TWO-PHOTON CALCIUM IMAGING EXPERIMENTS TO INVESTIGATE THE PROPERTIES OF NEURONAL MICROCIRCUITS: THE EXAMPLE OF CEREBELLAR NETWORK ACTIVITY

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Abstract We have developed a custom-made two-photon microscope integrating a spatial light modulator that can modulate the phase of a laser beam and divide it into multiple diffraction-limited beamlets configurable in real time. We used this system to study cerebellar circuit activity and long-term synaptic plasticity expression by simultaneously acquiring stimulus-induced calcium signals from neurons located in different cerebellar layers.

***Keywords*:** Two-photon Microscopy, Holography, Neuronal Microcircuits, Cerebellum, Neuronal Synaptic Plasticity

1. INTRODUCTION

Brain microcircuits are defined as localised neuronal networks organised in specific patterns, in which different cells interact dynamically, playing a key role in information processing[1]. To study their activity, it is necessary to acquire data from several neurons, which must be uniquely identified and whose activity must be monitored over time.

We used a computer-guided holographic microscope, the spatial-light modulator two-photon microscope (SLM-2PM), to investigate the cerebellar cortex microcircuit activity. In this circuit, afferent mossy fibers (MFs) are the primary excitatory input that transmit to the granular layer (GL), where Golgi cells (GoCs) modulate granule cells (GrCs) activity by feed-forward and feed-back inhibition. Different forms of plasticity further recode input signals transmitted to the upper layers, where stellate and basket cells (SCs, BCs) modulate Purkinje cells (PCs) activity, which in turn generate the cortex output[2]. To investigate this network in depth, we performed calcium imaging experiments in acute cerebellar slices, simultaneously acquiring data from neurons located in different layers of the cerebellar cortex, before and after the induction of long-term synaptic plasticity at the MFs bundle, revealing for the first time the expression of plasticity in the entire cerebellar network with single-cell resolution.

2. METHODS AND PROCEDURES

Acute parasagittal cerebellar slices (230 µm, p18-p24, C57/BL6 mice) were first bulk loaded with Fura-2 AM (30 µM, Molecular Probes) at 35°C for 40 minutes in the dark and then positioned in the recording chamber. Oxygenated Krebs solution (containing in mM: 120 NaCl, 2 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 1.18 KH_2PO_4, 26 $NaHCO_3$, and 11 glucose, equilibrated with 95% O2–5% CO_2, pH 7.4) was perfused during the whole experiment (2 mL/min, 32°C)[3].

The spatial light modulator two-photon microscope (SLM-2PM) was used to perform calcium imaging experiments. In contrast to conventional two-photon systems that employ galvo scanners for beam steering, the SLM-2PM employs only the SLM (X10468-07, Hamamatsu) to modulate the phase of a coherent laser light source (Chameleon Ultra II, Coherent) and generate different spatial illumination patterns in the sample plane through custom Python (PSF, 9450 SW Gemini, USA) routines based on the iterative-adaptive Gerchberg-Saxon algorithm[4]. This design eliminates the need for galvo scanners, simplifying the optical setup.

Experiments were performed at depths of ~50-60 µm, using a 20X 1.0 NA water-immersion objective (Zeiss Plan-APOCHROMAT); the laser power was set ≤ 6 mW/laser beamlet. Two-photon images were acquired through a high-spatial resolution CCD camera (CoolSnap HQ, Photometrics) to identify neurons somata positions and to select cells for the subsequent calcium signals acquisition (field of view of ~220x300 μm^2). Cerebellar circuit activity was elicited by electrical stimulation of the mossy fiber bundle (10 pulses@50 Hz repeated 3 times at 0.08 Hz) with a large-tip patch-pipette (10-20 µm tip) filled with extracellular Krebs' solution, via a stimulus isolation unit. Stimulus-induced fluorescence calcium signals were acquired at 20 Hz through a high-temporal resolution CMOS camera (MICAM Ultima, Scimedia), connected through an I/O interface (BrainVision, Scimedia) to a PC controlling illumination, stimulation and data acquisition (final pixel size of 4.6x4.6 μm^2) and analyzed off-line using custom Matlab routines (MathWorks, Natick, MA). Calcium responses from several neurons located in different cerebellar cortex layers (GL, PCL, and molecular interneurons layer, MLI) were acquired before and after long-term synaptic plasticity induction (high-frequency stimulation of MFs, HFS, 100 pulses@100 Hz). Persistent changes in $\Delta F/F_0$ (peak fluorescence amplitude normalized to mean resting fluorescence) >± 20% after HFS were considered as long-term changes. The related cumulative plasticity map was computed by centering individual experiments maps on the maximum potentiation value, aligning them along MFs bundle, and smoothed with a sliding box filter (3×3 pixels). Data are reported as mean ± standard error of the mean (SEM).

