FUNCTIONAL COOPERATIVITY OF FINE-SCALE INPUT PATTERNS IN THE DENDRITES OF HIPPOCAMPAL CA1 PYRAMIDAL CELLS

Ph.D. thesis

Ádám Magó

János Szentágothai Doctoral School of Neurosciences

Semmelweis University

Supervisor: Judit K. Makara MD, PhD

Official reviewers of the Ph.D. dissertation:
Péter Enyedi MD, DSc
Viktor Szegedi PhD

Head of the Final Examination Committee:
Anita Kamondi MD, DSc

Members of the Final Examination Board:
Lucia Wittner PhD
Márk Kozsurek MD, PhD

Budapest
2021

Introduction

Cortical pyramidal cells (PCs) receive thousands of excitatory input patterns with different spatiotemporal profile on their dendrites. The different input patterns are integrated by the dendrites depending on their active and passive properties which are determined by the expressed ion channels and dendritic morphology, respectively. There are three fundamental summation modes whereby dendrites can integrate different input patterns, defined by the relationship of the actual voltage response and the arithmetic sum of inputs: a) sublinear mode, where the measured EPSP is lower than the individual EPSP amplitudes' arithmetic sum; b) linear mode, where the two amplitudes are nearly the same; and c) supralinear mode, where the measured EPSP amplitude is higher than the summated amplitude of individual EPSPs. Supralinear integration mode can be achieved when larger numbers of inputs are synchronously active that can lead the generation of a local dendritic regenerative voltage event. These dendritic 'spikes' have three types, Na^{+}, NMDA and Ca^{2+} spikes, based on the main ion channels responsible for their generation.

The highly branching structure of the dendritic tree raised the idea that dendritic branches may serve as individual integration compartments. For example, branchpoints limit the propagation of regenerative voltage signals, such as Na^{+} spikes, into other branches. In addition, even shorter dendritic segments, with a group of coactive synapses may also represent integration compartments by producing local NMDAR-mediated spikes, which is a key modulator of synaptic plasticity . This compartmentalization is supported by

in vivo studies using Ca^{2+} imaging techniques, which suggest correlated, spatially clustered synaptic activity on relatively short dendritic segments both in developing and adult hippocampus, as well as other brain regions. Some studies also suggest that neighbouring synapses, with less than ~16 micrometer interspine distance are more likely to be coactive. In addition, not only functional clusters were observed *in vivo,* but there is also evidence of small plasticity clusters, where inputs within a short dendritic segment showed concerted signs of long-term plasticity (LTP).

As such small input clusters, consisting of few coactive synapses are not well-studied, little is known about their local interactions and their potential long-term effects on synaptic functions. Thus, our primary goal was to understand the local postsynaptic interactions of such input patterns, and map the local functional long-term changes of strength at CA3-CA1 Schaffer collateral synapses in the rat hippocampus.

Objectives

I aimed to address the following specific questions:

1. How sensitive is the function of a synapse to the coactivation of other closely located synapses? How does this property depend on the position of the synapse in the dendritic tree?
2. Do local interactions between spatially clustered coactive synapses lead to synaptic plasticity? How does this phenomenon depend on the number and spatial pattern of the inputs?
3. How are local synaptic plasticity rules affected by the dendritic integration mode of input patterns, in particular by the generation of dendritic spikes?

Methods

Electrophysiology: Adult male Wistar rats (7-11 week old) were used to prepare 400 µm thick transverse slices from the hippocampus. The slice preparation was performed according to methods approved by the Animal Care and Use Committee of the Institute of Experimental Medicine, and in accordance with the Institutional Ethical Codex, Hungarian Act of Animal Care and Experimentation.

Animals were deeply anaesthetized with 5% isoflurane (~5 minutes) and quickly perfused through the heart with ice-cold cutting solution, saturated with 95 % O_2 and 5 % CO_2. After the perfusion, the brain was quickly removed and 400 µm thick slices were prepared in cutting solution using a vibratome. Slices were incubated in a submerged storing chamber containing ACSF at 35 °C for 30 min, and then stored in the same chamber at room temperature. For recordings, slices were transferred to a custom-made submerged recording chamber under the microscope where experiments were performed at 32–35 °C in ACSF containing, saturated with 95 % O_2 and 5 % CO_2.

