c-Fos immunoreactivity and variation of neuronal units in rat's motor cortex after chronic implants

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Abstract— **Recovering of people suffering from spinal cord and brain lesion is a medical challenge. Brain-machine interface (BMI) emerges as a potential candidate, by allowing patients to use their own brain activity to reestablish sensorimotor control of paralyzed body parts. BMI can be divided in two main groups: non-invasive, based in the capture of the neuronal signal over the cranium, and invasive, much more effective in generating high resolution brain-derived motor control signals, despite requiring a brain surgery for implantation of recording microelectrodes. Accordingly, chronic multielectrodes implants define the fundamental component of an invasive BMI. However, it is important to characterize the impact of microwire arrays' implant on the nervous tissue before this technique can be available to human clinical trials. Here we evaluated the expression of immediate early-gene *c-fos* and inflammatory response (astrogliosis), as well as the quality of the neuronal signal comparing the variation of the total number and the amplitude of the recorded units after long-lasting chronic multielectrode implants. Electrode recordings remained viable for 6 months after implant, and did not alter the general physiology of the implanted tissue, as revealed by normal c-Fos expression in implanted sites. Moreover, there was a small inflammatory response across implanted regions. Our findings suggest that tungsten microwire arrays can be viable candidates to future human BMI interventions.**

***Index terms*—Brain-machine interface; chronic implants; electrophysiology; multielectrode; tissue integrity.**

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I. Introduction

TRAUMATIC spinal cord and brain injuries impose a serious impairment in the quality of life of millions of individuals [1]-[3]. For instance, every year, around 1.7 million people seek medical care with brain/spinal cord lesion only in the United States of America [4], generating, further to the direct impact in the patient's quality of life, a severe socioeconomic burden to the health care system [5], [6]. In Latin America and the Caribbean, traumatic brain injury also appears as a critical public health concern [7]. In light of this, one of the main medical challenges in this field involves the development of therapeutic methods capable to ensure a better quality of life for those suffering with body paralysis. Brain-machine interface (BMI), which provide a new approach for establishing direct communication between the brain and an external apparatus [8], emerges as a potential candidate to improve the lives of people suffering with devastating neurological disorders [9].

Based in the location of apparatus used to collect neural signal, BMIs can be divided in two main groups: non-invasive and invasive. A non-invasive BMI, such as the electroencephalogram (EEG), is based in the capture of the neuronal signal over the cranium. The advantage of this approach is exactly the non-invasiveness of the method, excluding any kind of brain surgery and allowing its use by any patient. Though, this technique has some limitations, since the signal collected is not entirely free of artifacts and lack on the spatial resolution required to extract motor signals necessary to reproduce fine movements in an artificial device. Invasive techniques, conversely, even demanding a brain surgery for implantation of recording microelectrodes, are much more effective in generating high resolution brain-derived motor control signals [10].

Chronic multielectrodes implant is the major component of an invasive BMI apparatus. Its use assurances a precise assessment of the neuronal activity in awake, behaving animals [11], [12], representing a crucial tool for improving our understanding about the neurophysiological principles that govern the interactions of large populations of neurons. Accordingly, BMIs have been employed successfully in animal models by several groups around the world [13]-[16]. However, before microwire arrays can be effectively available to human clinical trials, it is necessary to draw a whole picture of their impact on the nervous tissue.

Here we examined the impact of chronic implant of tungsten multielectrodes in the rat's motor cortex by evaluating the pattern of expression and distribution of c-Fos

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immunoreactive cells, an immediate early-gene employed as a marker of neuronal activity [17], and inflammatory response (astrogliosis). We also assessed the quality of electrophysiological recordings by analyzing the variation of the total number of neuronal units, the mean amplitude of units sorted throughout the recording sessions and the Local Field Potential (LFP) across 1, 3 and 6 months of implant.

II. METHODS

A. Experimental procedures and electrophysiological recordings

Fifteen adult male Wistar rats (325±25g) obtained from the Edmond and Lily Safra International Institute for Neurosciences of Natal (ELS-IINN) Animal Facility were used. All experimental procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), and were approved by the ELS-IINN Committee for Ethics in Animal Experimentation (ID # 13/2011). All efforts were employed to avoid animal suffering and to reduce the number of subjects used.

