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Influence of Microgravity on Spontaneous Calcium Activity of Primary Hippocampal Neurons

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Abstract

The influence of variations of gravity, either hypergravity or microgravity, on the brain of astronauts is a major concern for long journeys in space, to the Moon or to Mars, or simply long-duration missions in the ISS. Monitoring brain activity, before and after ISS missions already demonstrated important and long term effects on the brains of astronauts. In this study, we focus on the influence of gravity at the cell level on primary hippocampal neurons. A dedicated setup has been designed and built to perform live calcium imaging during parabolic flights. Using this technology, throughout the CNES parabolic flights campaign, organized by Novespace in spring 2022, we were able to observe neurons inside microfluidic devices and monitor their calcium activity during gravity changes over different parabolas. Our preliminary results clearly indicate a modification of the calcium activity associated to variations of gravity.

Keywords: Microgravity, Neuronal activity, Microfluidic

1 Introduction

Knowing how space flight and associated stress stimuli - hyper or microgravity as well as radiation - impact the development of biological organisms is a main concern regarding the future of space exploration [1]. From crops to astronauts, it is now essential to understand how the particular environmental conditions of space flight can affect the health of cells and tissues. Upon their return from the ISS, astronauts showed pathological symptoms linked to their prolonged stay in microgravity such as bone density loss, muscle atrophy, cardiovascular and hematic changes, metabolic, endocrine and sleep disturbances [2]. The main physiological effect identified and linked with microgravity that impacts most organs is the blood redistribution across the whole organism. Indeed, there is no more privileged direction for blood flow, making the whole blood circulation more efficient. Thus the vascular system, including the heart, is under less pressure as less work is necessary to oxygenate primary organs. Aside from this, many other physiological functions can be affected by space-flight conditions.

From a neurological point of view, medical studies on astronauts also showed that space flights have effects on the brain both on the short term (motion sickness, vestibular dysfunction and Spaceflight-associated neuro-ocular SANS syndrome) and on the long term (increased risk of brain malignancies, premature ageing, accelerated neurodegeneration) [3, 4]. Alteration of brain structure has recently been substantiated by fMRI analysis of astronauts evidencing subtle yet significant modification of brain connectome [5]. These phenomena are either linked to microgravity itself, via mechanical unloading, or to the intracranial fluid redistribution, or to space radiation. One way or another, understanding the mechanisms underlying the appearance of those symptoms remains an important health concern.

To this end, we designed an experimental setup to record live calcium transients of living primary hippocampal neurons, grown inside microfluidic chips, during hyper and microgravity phases of parabolic flights. To adjust to these particular experimental conditions, conventional laboratory equipment were adapted and automated. An inverted fluorescence microscope equipped with a miniaturized cell culture incubator and fitted with a fast CMOS camera was packaged in a closed and secured box and remotely controlled through a control rack. Microfluidic chips were chosen as culture devices for the primary neurons because they show several advantages (for review see [6]). First, they recently emerged as a powerful tool allowing spatial control of neuronal connectivity together with exquisite control of fluidic and chemical microenvironment of the cells. Second, microfluidic chips cell culture platforms are perfectly suited for parabolic flight campaigns as they allow to limit redistribution of fluids and shear stress during the different phases of the flights in order to preferentially record the neuronal activity affected by changes in gravity. Finally, calcium imaging was chosen as readout because of the fast variation in signal due to adaptation to external stimuli. Indeed, during a parabola, the duration of each

acceleration (1, 1.8, 0g) is on the order of tens of seconds while most biological phenomena occur in the range of minutes, making the observation of their fluctuations impossible to link with changes in gravity conditions. Remarkably, calcium activity of neurons varies on a few seconds span, allowing to record enough spikes for every gravity phase so as to discriminate their corresponding firing rates. [7]

For the first time, thanks to a refined setup boarded on the Novespace plane during the 164th CNES parabolic flights campaign, live calcium activity of primary hippocampal neurons was successfully extracted, for different gravity conditions, and compared with on-Earth data.

2 Methods

2.1 Microfluidic chip for 2D neuronal network

To study the activity of neurons, the first step consisted in designing a proper microfluidic chip allowing for fluid confinement as well as growth of primary cells leading to the creation of a complex 2D network of connected and active neurons. Similar to [8], a microfluidic chip comprising of two independent cell culture chambers separated by an array of “axonal diodes” was used. The diodes are asymmetric micro-channels imposing unidirectional axon connectivity with 97% selectivity supporting the construction of a complex, oriented neuronal network. Nevertheless, as first proof of concept in the present experiment, only one chamber seeded with primary hippocampal neurons was used (Fig. 1).

