributing to diverse synaptic integration and firing behaviors.

4.3. Compound Ca^{2+} spikes

4.3.1. Cell-to-cell heterogeneity of compound dendritic Ca^{2+} spikes

To examine how dendritic Ca^{2+} spikes across multiple branches contribute to somatic activity, we recorded at the soma while blocking Na^{+} channels with TTX and applied long (1 s) depolarizing steps to evoke widespread dendritic Ca^{2+} activity. These stimuli reliably triggered regenerative compound Ca^{2+} spikes in 91.7% of CA3PCs. While the shape of these spikes

remained consistent within individual neurons, substantial cell-to-cell heterogeneity was observed, particularly in spike halfwidths.

Two major spike classes emerged: short-duration (~9.4 ms) and long-lasting (~46.2 ms) events. The latter frequently exhibited complex, multipeak waveforms with repetitive components (10–70 Hz). Both spike types were associated with time-locked dendritic Ca^{2+} signals >300 μm from the soma. Hierarchical clustering analysis (based on the most distinctive spike parameters, including dV/dt_{total}, duration, and number of peaks) revealed three distinct waveform groups: short, simple long, and complex long spikes.

To directly determine the relationship between compound Ca^{2+} spikes and somatic firing pattern, we measured CSB rates and durations under control conditions in ACSF and subsequently in TTX to eliminate APs and isolate Ca^{2+} spikes. Cells with short-duration spikes showed low CSB propensity and mostly regular spiking, whereas cells with long-lasting spikes frequently produced prolonged CSBs. Thus, only CA3PCs capable of generating prolonged compound Ca^{2+} spikes could support sustained depolarizations and CSB activity.

4.3.2. Morpho-topographic mapping of compound Ca^{2+} spike heterogeneity

We observed correlations between topographic location and CSB propensity, as well as between dendritic morphology and dendritic Ca^{2+} spike properties in CA3PCs. We therefore also aimed to investigate comprehensively how compound Ca^{2+} spike phenotypes relate to these anatomical features. By mapping Ca^{2+} spike phenotypes onto the proximo-distal and radial (taking primary dendrite length as a proxy, position of the cells, we discovered a non-uniform distribution of spike types across the CA3 area. Cells with short-duration Ca^{2+} spikes were mainly located in proximal CA3, whereas long-lasting spikes were enriched in distal CA3, particularly in deeper layers associated with longer primary apical dendrites. In distal CA3, short spikes were mostly restricted to PCs with short primary trunks, often heavily decorated with thorny excrescences (TEs).

Morphological analysis showed that cells with short-duration spikes often had multiple apical trunks and more complex proximal arborization, whereas long-spike cells typically had a single primary trunk and simpler apical structure. Sholl analysis confirmed that long-lasting Ca^{2+} spiking cells had fewer intersections near the soma (50–200 μm), indicating reduced dendritic complexity. Compared to earlier results on

CSBs, where only minor morphological differences were found, this analysis revealed stronger correlations between compound Ca^{2+} spike type and dendritic architecture, particularly in primary trunk structure and branching patterns.

In our extensive dataset of 2P-imaged CA3PCs, we consistently observed TEs on proximal dendrites, though at varying densities. We did not encounter any cells completely lacking TEs, even among deep distal CA3PCs, where only sparse TE-like structures were occasionally present. To confirm this finding given the resolution limits of 2P imaging, we performed STED super-resolution imaging on a subset of cells (n = 10), focusing on deep distal CA3PCs. In all cases, well-defined TEs were present, including in distal regions. While TE density was somewhat reduced distally, their consistent presence suggests that CA3PCs across all regions in adult rats receive mossy fiber input.

4.3.3. Ion channels underlying diverse Ca^{2+} spike forms

To identify the mechanisms shaping Ca^{2+} spike kinetics, we investigated the roles of VGCCs and K^+ channels. Pharmacological experiments showed that blocking T-type, R-type, or N/P/Q-type VGCCs produced only modest effects. In contrast, L-type VGCC inhibitors (nimodipine or nifedipine) robustly suppressed both short- and long-duration spikes,

indicating that L-type channels are the primary drivers of dendritic Ca^{2+} spikes in CA3PCs.