Cells were visualized using an epifluorescent microscope equipped with differential interference contrast (DIC) optics under infrared illumination and a water immersion lens (60X or 63X). Current-clamp whole-cell recordings from the somata of hippocampal CA1PCs were performed using an amplifier in the active „bridge" mode, filtered at 3–5 kHz and digitized at 50 kHz. Patch pipettes (2–6 MΩ) and puffer pipettes were pulled with a P-97 Flaming/Brown Micropipette Puller. Patch pipettes were filled

with a low Cl^- intracellular solution (pH=7.25) complemented with Alexa Fluor 488 (100 µM) in order to visualize neurons. In some experiments where Ca^{2+} imaging was performed, intracellular solution contained the Ca^{2+} dye Oregon Green BAPTA 1 (OGB-1, 100 µM) and Alexa Fluor 594 (50 µM). Only CA1PCs with a resting membrane potential (V_{rest}) more negative than -55 mV were used. Cells were kept at -63– -65 mV.

Two-photon imaging and uncaging: A dual-galvanometer-based two-photon scanning system was used to image the neurons and to uncage glutamate at individual dendritic spines. Two ultrafast pulsed laser beams were used: one for imaging of fluorophore (at 920 or 860 nm) and the other to photolyse MNI-caged L-glutamate at 720nm (10 mM), applied through a puffer pipette, downward-tilted aperture above the slice, using a pneumatic ejection system. Laser beam intensity was independently controlled with electro-optical modulators.

Individual spines with an average phenotype and separated from their neighbors were selected for stimulation. Stimulation was performed by uncaging glutamate $\leq$0.5 µm lateral to the head of visually identified spines, using 0.5 ms uncaging duration. The uncaging points were placed more than ~1.1 µm apart. Time interval between the stimulated spines in a trace (termed interspine stimulus interval) was 200 ms (for recording individual voltage responses of spines) or 0.1 ms (quasi-synchronous activation during the LTP protocol). Unitary EPSPs were measured repeatedly (usually 6–12 times, repeated every 5 min).

Ca^{2+} measurements: In experiments measuring spine Ca^{2+} signal nonlinearity, the bath solution contained 0.5–1 mM

TTX to eliminate nonlinearities arising from Na^+ spike activation, except where indicated. Freehand linescan imaging through spines was performed at 200–500 Hz with 8 μs dwell time. At the beginning of the experiment, the set of two to four spines were first stimulated individually (200–305-ms intervals) and the laser power was adjusted to yield physiological unitary EPSPs and reliable associated spine Ca^{2+} signals. Next, stimulation of various numbers of the selected spines was performed with the same laser power synchronously (0.1 ms ISI for galvo movement, plus 0.2 ms uncaging duration per spine). In experiments examining the effect of larger proximal input clusters (up to 12 spines), Ca^{2+} signals were measured only in the first four spines. Following synchronous stimulation, spines were stimulated individually again to confirm the stability of single spine responses.

LTP experiments: To measure changes in synaptic function induced by LTP protocols, we recorded EPSPs evoked by 2PGU in whole-cell current-clamp mode. We used a method where LTP protocol could be started within 10 min after establishing the whole-cell configuration, as previously developed (Weber et al., 2016). After establishing whole-cell configuration and measurement of V_{rest}, uncaging started immediately. A set of four individual spines were first stimulated separately (200 ms between stimulation of individual spines) and the uncaging laser power was adjusted to yield physiological-sized EPSPs (~0.1-0.9 mV) at each stimulated spine. After the test recording, an LTP induction protocol (50 stimulations at 3 Hz at a group of spines, unless otherwise indicated) was applied as soon as possible (within 2 min from the last test stimulus and within 10 min after break-in). In all homosynaptic LTP experiments, the same laser

power was used for the LTP induction protocol as that for monitoring the test spines throughout the experiment. In some of the heterosynaptic LTP experiments, the laser power was increased by ~15% during the LTP induction protocol, to increase the likelihood to evoke d-spikes by the LTP induction spines; then we commenced monitoring the test spines with the original test laser power.