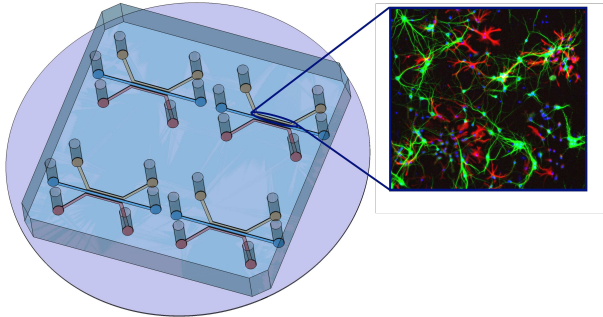


Fig. 1: CAD (Computer Assisted Drawing) view of the microfluidic chip used to grow the neuronal networks. The height of the chambers is $50 \mu\text{m}$, thus confining the cells in a monolayers.

2.2 Microfluidic Chip Fabrication

To fabricate the microfluidic chips suitable for the calcium imaging experiment in the parabolic flight, standard photolithography process was used, based on

previous work from Lassus et al 2018 [9]. Briefly, microfluidic silicon wafers were generated through spin coating of photosensitive resist SU8-2002. They were baked at 65 °C for 30 s and at 95 °C for 2 min. They were then exposed to UV light through a mask for photopolymerization of the resist. The resulting silicon wafers encode 4 microfluidic chips composed of 2 cell culture macro chambers (length 4 mm, width 1 mm, height 50 μ m), each of the chambers being connected to an inlet and outlet reservoir. Silicon wafers (or epoxy replicated molds) were used to produce the cell culture PDMS chips. A mixture of Poly Dimethyl Siloxane (PDMS) elastomer and curing agent with a 9:1 ratio was degassed under vacuum to remove air bubbles. The PDMS mix was then cast onto the molds and baked for 4 hours at 70°C. PDMS microfluidic chips were peeled off the wafers, wells for macro chambers were punched manually and the surface of the PDMS was cleaned with tape. PDMS pads were then bonded to glass slides, previously cleaned with iso-propanol, by using a plasma cleaner (100% power, 0.6 mBar, Diener Electronic). One inlet per chamber was immediately filled with distilled water maintaining the hydrophilicity of PDMS acquired during the plasma treatment. Finally, the microfluidic chips were sterilized under UV for 30 min. Prior to cell seeding, they were coated with poly-D-lysine (PDL-10 μ g/mL, Sigma) overnight at 37°C and then with Laminine (2,5 μ g/mL, Sigma) for 2 hours at 37°C, allowing cells to adhere to the glass slide.

2.3 Cell culture of Primary Hippocampal Neurons

Hippocampal neuronal cultures were generated from E18 swiss mouse embryos by building upon standard cell-culture techniques, as follows. SWISS pregnant mice were purchased from Janvier (Le Genest Saint Isle, France). Animal care was conducted in accordance with standard ethical guidelines (U.S. National Institutes of Health publication no. 85–24, revised 1985, and European Committee Guidelines on the Care and Use of Laboratory Animals). The cell culture preparation was done in the Neurocentre Magendie laboratory in Bordeaux. Hippocampi were micro-dissected from E18 embryos with all steps performed in cold Gey's Balanced Saline Solution (Sigma G9779). Dissected structures were digested with papain (50 U/mL, Sigma 76220) in DMEM (Thermofisher 31966 021). After papain inactivation with FBS (GE Healthcare), structures were mechanically dissociated with a pipette in presence of DNase type IV (20U/mL Sigma D5025). Cell viability was determined by Trypan Blue exclusion assay. The resulting cell pellet was resuspended in the culture medium containing DMEM, 10% FBS, 2% B27, 0.5mM Glutamate and 1% Penicillin/Streptomycin (Gibco) at a concentration of 20 million cells/mL. Cells were seeded in macrochambers and allowed to adhere to the glass surface for 5 to 10 min before filling the rest of the chambers with culture medium. All cell cultures were kept in an incubator at 37°C and 5% CO₂.

2.4 Immunofluorescence staining

To evaluate the survival of the primary hippocampal neurons inside our microfluidic chips, some chips were fixed in PBS containing 4% paraformaldehyde (PFA, Electron Microscopy Science 15714S) and 4% sucrose (Sigma S9378) for 8 min and rinsed twice in PBS for 5 min. Then, they were permeabilized for 30 min at room temperature with 0.2% Triton (Sigma X100) and 1% bovine serum albumin (BSA, Thermofisher 15561020) in PBS. After removing the permeabilizing solution, primary antibodies were added in PBS with 1% BSA and the samples incubated at 4°C overnight. The samples were rinsed for 5 minutes with 1% BSA in PBS and further incubated with the corresponding secondary antibodies for 2 hours at room temperature. Finally, the chips were rinsed first with 1% BSA in PBS and last only with PBS. The following primary antibodies were used: DAPI (1050, Euromedex, 1/2000), , MAP2 (M4403, Sigma, 1/1000), β 3-tubulin (MA1-118, Thermofisher, 1/1000) and GFAP (Z0334, Dako, 1/1000). Species-specific secondary antibodies coupled to Alexa 350, 488, 555 or 633 were used (1/1000, Invitrogen) to visualize bound primary antibodies.