Multielectrode implantation was performed in animals anesthetized with a mixture of 100 mg/kg of ketamine chlorhydrate and 5 mg/kg xylazine chlorhydrate (i.p.) as detailed elsewhere [11]. In brief, the animals were positioned in a stereotaxic apparatus, and a craniotomy was made over the implant target area (primary motor cortex) based in the following coordinates: 1.0-3.0, anteroposterior (AP); 2.0-3.0, mediolateral (ML); and 1.8-2.0, dorsoventral (DV) (in millimeters relative to bregma) [18]. After craniotomy, multielectrode arrays (4x8 with 500 µm spacing) made of 32 Teflon-coated tungsten electrodes (50-µm microwire diameter, 1.5 MOhm at 1.0 KHz), attached to an Omnetics connector (Omnetics Connector Corp., Minneapolis, MN, USA) (Fig. 1a) were gently implanted in order to avoid bleeding and mechanical injury of the tissue. Electrode impedance was tested before implantation to ensure their functionality. Stainless steel screws and dental acrylic were used to hold the implant. After one week of surgical recovery, animals started to be recorded weekly. Three survival time groups were defined, according to the total time of recording: 1, 3, and 6 months after implantation, which allowed us to compare the progression of the impact of electrode persistence on the nervous tissue.

A 32-channel multi-neuron acquisition processor (MAP, Plexon Inc., Dallas, TX, USA) was employed for digital spike waveform discrimination and storage (Fig. 1b). Single-unit recording sessions lasted for at least 45 minutes weekly, while the rats were allowed to move spontaneously into their cages (Fig. 1c). Online spike sorting was performed with SortClient 2002 software (Plexon Inc., USA). A maximum of 4 neuronal action potentials per channel were sorted online and then validated by offline analysis (Offline Sorter 2.8, Plexon Inc., USA) according two criteria: signal-to-noise ratio >2.5 (as verified directly on the oscilloscope screen) and general stereotypy of waveform shapes, as determined by a waveform template algorithm and principal component analysis (PCA). Local field potentials (LFP) were simultaneously recorded from the wires and pre-amplified (500x), filtered (0.3-400 Hz), and digitized at 500 Hz using a Digital Acquisition board (National Instruments, Austin, TX, USA) and a MAP box (Plexon Inc., USA).

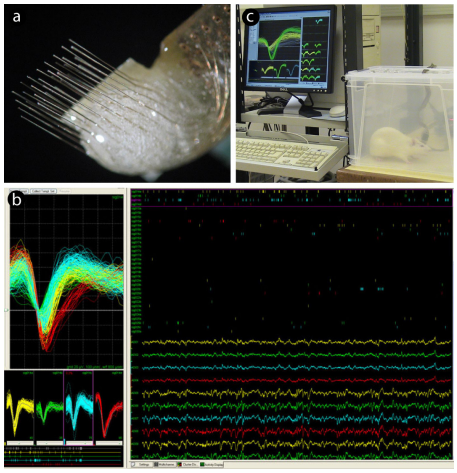

Fig. 1. General features of multielectrode array and electrophysiological signal recording. (a) Example of a tungsten 32 multielectrode array. (b) Selection of four neurons during real-time recording three months after electrode implant, represented by sorted waveforms of distinc colors. (c) Recording session after 1 month of electrode implant, with the animal moving freely in its cage.

B. Perfusion and tissue processing

After the final section of electrophysiological recording, animals were anesthetized with 5% isoflurane and overdosed with sodium thiopental (90 mg/kg), being perfused through the left ventricle with 0.9% warm heparinized saline followed by cold 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4. Afterwards, the brains were removed from the skull and immersed in 20% sucrose in 0.1M PB for 12h. The brains were then frozen in a embedding medium (Tissue Tek, Sakura Finetek, Japan) and sectioned frontally at 30µm in a cryostat (Carl Zeiss Micron HM 550, Germany). The sections were then mounted on electrically charged glasses (Super Frost Plus - VWR International, USA) and submitted to c-Fos immunohistochemistry as follows: the slides were washed for 20 minutes (2x, 10 minutes each) in 0.1M PB and then incubated in a blocking buffer solution (0.5% fresh skimmed milk and 0.3% Triton X-100, in 0.1M PB) during 30 minutes to block non-specific binding. Next, sections were incubated overnight in c-Fos primary antibody (1:100, Santa Cruz Biotechnology, USA; diluted in blocking buffer) at 18°C, washed in 0.1M PB during 10 minutes (2x, 5 minutes each), incubated in a biotinylated secondary antibody (1:200, Vector Labs, USA; diluted in 0.1M PB) for 2h, washed during 10 minutes (2x, 5 minutes each) in 0.1M PB, and incubated in avidin-biotin-peroxidase solution (Vectastain Standard ABC kit, Vector Labs, USA) for 2h. Sections were finally incubated in a solution containing 0.03% 3,3' diaminobenzidine (DAB) (Sigma Company, USA) and 0.001% hydrogen peroxide in 0.1M PB, being intensified with 0.05% nickel ammonium sulphate. To certify the specificity of the labeling, the