Chemicals: D-AP5 and $NiCl_2$ were dissolved in distilled water; nimodipine and U0126 were dissolved in DMSO. Aliquots of the stock solutions were stored at -20°C and dissolved into ACSF on the day of the experiment. Inhibitors were applied by perfusing the slice with ACSF containing the blocker(s) for 10–15 min before repatching the cells. Solution containing nimodipine and Ni^{2+} was protected from light.

Computational modeling: We used a detailed biophysical CA1 PC model, optimized for reproducing the dendritic processing of synaptic inputs in CA1 pyramidal neurons. The model captures several somatic and dendritic properties of these cells measured under in vitro conditions, including the generation and propagation of Na^+ action potentials at the soma and along the apical dendritic trunk; the generation of local Na^+ spikes in thin dendritic branches; amplitude distribution of synaptic responses; nonlinear integration of inputs via NMDARs; the similar voltage threshold for Na^+ and NMDA nonlinearities; and the major role of A-type K channels in limiting dendritic excitability. When stimulated with in vivo-like synaptic inputs distributed throughout the entire dendritic tree, the same biophysical model shows place-selective activity, with several features of the somatic membrane potential activity falling in the

physiological range. The simulations were performed with the NEURON simulation environment.

Results

Cooperative spine Ca^{2+} signaling along CA1 pyramidal cell dendrites: In order to quantitatively investigate potential cooperative nonlinearities of interactions between nearby synaptic inputs, we first determined how voltage integration and spine Ca^{2+} signals were affected by coactivation of small clusters of synapses (Weber et al., 2016). We measured the somatic voltage and spine Ca^{2+} responses (within a 3-6 μm long dendritic segment) of individually or synchronously stimulated 4 nearby synapses, and compared the measured signals with that expected from independent (arithmetically summated) synapses. When stimulating the four synapses quasi-synchronously, at distal dendritic locations we observed a pronounced amplification of peak spine Ca^{2+} signals, with a gradual nonlinear increase by activation of each additional nearby spine. Surprisingly, the large Ca^{2+} signal amplification was not associated with suprathreshold voltage integration of EPSPs: summation remained linear. On the other hand, coactivation of similar synapse clusters at proximal dendritic locations led to little if any nonlinearity of spine Ca^{2+} signals along with linear voltage summation.

In summary, the threshold sensitivity of synaptic Ca^{2+} cooperativity increases gradually along thin dendrites from their base to their tip systematically in the dendritic target area of Schaffer collaterals, a pattern well matching the passive impedance profile of the dendritic arbour. In contrast, the corresponding EPSPs sum at the soma largely linearly with little location dependence.

Pharmacological experiments using blockers of various possible sources of spine Ca^{2+} signals revealed that the Ca^{2+} nonlinearity evoked in spines and the dendrite at distal locations was mediated by NMDARs.

Thus, the results showed that NMDAR-mediated spine Ca^{2+} signals are highly sensitive to coincident activation of even low number of spatially close synapses in distal dendritic compartments. This local cooperative function takes place in the linear electrical integration regime, where voltage recordings at the soma remain uninformative about the spatial distribution or cooperation of the synapses involved.

Location-dependent cooperative synaptic LTP: We hypothesized that cooperative enhancement of spine Ca^{2+} signals in coactive synapses even in the linear voltage integration regime, as shown above, may promote clustered forms of synaptic plasticity. To examine this hypothesis, we measured peak amplitude changes of EPSPs in response to a cooperative 2PGU LTP induction protocol that involved synchronous stimulation of various number of spatially clustered spines depending on the experiments, repeated 50x at 3 Hz in normal ACSF near the resting membrane potential. In most experiments, EPSPs were also measured at an additional nearby (<15 μm) reference spine that was not stimulated during the cooperative LTP protocol.