2.5 Calcium Imaging

The calcium dye Fluo4-AM was obtained from ThermoFisher Scientific (ref F14201). It was dissolved in dimethyl sulfoxide (DMSO) in a ratio of 1 μ g: 1 μ L to obtain a stock solution of 1 mM. This stock solution was frozen in 2 μ L aliquots. Ca^{2+} free wash buffer was made according to previous protocols and the stock solution of Fluo4-AM was added in order to load the cells with a final concentration of approximately 2 μ M. Each day of flight or classical on-Earth experiment, a chip with good cell survival was chosen, loaded with Fluo 4 AM and incubated at 37°C and 5% CO₂ for 25 min. It was then rinsed with Ca^{2+} free wash buffer for 5 min at room temperature and finally transported in a closed box to the plane or the on-Earth laboratory imaging facility. For both parabolic flights and on-Earth experiments, the exposure time was of 100 ms every 400 ms.

2.6 Image analysis and detection algorithm

Calcium imaging allows for the visualization of neuronal activity within the neuronal network. In general, segmentation of cells inside a field of view and analysis of fluorescent traces were made with Calima software [10]. For the flight experiments, some parts of the video were too noisy to be analyzed due to an unwanted motion of the microfluidic chip. Thus, a dedicated correction algorithm was applied, based on the Lucas-Kanade method using the optical flow functions of the OpenCv library on Python. This part is described in more details in section 3.1.2.

3 Results

3.1 Monitoring calcium activity

To evaluate the influence of gravity variations on a network of neurons during parabolic flights, one needs a readout for every gravity phase which can vary fast enough in a short time period, i.e. typically 30 s. Calcium imaging is particularly suitable because of the expected frequencies (around a few Hz [11]) and it allows real-time recording . Nevertheless, running these kind of measurements in the noisy environment of a plane remains challenging.

3.1.1 Standard activity in the laboratory

The standard activity of 10 days old hippocampus neurons was measured on earth in classical laboratory conditions with the exact same experimental setup that was used during the parabolic flights. Five different chips from different cultures were analyzed, with fields of view containing around 20 active neurons. A typical snapshot of a neuronal network is shown on Fig. 2 b). If the network was active, specific areas corresponding to neuron cell bodies were segmented to extract the time-evolution of the fluorescence signal i.e. calcium activity (Fig. 2 c)) and further evaluate their period of oscillation. The process was repeated for many neurons leading to a distribution of periods of oscillations observed in the network. On Fig. 2 d), one can see a wide distribution of periods of activity, centered around 5 s, and ranging from 2 s to 8 s, meaning we could expect to record a few oscillations during each gravity phase. The networks were labeled via immunohistochemistry to assess the viability and ratio between neurons and astrocytes (Fig. 2 a)). The viability was evaluated, with homemade FIJI routines, at 65% of living cells with 2 times more neurons than astrocytes.

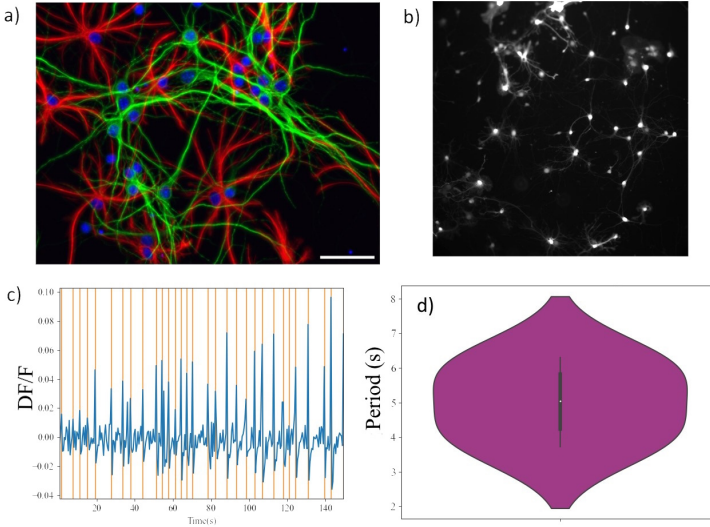


Fig. 2: a) Immunostaining of hippocampus neuronal network with DAPI (blue), MAP2 (green), GFAP (red). Scale bar is $50 \mu\text{m}$. b) Typical view of a 10-day-old hippocampal neuronal network. c) Example of the calcium activity of one hippocampal neuron. d) Distribution of the oscillation period of calcium activity of immature hippocampal neurons in 1g laboratory conditions. The average period is 5 s.