All experimental protocols were conducted in accordance with international guidelines on the ethical use of animals (2010/63/EU) and approved by the ethical committee of Italian Ministry of Health (639.2017-PR; 7/2017-PR).

3. RESULTS AND DISCUSSION

To study the cerebellar microcircuit activity, we first acquired SLM-2PM images of acute cerebellar slices and then recorded stimulus-induced calcium signals from neurons in the entire cerebellar cortex. The simultaneity of signals acquisition provided by the SLM-2PM allowed us to observe different temporal activation in neuronal responses. PCs calcium signals show different trends when acquired from the soma or the dendrites. In the former case, the $\Delta F/F_0$ response is characterized by a rapid peak concurrent with MFs stimulation (first peak) followed by a slow negative deflection (second peak), probably due to activation of metabotropic glutamate receptors[5]. The dendrites showed only a slow negative activation, simultaneous with the second peak observed in the soma (Figure 1).

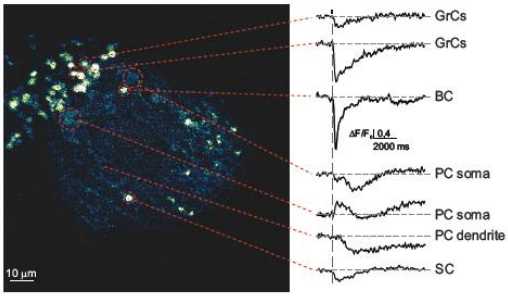

Figure 1. Left: SLM-2PM image of a sagittal cerebellar slice bulk loaded with Fura-2 AM (20X objective). Right: examples of stimulus-induced Ca^{2+} signals simultaneously acquired from GrCs, PCs, SC and BC, showing different trends and temporal activations. In this experiment, inhibition was blocked by adding 10 µM gabazine to the Krebs solution.

To study the expression of long-term synaptic plasticity, we delivered HFS to the MFs bundle. Robust activation of the cerebellar primary input elicited sustained changes in $\Delta F/F_0$ responses across GrCs, PCs and MLIs, in line with *in vivo* observations[6,7]. These changes consisted of either calcium-related potentiation (CaR-P) or calcium-related depression (CaR-D), while a subset of neurons exhibited no significant variation in signal amplitude post-plasticity, as shown by the relative time courses in Figure 2. Interestingly, both the second peak of PC somatas calcium responses and that of PC dendrites show short-term depression in the first five minutes after induction, followed by either no change or CaR-P expression. (GrCs. No change: -2.87±2.88 n=34; CaR-D: -39.24±2.15 n=47; CaR-P: 79.42±14.70 n=19; MLIs. CaR-D: -60.87±9.18 n=4; CaR-P: 294.75 n =1; PC somata. First peak: No change: -1.59±4.88 n=2; second peak: No change: -4.11±7.74 n=2; PC dendrites. No change: -1.97±7.35 n=2; CaR-P: 24.06±4.50 n=3).

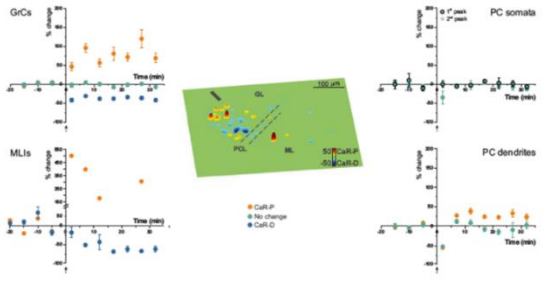

Figure 2. Time courses of $\Delta F/F_0$ responses of different neuronal types in the cerebellar cortex before and after plasticity induction (arrows). In the middle, cumulative plasticity map showing the spatial expression of long-term plasticity in the entire cerebellar cortex.

To visualize these results, we computed a cumulative plasticity map (n=5 independent slices) showing on a colour scale the spatial organization of plasticity expression in the cerebellar cortex (Figure 2, middle).

4. CONCLUSIONS

Our efforts have been focused on the development of a two-photon scanless microscope employing a spatial light modulator, for neurophysiological applications. We used this system to study the functioning of the cerebellar cortex by recording stimulus-induced calcium signals from different types of neurons that constitute the cerebellar microcircuit. We acquired data from multiple neurons simultaneously while maintaining single-cell resolution. This allowed us to observe the propagation of the signal from the main cerebellar input to the entire cortex and to observe how the induction of long-term synaptic plasticity at the primary circuit input reverberates in the microcircuit. The characteristics of the system and the reliability of these recordings will help to understand the complex neuronal interactions and the specific role of different neurons in brain microcircuits. The integration of these advanced experiments with computational models will provide an efficient approach to formulate comprehensive assessments of microcircuit activity and to answer open questions about microcircuit function and dysfunction.

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