The costimulation of 4 spines during the LTP protocol led to an increase of somatic EPSP amplitude to 139±10% of control values at the four LTP-induced spines at distal dendritic locations (n=17). Importantly, EPSP amplitude did not increase (in fact, slightly decreased) in the reference spine that was not stimulated during the LTP protocol, indicating

input specificity of potentiation. In contrast to distal locations, no cooperative LTP could be induced at proximal dendritic locations using the same protocol even by stimulating 12-15 spines.

Cooperative LTP in distal dendritic segments was eliminated by the NMDAR inhibitor D-AP5 (50 μM), but was not significantly affected by the voltage gated Na^+ channel (VGNC) inhibitor TTX (1 μM), demonstrating that NMDARs but not VGNCs are required for LTP.

Regenerative d-spikes are required for efficient cooperative LTP at proximal dendritic locations: Since we did not observe LTP neither by subthreshold coactivity of small nor larger input clusters at the proximal sites of perisomatic dendrites, we next asked whether proximal synapses can undergo LTP if they contribute to input patterns triggering d-spikes. Generation of d-spikes was indicated by either a transient increase in the rate of rise (dV/dt) of the compound EPSPs, a sign of dendritic Na^+ spikes, and/or a peak somatic nonlinearity ≥2 mV, indicating NMDA spikes. Triggering d-spikes induce robust long-lasting increase in the mean EPSP amplitude of the four proximal test spines (1.98±0.43, n=7 experiments), and potentiated synapses were found in every experiment. Similar LTP was measured when APs were also evoked by at least 1 of the 50 LTP stimulus pulses: EPSP amplitude increased to 2.15±0.44 (n=8), and potentiated synapses were found in all experiments. These data suggest that large depolarization, involving regenerative dendritic spikes (local or backpropagating AP), is needed for cooperative LTP induction of synapses located in proximal segments of perisomatic dendrites.

Subthreshold LTP at distal dendritic locations depends on fine-scale input configuration: Next we explored the rules of subthreshold cooperative LTP at distal segments of perisomatic dendrites. First, to determine the minimum cluster size required for subthreshold cooperative LTP, we reduced the number of test synapses coactivated during LTP induction. With three synapses, we still observed input-specific increase in EPSP amplitude (1.35±0.13, n=8), similar to that measured with four inputs (1.32±0.11, n=14). In contrast, LTP protocol with only two synapses did not induce their potentiation (0.81±0.11, n=8).

Next, we examined the spatial pattern requirements for subthreshold LTP. LTP protocol with four coactivated test spines that were distributed evenly on longer dendritic stretches (17.4±0.5 μm, ISD: 5.8±0.2 μm) did not produce subthreshold LTP effectively (EPSP amplitude: 0.91±0.05, n=21), with strong negative correlation between ISD and EPSP change.

Together, the above results show that tight clusters of ≥3 coactive distal inputs can be strengthened by subthreshold cooperative LTP, even without regenerative dendritic activity.

D-spikes alleviate the tight clustering requirements of LTP: Are the strength and/or the spatial rules of plasticity at distal dendritic segments different if synapses participate in a stronger input pattern that can evoke local d-spikes? To address this question, during the LTP protocol, additional neighbour spines were coactivated together with the four clustered test inputs to trigger d-spikes. This clustered, locally suprathreshold input pattern induced LTP in at least one test

spines in all experiments (n=9). Surprisingly, neither the magnitude of LTP (1.37±0.11) nor the ratio of potentiated synapses (47%) was significantly different from that measured with subthreshold LTP by 3 or 4 clustered inputs.

We next explored how LTP with d-spikes depends on the spatial arrangement of the inputs. To test this, we repeated the experiments above with distributed arrangement of the four test spines. In most experiments (4 of 5), we found at least one synapse to be potentiated, and an average LTP of 1.52±0.20 was induced.

Previous reports using electrical stimulation indicated that d-spikes can trigger synaptic potentiation with fewer stimulus repetitions than other LTP-inducing activity patterns. To test whether there is a difference in this regard between locally subthreshold and suprathreshold input patterns, we performed experiments with a short LTP protocol, consisting of only 5 coactivations of 4 (subthreshold) or 8 (suprathreshold for d-spikes) clustered spines. We found that suprathreshold clustered inputs did develop robust LTP (1.50±0.15, n=4), whereas only 5 synchronous activations were not sufficient to induce LTP with subthreshold clustered inputs (0.79±0.05, n=5).