3.1.2 Corrections of image distortion

Running microscopy measurements during parabolic flights is challenging because of the many vibrations leading to possible loss of focus or undesired side motions of the samples. To remove undesired motions (vibrations) and distortions due to partial mechanical deformation of the glass wall during the hypergravity phase, a dedicated correction algorithm was developed. The algorithm used is based on the Lucas-Kanade method developed by Bruce D. Lucas and Takeo Kanade in 1981 [12] for computer vision. This method assumes that the displacement between two consecutive images is small and varies little around a given value, but also that the neighboring pixels have a similar speed. It is now commonly used to monitor the motion of objects or even as a visual sensor in closed-loop flow control experiments because of its high-efficiency leading to real-time velocity measurements [13, 14]. In the present study we used the optical flow functions of the OpenCv library on Python [15]. First step was to detect suitable points for the tracking algorithm, in order to follow their movement. In our case, this first step was very efficient thanks to the image resolution and the shape of the objects, which made it possible to have a very good measurement of movement. The second step consisted in tracking the objects and deducing the speed associated with the movement. A typical example is shown on Fig. 3 where the undesired motion induced by the

vibrations of the experimental setup was measured. This allowed to stabilize the snapshots by substracting the measured displacements to the time-series.

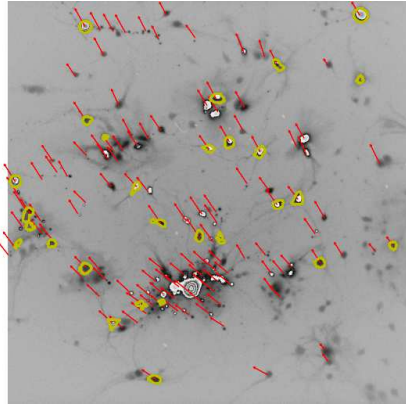


Fig. 3: Measurement of the unwanted motions of the sample during parabolic flights leading to corrections before processing the time-series of calcium activity.

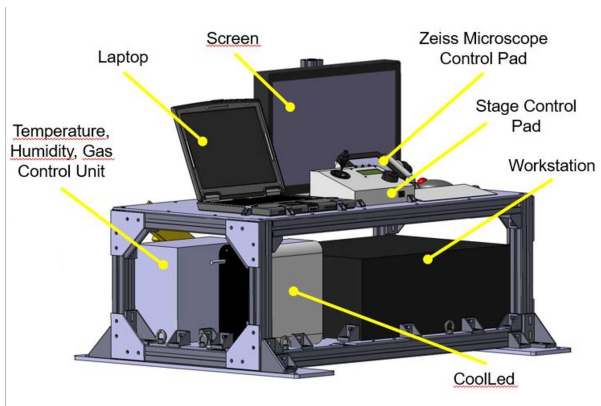


Fig. 4: 3D view of the monitoring rack (Rack 1). It allows for the control of all important parameters of the experiment as well as the recording of all data, especially visualization of the calcium activity.

3.2 Experimental setup adapted to Zero-G flights

To run the calcium imaging experiments in the Airbus Zero-G, we designed a dedicated setup composed of three racks, which is an evolution of a previous

setup used to study nanorods' self-propulsion [16]. The main constraints were related to the manipulation of living cells in a highly confined environment.

First, the monitoring rack was conceived to easily control every important parameter of the experiment (acoustic frequency and amplitude, optical illumination, etc). It also required to allow for quick data acquisition from a high-speed and highly sensitive camera, and digital microscopes as well as various sensors, such as an accelerometer (Fig. 4).

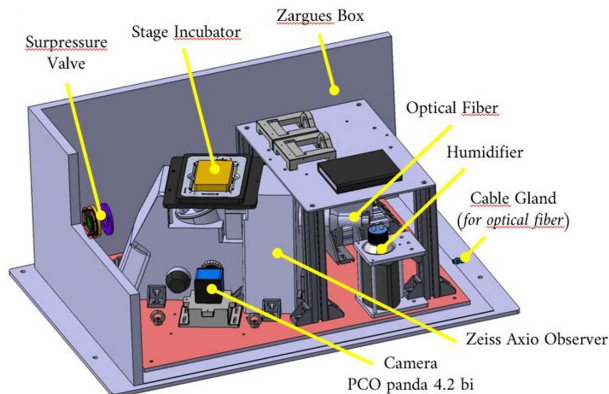


Fig. 5: 3D view of the experimental box (Rack 2). Inside a sealed Zargues box, we installed a Zeiss inverted microscope with a motorized stage. A stage incubator from TOKAI HIT was used to ensure a well-controlled environment for the cell cultures during the flights. The fluorescence images were recorded using a PCO Panda Bi camera.

Second, the experimental setup was designed to fit into a sealed Zargues box to insure a double confinement of liquids (Fig. 5). As previously described, the microfluidic chips were designed to contain a large number of cells in a minimal volume and to be easily sealed with a PDMS layer on top of the inlets. To be able to record the calcium activity of living neurons during the flight, a stage incubator (TOKAI HIT) was used to control the environmental conditions of the samples (37°C and $5\% \text{CO}_2$). The stage incubator was placed on an ASI imaging stage, remotely controllable with a computer installed on Rack 1. Both the stage and stage incubator were fixed on an inverted Zeiss microscope. A CoolLED PE4000 was used for illumination and a PCO Panda Bi camera for image acquisition. A 3D view of the whole experimental setup as installed in the Airbus ZeroG is shown in Fig. 6. The very same setup was used in the laboratory to record the 1g control experiment of the calcium imaging. The third rack was then used to secure the air/ CO_2 bottle on the floor of the plane (Fig. 6). Prior to each flight, the incubator was heated and filled with gas 1 hour before take-off.