We then tested the contribution of K^+ channels in regulating spike duration. Most tested blockers (targeting Kv1, Kv2, and Ca^{2+}-activated K^+ channels) failed to prolong spikes. However, inhibition of Kv4 (A-type) channels using AmmTx3 increased spike halfwidth, suggesting that these channels rapidly repolarize short Ca^{2+} spikes. Similarly, blocking Kv7 (M-type) channels with XE991 prolonged all spike types and often induced repetitive spikelets. Combined inhibition of both A- and M-type channels transformed short spikes into complex, long-lasting events, indicating that these K^+ channels are key regulators of Ca^{2+} spike kinetics.

4.3.4. Cholinergic regulation of Ca^{2+} spike kinetics

Since ACh modulates both VGCCs and A-type and M-type VGKCs, we tested its influence on dendritic Ca^{2+} spikes and CSB firing. Bath application of cholinergic agonist carbachol (CCh, 2 μM) in TTX significantly prolonged Ca^{2+} spike duration, reduced their amplitude and dV/dtmax, and transformed short spikes into long-lasting plateau-like events.

In the absence of TTX, carbachol facilitated CSB generation in response to both somatic I_{inj} and synaptic 2PGU stimulation, even in the presence of NMDAR blockade. The

CSBs triggered by carbachol were eliminated by the L-type VGCC inhibitor nifedipine (10 μM), confirming that L-type VGCCs mediate the observed cholinergic effects on Ca^{2+} spike kinetics and CSB firing.

Finally, to determine whether endogenous acetylcholine release could induce similar effects, we employed optogenetic stimulation of cholinergic axons in a transgenic mouse line (ChAT-Cre/Ai32) expressing ChR2-eYFP under the choline acetyltransferase (ChAT) promoter. CA3PCs in mice showed comparable heterogeneity in Ca^{2+} spike kinetics to rats. Phasic light stimulation of cholinergic fibers increased CSB rate and duration, consistent with carbachol effects.

Together, these results demonstrate that cholinergic input can dynamically convert CA3PCs from regular spiking to bursting mode by modulating dendritic Ca^{2+} spike properties. This may serve as a rapid, state-dependent mechanism to enhance synaptic plasticity and memory encoding in select CA3PC populations.

5. Conclusions

This dissertation investigated the mechanisms underlying dendritic Ca^{2+} spikes and CSBs in CA3PCs, addressing key

questions about their structural, functional, and neuromodulatory properties. The results demonstrate that:

1. CA3PCs exhibit a significantly higher CSB propensity compared to CA1PCs, with heterogeneity among individual cells, which can be categorized into CSB_H, CSB_L, and RS subtypes. This variability reflects diverse intrinsic properties and may be crucial for playing specific roles in recurrent network computations.
2. Two distinct forms of Ca^{2+} spikes were characterized in individual higher-order dendrites of CA3PCs: ADP and DI spikes. ADP spikes are slow, triggered by bAPs and promote somatic burst firing, whereas DI spikes are fast, arise independently of bAPs and generate only single APs.
3. Compound Ca^{2+} spikes measured at the soma exhibited large cell-to-cell variability and could be classified into short and long-duration forms. Long Ca^{2+} spikes could be further divided into simple and complex subtypes. The kinetics of Ca^{2+} spikes were closely linked to the bursting properties of CA3PCs: most cells with short Ca^{2+} spikes were RS or produced only brief CSBs, whereas long-duration Ca^{2+} spikes were associated with higher CSB propensity and sustained plateau potentials. The heterogeneity in compound Ca^{2+} spikes was related to proximodistal and radial positions as

well as dendritic morphology. Ion channel mechanisms were found to play a central role in regulating these processes. Ca^{2+} spikes are mediated mainly by L-type Ca^{2+} channels and modulated by A- and M-type K^{+} channels.

4. Cholinergic neuromodulation emerged as a critical regulator of dendritic and somatic activity. Pharmacologically or optogenetically enhanced cholinergic activity increased the duration of Ca^{2+} spikes converting short-duration events into prolonged plateau potentials, and thereby facilitated firing of prolonged CSBs.

These findings highlight the remarkable diversity and adaptability of CA3PCs, which enable them to perform complex computations necessary for memory encoding, retrieval, and pattern separation in the hippocampus. By elucidating the cellular and molecular mechanisms underlying these processes, this work provides a deeper understanding of the CA3 region's critical role in hippocampal function.

6. Bibliography of the candidate's publications

Publications related to the thesis:

Raus Balind, S., Magó, Á., Ahmadi, M., Kis, N., Varga-Németh, Z., Lőrincz, A., & Makara, J. K. (2019). Diverse synaptic and dendritic mechanisms of complex spike burst generation in hippocampal CA3 pyramidal cells. Nature Communications, 10(1), 1859.
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