These results together indicate that d-spikes, although not necessarily required for LTP at distal dendritic segments, can alleviate the tight spatial clustering requirements and reduce the number of coincident activity events needed to induce cooperative LTP.

Strong input patterns allow local plasticity crosstalk: D-spikes, evoking robust voltage and Ca^{2+} signals in the branch, may activate signalling mechanisms that affect the function of

not only those synapses that evoked them but other neighbour synapses as well. To examine this possibility, we coactivated a group of eight spines during LTP induction, in order to trigger d-spikes ("LTP induction spines"), and measured the impact of this stimulus on EPSP amplitudes of a different set of nearby four test spines (up to ~20 μm distance). The test spines were thus only activated before (≤2 min) and after (≥5 min), but not during the LTP protocol. Surprisingly, we observed variable effects: although the long-term change in test spines EPSP amplitude (1.25±0.12, n=30) was smaller than that by homosynaptic LTP with d-spikes, in a substantial fraction of experiments, we detected signs of potentiation in the test spines. First, in the majority of experiments (20 of 30), EPSP increased >30% in at least one of the test spines. Second, in 12 of 30 experiments, the mean EPSP amplitude change in the test spines was larger than the mean +2 SD measured in control experiments with no LTP protocol.

To better understand the nature of this effect, we tested whether crosstalk potentiation is evoked when test spines and LTP induction spine groups are located at short Eucledian distance, but on different dendrites of the cell. Under these conditions, no LTP was found in any of the test spines, and their EPSP amplitude rather decreased (0.77±0.05, n=6), similar to the control experiments with no LTP protocol (0.86±0.05, n=11). This indicates that the crosstalk mechanism involves intracellular rather than extracellular signal(s), and affects only the activated dendritic segment. These results also excluded that the effect could be attributed to the diffusion of uncaged glutamate or other nonspecific effects of 2PGU.

We considered the possibility that the LTP induction protocol, triggering repeated dendritic spikes, perhaps

produced a general change in the electrical properties of the stimulated dendrite, leading to a virtual increase of synaptic voltage signals at the soma. However, the somatic strength of dendritic Na^+ spikes (dV/dt), a parameter expected to increase by enhanced dendritic excitability, did not systematic change from the value measured during the first pulse of the LTP induction protocol to that evoked again at the end of the experiments. To further explore whether changes in the dendritic excitability can explain our data, my colleague Balázs Ujfalussy implemented a detailed biophysical model of a CA1PC and measured the somatic response amplitude to near-synchronous stimulation of 1-30 excitatory synaptic inputs. We used this model to explore which mechanisms can increase synaptic EPSP amplitudes without changing the strength of the Na^+ spikelets as measured in the soma. Increasing the local excitability of the branch by changing passive parameters (increasing the local membrane resistivity [R_m] and decreasing axial resistivity [R_a]) within the branch increased the amplitude of individual EPSPs, but it also significantly increased dV/dt of the somatic spikelets. Changing the local excitability by locally eliminating K^+ channels also increased dV/dt of the somatic spikelets but failed to increase EPSP amplitudes. On the other hand, increasing the AMPA conductance of the synapses by 40% (mimicking LTP) increased the amplitude of the EPSPs without changing the spikelets. These effects were robust against changing the passive parameters in the model. The simulations above made it unlikely that changes in dendritic excitability by so far described mechanisms could alone explain the increase in somatically measured amplitude of the

test spine EPSPs, and suggest that crosstalk was most likely mediated by synaptic mechanisms.