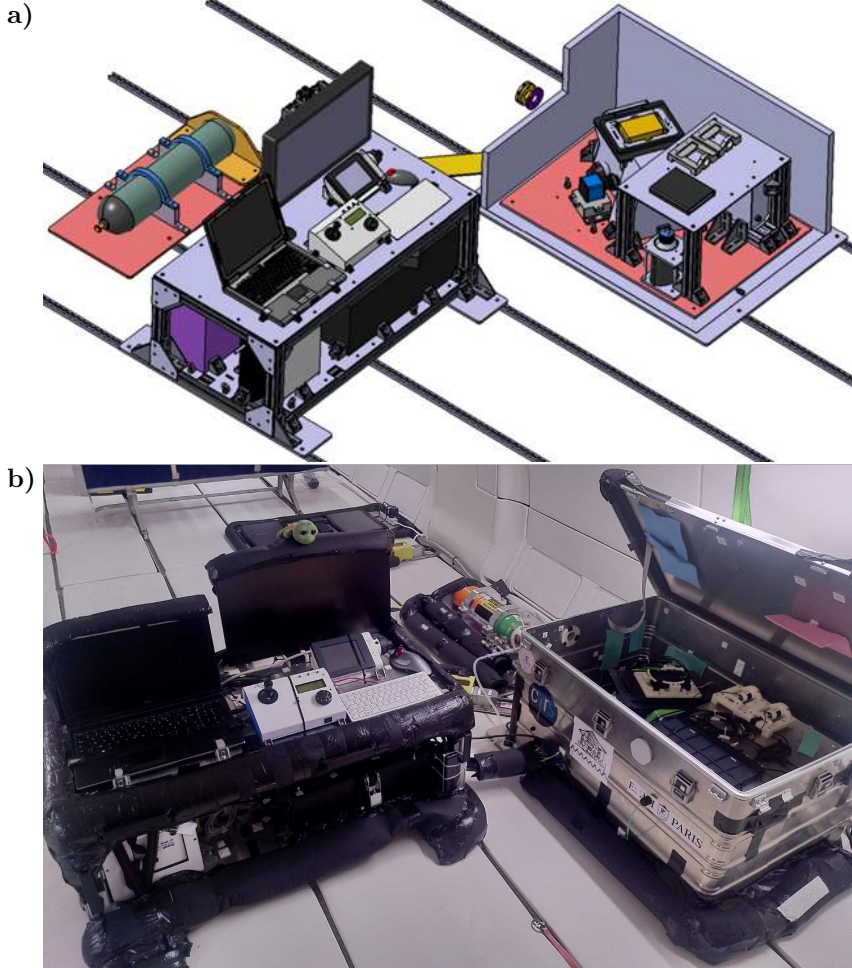


Fig. 6: a) 3D view of the full experimental setup (3 racks) used for calcium imaging during the parabolic flights. The racks were fixed on the floor in the Airbus ZeroG as shown on this 3D view. The two racks described above can be operated by two flying experimenters while the third rack in the back is used to fix the air/CO₂ gas bottle. b) Picture of the setup installed inside the Airbus AirZero G.

3.3 Monitoring Network calcium activity in Zero-G flights

During the three days of flights, we were able to image and record the calcium activity of 29 fields of views, each containing, in average, 6 active neurons. The recordings were started 10 s before the onset of the parabola, i.e. before the hypergravity phase (1.8g) and ended 12 s after the offset of the parabola, i.e. the second hypergravity phase (Fig. 7). Every parabola was composed of

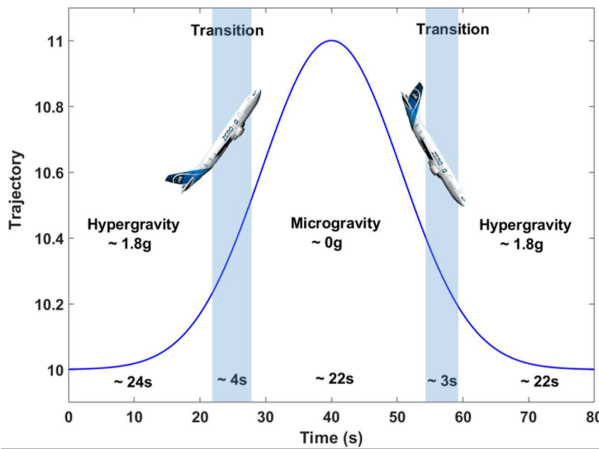


Fig. 7: Sketch showing the three phases of gravity during a parabolic flight. It starts with an hypergraviaty phase, followed by the zero(micro)gravity phase before a final hypergravity phase. From Dumy et al [16].

approximately 20 s of hypergravity followed by 22 s of microgravity and finally ending with 20 s of hypergravity. Thus, every recording was approximately 84 s long. Throughout the campaign, we managed to analyze in total around 10 min of calcium activity of hippocampal primary neurons in hypergravity and more than 10 min in microgravity. Considering that the second hypergravity phase, after the microgravity, was never stable enough in acceleration due to adjustments of the plane trajectory, we only took into account the first phase of hypergravity in the results thereafter. In the end, out of all the imaged neurons, around 60% showed a spontaneous activity and half of them reacted to changes in gravity.

Interestingly, many neurons didn't show activity at first, in the 1g phase, but started being active during the hypergravity phase and then presented activity for the rest of the parabola duration. Two typical time-series of calcium activity variations during the three phases of gravity are shown on Fig. 8a). One can qualitatively see the modifications of the activity, both in amplitude and period, for the hyper and microgravity phase compared to the 1g case. Fig. 8.b) shows a more quantitative analysis through violin plots. One can clearly see an increase in the averaged period of oscillation (from 3s in 1g to 4s in 1.8g and 0g), associated to a much wider distribution of the period of oscillation (reaching nearly 12 s for the 1.8g and 0g, compared to nearly 7 s for the 1g case).

We also observed a modification of the envelope of the violin distribution. Indeed, a bi-modal distribution of the neuronal activity period arises during both the microgravity and hypergravity phases (Fig. 8b)), which can be explained by the activation of neurons with a slower activity. However, it is difficult in the present experiment, due to the constraints of parabolic flights,

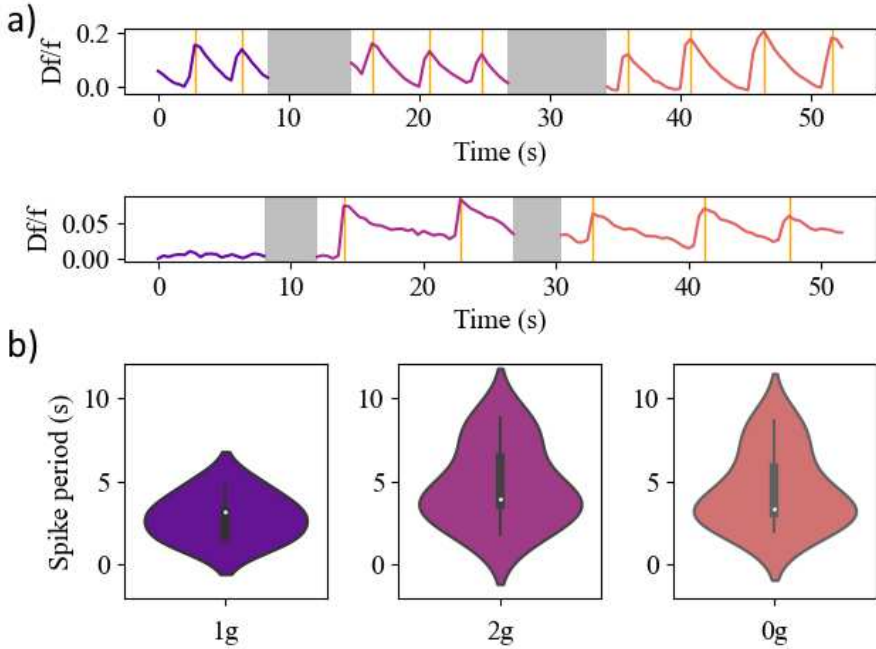


Fig. 8: a) Typical calcium activity of neurons imaged during a parabola. Grey areas correspond to blurred images associated to transition phases in the parabola shown in Fig. 7. The upper graph corresponds to a neuron already active during the 1g phase while the lower graph corresponds to the activity of a neuron triggered by the 2g phase. b) Violin plot of hippocampal neuronal network activity for each of the three phases of gravity.

to evaluate whether this change of activity was triggered by the 1.8g phase and maintained in the 0g phase or if it was also a response caused by the 0g phase itself.

Nevertheless, Fig. 9 shows a more detailed analysis of the activity of the neuronal network. Fig. 9.a) shows the evolution of the mean spike periods for different sets of neurons during the three phases of gravity. From Fig. 8, we first observed that the spike period seemed to increase in the hyper and microgravity compared to the 1g case. It was confirmed quantitatively in the Fig. 9b) where we show the ratio of spike activity compared to the 1g case. The median variations of spike period between 1g and 1.8g phases $\alpha_{1 \rightarrow 1.8g}$ and between 1g and 0g phases $\alpha_{1 \rightarrow 0g}$ are both superior to 1, showing that the global network activity indeed slows down. We also notice that the dispersion of $\alpha_{1 \rightarrow 1.8g}$ is bigger than $\alpha_{1 \rightarrow 0g}$ even though the two distributions are statistically equivalent.