Previous studies showed that LTP at a single spine can lower LTP induction threshold at nearby spines for several minutes, so that even weak stimuli can induce potentiation. Thus, we asked whether the crosstalk plasticity may be related to the weak test stimuli applied to monitor EPSPs. Since the initial selection of the four test spines involved variable numbers of pre-LTP stimuli at different spines, we first analysed whether pre-LTP stimulation was related to the ability of spines to develop potentiation. Although we did not find a correlation between the number of pre-LTP stimuli and the magnitude of LTP by the individual spines, when we separated test spines based on the number of received pre-LTP stimuli into two groups divided near the median (16 stimuli, range: 6-43), we found a trend for smaller EPSP amplitude change and fewer potentiated spines in the spine group receiving ≤15 pre-LTP stimuli (1.02±0.08; n=50; 22% of test spines potentiated) than in those receiving ≥16 pre-LTP stimuli (1.43±0.13; n=63; 40% of test spines potentiated). This raises the possibility that synapse activation before LTP induction by other synapses may facilitate crosstalk potentiation. Suspending test stimulation for 30 min after LTP induction did not eliminate the crosstalk (EPSP amplitude measured at 30-40 min: 1.26±0.11; 42% of test spines potentiated, n=14).

Biophysical mechanism of crosstalk: The crosstalk mechanism was NMDAR-dependent because no potentiation developed in the presence of D-AP5 (50 μM; 0.90±0.07, n=10). In the presence of T-,R-, and L-type VGCC inhibitors

(100 μM Ni^{2+} and 10 μM nimodipine), we observed an initial increase in EPSP amplitude in test spines, followed by gradual decline toward the baseline, but the EPSP change at 30-40 min was not significantly different from that in ACSF (1.07±0.05, n=8). Thus, VGCCs are not indispensable for crosstalk but they may support stabilization of the process. Finally, inhibition of the MEK/ERK pathway by U0126, which was proposed to mediate local metaplasticity of nearby spines via small GTPases eliminated the crosstalk (EPSP amplitude at 30-40 min in 20 μM U0126: 0.88±0.05, n=9).

Together, the results suggest that crosstalk potentiation of synapses by nearby activity pattern evoking d-spikes may provide a less effective, but not negligible, mechanism to increase synaptic strength, via signalling mediated by NMDARs and MEK/ERK activation.

Conclusions

Using a combination of various technical approaches such as *in vitro* 2P imaging, 2P glutamate uncaging combined with whole cell current-clamp recordings, as well as modelling, we elucidated the local interactions of spatially colocalized coactive synaptic inputs along the dendritic branches of CA1 pyramidal cells in acute adult rat hippocampal slices.

We first showed that subthreshold activation of small input clusters can lead to cooperative NMDAR dependent supralinear Ca^{2+} signal amplification in the co-active synapses in a location-dependent manner, observed at distal but not at proximal dendritic segments.

Based on the local large spine Ca^{2+} signal produced by cooperativity at distal synapses, we investigated whether

synaptic plasticity of small clusters of coactive inputs can be induced. Repetitive subthreshold co-stimulation (50x at 3 Hz) of 3-4 synapses by 2PGU evoked input specific cooperative LTP. Similar to the Ca^{2+} amplification of clustered co-active synapses, the efficacy of subthreshold LTP also matched the passive impedance profile of dendritic branches. The required interspine distance was short (≤~5 µm), suggesting that large local intracellular Ca^{2+} concentration is needed to induce LTP.

Evoking dendritic spikes using stronger input patterns during LTP stimulation changed the local plasticity rules in several aspects. We have shown that regenerative voltage signals were crucial for inducing LTP at proximal dendritic locations. At distal locations, the magnitude of LTP was not affected by d-spikes, but d-spikes alleviated the tight spatial requirement of input distribution, transforming the potentiation from local restricted plasticity to a more extended, perhaps branch specific form. In addition, substantially smaller number of synchronous stimulations (5 instead of 50) were sufficient for potentiation in the presence of d-spikes.

In addition to homosynaptic plasticity of the coactive synapses, d-spikes led to local heterosynaptic crosstalk as well, resulting in potentiation of nearby non-synchronous synapses. This potentiation was less efficient compared to homosynaptic LTP, but was above chance level. Heterosynaptic LTP was mediated by NMDARs and the MEK/ERK pathway, and our data suggest that VGCCs may have a stabilizing role. In addition, using in silico modelling we reinforced our findings as the long-term increase of EPSPs during heterosynaptic LTP could not be explained by changes in dendritic excitability.

The bibliography of the candidate's publications

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