The second important observation was the triggering of the activity of neurons that were not previously active in 1g. On Fig. 9a), we can see nearly three

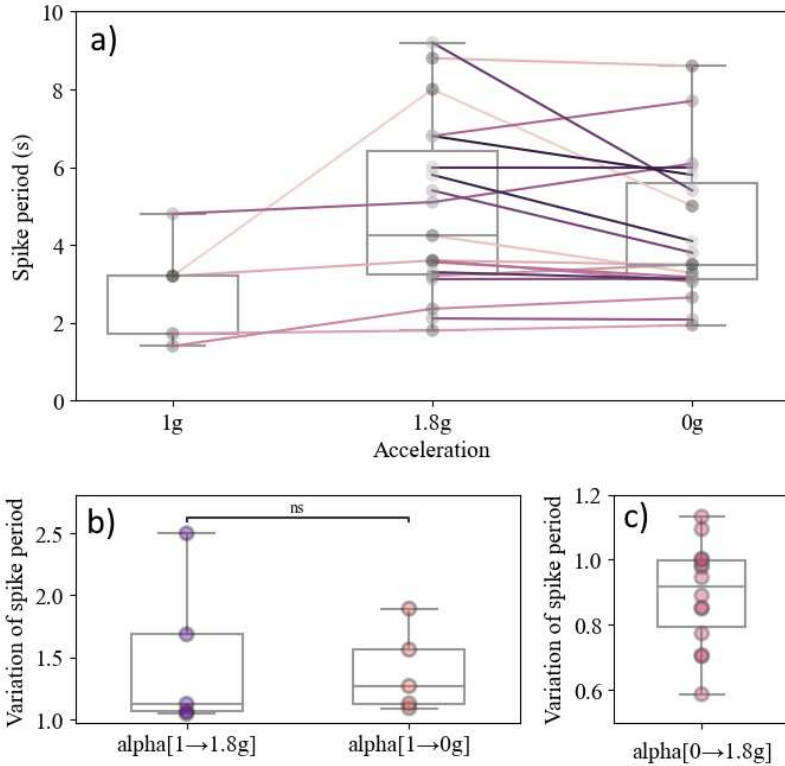


Fig. 9: Activity of primary hippocampal neurons during parabolic flights. (a) Diagram showing the spike period of active neurons computed for each of the three phases of gravity. Each point corresponds to the average spike period for one field of view containing between 2 and 10 neurons. The points that are linked are associated to the same group of neurons. (b) Relative spike period variation of the neurons compared to the 1g phase for those which already presented activity at 1g. (c) Relative spiking period variation of the neurons between the 1.8g and 0g phases, for those which did not present activity at 1g.

times more active networks in the 1.8g and 0g phases than in the 1g phase. Indeed, 5 neuronal networks were active during the 1g phase, compared to 14 which only got activated during the hypergravity phase. For all of those networks, once the neuronal activity was triggered in the 1.8g phase, the neurons remained active in the 0g phase. Furthermore, from Fig. 9c), one can also infer that the spike period of these newly activated neurons was, in average, reduced in the 0g phase compared to the 1.8g phase, as the median of $\alpha_{1.8 \rightarrow 0g}$ is below 1. This means that the networks activated by the hypergravity phases mostly

had a faster activity in microgravity. Overall, these results confirm the influence of the gravity variations on the calcium activity of immature hippocampal neuronal networks.

4 Discussion

There have been relatively few studies on the effects of weightlessness on the brain, and yet decades of observations on space-flown animal models and human astronauts have confirmed that physiology is profoundly impacted by spaceflight [17–19]. Growing evidences show that prolonged exposure to spaceflight and microgravity environment leads to alteration of brain function and structure, as suggested by recent analyses of astronauts’ brain fMRI[5]. Even at a smaller scale, microgravity is known to have a direct effect on cells, such as themodification of their cytoskeleton or changes in their physical properties [20, 21]. While neuronal connectivity and brain activity are highly dynamical and intermingled processes, this calls for the detailed study of the impact of microgravity and/or space environment on the intimate functions of brain networks.

Probing alteration of brain connectome and neuronal networks functions under microgravity conditions remains a very challenging task. This, indeed, relies on the recording of fast and transient signals generated by brain networks typically recorded trough EEG at the whole brain level, or through electrophysiological or calcium imaging studies at the cellular (neuronal) level. While many studies have been developed to monitor the differences in the behavior of mammalian cells maintained in cell culture under simulated or real microgravity environment [22–25], most of those have been conducted without live imaging at the cell level.

In order to tackle these issues, our goal was to setup and validate an integrated and automated live cell imaging platform for recording fast calcium transients in living neuronal networks during parabolic flight campaigns. This encompassed the adaptation to Zero-G airplane confinement and security prerequisite, like fluid management, gas leaking security, and vibration. It required the engineering of a conventional setup composed of an inverted automatic microscope setup, fitted with a cell culture incubator, fast LED illumination and CMOS camera.

One issue was to choose the proper cells to grow a mature neuronal network, taking into account the constraints of zero-g flights. In this very specific environment, while manipulating human derived neurons would be of great interest, handling the cells during a parabolic flight remains an issue for security reasons. To benchmark our experimental platforms and still obtain relevant results, we therefore chose to handle mouse-derived neurons. A microfluidic cell culture environment presented several advantages compared to conventional ones. Indeed, beside its high versatility and fast prototyping capacity allowing to tailor cell culture chambers for specific experiments, microfluidic chips allowed excellent control of the fluidic environment during

parabola. Sealing the microchips inlet and outlets allowed to avoid back and forth cell medium movement and therefore avoided any risk of fluid spillover and allowed mitigation of fluid shear stress during tilting of the plane.

On top of this, at a cellular level, a limitation in our experiments was linked to the photobleaching and phototoxicity of the calcium fluorescent probe (Fluo4-AM) used to image calcium activity. With a high dynamic range and no photobleaching, genetically encoded Ca^{2+} sensors such as GCaMPs exhibit large increases in fluorescence sensors and could therefore drastically improve the quality of the signal recorded during the 4 hours sessions of parabolic flights [26]. This option will be evaluated during future parabolic flight campaigns.

Furthermore, while we matched media and environmental conditions for ground and flight samples as closely as possible, we note additional variables. Different activity on the ground compared to 1g in the plane, may be due to take-off accelerations, as well as temporary changes in experimental conditions (heating and CO₂ are turned off for safety reasons during take-off which takes around 30 min). These are limitations related to the particular conditions of parabolic flights that can not be avoided.

Overall, using the aforementioned newly developed platform and this experimental protocol, we succeeded in recording the spontaneous activity of neuronal networks derived from rodent primary neurons, grown in microfluidic chips, during parabolic flights. We observed important variations of neuronal behavior that encompassed two distinct phenomena. First, a slowdown of the spontaneous activity frequency due to the variation of gravity, as the period between spikes increased in 1.8g and 0g conditions compared to 1g. Second, we observed significant changes in the percentage of active neurons. Indeed, the transition from 1g to 1.8g led to activation of silent, non oscillating, neurons that remained active during the rest of the parabola. While this substantiates the notion that changes in gravity modify neuronal oscillatory behavior, our results raise several questions. First, we noticed that both neuronal firing probability and frequency of oscillation were similarly affected by 1.8g and 0g conditions. This is in line with previous observations that showed that while 0g led to membrane hyperpolarization and 1.8g led to depolarization, intracellular calcium levels of immortalized neuronal cell lines rose as well under hyper as under microgravity [27]. More recently, a study performed on human iPSC derived neurons interfaced with MEA recording platforms evidenced slight increases in network's bursting frequency during both 0 and 1.8g phases in a dropping tower experimental paradigm [24]. This raises the possibility that a memory or hysteresis effect occurs at the cell membrane level that lasts during the following gravity phases.

Second, our results shows that 0g (and 1.8g) conditions lead to a decrease in neuronal firing rates. While this may seem contradictory with results showing that intracellular calcium level increases in SY5Y immortalized cell lines and iPSC human neurons, our experimental platform significantly differs from the previously described one. Indeed, we recorded the single cell activity of relatively young, immature, primary hippocampal neurons. At that developmental

stage, hippocampal neurons behave as self-sustained oscillators [28] in a non-synchronous manner regarding the rest of the network. On the contrary, at later developmental stages, massive networks show synchronous activity which may arise through a percolation process and the setting up of both feedforward and feedback activity loops between excitatory and inhibitory neurons contained in the culture [29, 30]. Yet and importantly, both our study and the one performed in dropping tower show that changes in gravity significantly activate silent or very slow firing rate neurons. In their review, Florian P.M.Kohn and Ramona Ritzmann [31] discuss a first model of neuronal short-term adaptation to changes in gravity which seems adapted to the timescale of parabolic flights. This takes into account the findings on the different levels of organization predicting opposing results on neuronal firing pattern depending on network connectivity. Acknowledging that long term changes in gravity lead to modification of synaptic connectivity [32], longer recording time over several parabolas, would allow to study how neuronal networks integrate and self-adapt to such transient and periodic changes at the whole population level. One improvement for future parabolic flight campaigns could be to observe the activity of more mature cells in order to be able to analyze the influence of gravity variations on network dynamics and not only on isolated neurons.

As humans will spend more time in space, we must better understand the effects of spaceflight on human physiology at the cellular level. In the present study, we demonstrated that live-imaging of primary neurons can be achieved in parabolic flights leading to a clear demonstration of the modification of neuronal activity by changes in gravity. Further investigations into neuronal response to spaceflight are necessary to confirm these results and elucidate the mechanisms of response to gravity modifications. Improvements can be achieved by using three-dimensional, tissue-like structures, such as cerebral organoids, which would provide a more physiologically accurate model and allow the study of interactions between multiple cell types [33]. At longer time scales, astronauts do not suffer from dramatic modification of neuronal activities. Certainly, even though gravity variation induce transient changes in rhythmic activity, several modifications of cells homeostasis occur at whole network levels to reset normal global activity through molecular adaptations.

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