primary antibody was replaced by normal serum in some test sections. At the end of the procedure the sections were dehydrated in alcohol gradient, cleared in xylene and coverslipped with Entellan (Merck, Darmstadt, Germany).

Inflammatory response is a crucial event underlying chronic implants. In order to evaluate the pattern of astrocytosis around microelectrode's implant sites in the present work, some sections were stained with an antibody against the glial fibrillary acid protein (GFAP) using fluorescent labels. In brief, sections were washed during 10 minutes in 0.1M PB-Tween (PB-T) and pre-incubated in 10% goat normal serum during 30 minutes. The sections were then incubated during 24h with primary antibodies (GFAP; 1:500; DAKO, Glostrup, Denmark) at 18ºC. Sections were washed in 0.1M PB-T during 5 minutes and incubated with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody overnight (1:700 in 1% normal serum, Invitrogen, Grand Island, NY, USA). Finally, the sections were mounted using Vectashield mounting medium for fluorescence (Antifade solution) (Vector Laboratories, Burlingame, CA, USA).

C. *Qualitative and quantitative analyses*

c-Fos-immunoreactive cells in the region around electrode tracks in all survival time points evaluated (1, 3, and 6 months after implant) were counted with the help of the Neurolucida software (MBF Bioscience Inc., Williston, VT, USA). Three sections per animal were quantified (in an interval of 100µm from each other), encompassing tissue from regions where the electrode tracks could be clearly observed (n = 5 animals by group). Cell density (cells/10,000 μm^2) was measured using an automatic grid from Neurolucida. The non-implanted contralateral hemisphere was adopted as an intrinsic control in all groups. Average values for all measurements were obtained with non-parametric Kruskal-Wallis test followed by a Bonferroni *post hoc* test (level of significance 0.05). Average values were expressed as mean ± standard error of mean (SEM). Digital images were acquired with a CX9000 camera (MBF Bioscience Inc., USA), attached to a Nikon Eclipse 80i optical microscope (Nikon, Japan - 4x, 10x and 20x objectives). Fluorescent images were obtained in a Carl Zeiss Laser Scanning Microscope (LSM 710, 10x and 20x objectives, Carl Zeiss, Jena, Germany). Contrast and brightness of pictures were adjusted using Photoshop CS5 software (Adobe Systems Inc., San José, CA, USA).

The pattern of the electrophysiological recording was evaluated comparing the total number of neuronal units detected in each group, the mean amplitude of units sorted throughout the sessions, and the measurement of LFP, usually used to detect variations in recording quality over time. To measure LFP, we performed an analysis of power spectrum for different bands of frequency in function of time for channels with and without spikes. The LFP signal was initially separated in standardized spectral bands (0-4, 4-8, 8-12, 12-24 and 24-60Hz). For every animal the band power was further divided by the total LFP power. Channels with spikes existent were then compared to channels without spikes at different time points.

III. RESULTS

A. *Variation of neuronal units over time*

The evaluation of the temporal variation in the number of neuronal units in each group during its time of implant, comparing the first and the last week of implantation in all survival times, allowed us to evaluate the level of signal improvement in chronically implanted animals. Our analysis showed an increase in the total number of neuronal units recorded 3 months after implant as compared to 1 month of implant. In latter survival time (6 months of implant) the number of recorded cells decreased (Fig. 2a). The average number of recorded neurons across animals demonstrates the presence of neurons functionally active after 6 months of electrode implantation (Fig. 2b). Mean amplitude of neuronal units recorded across time points evaluated followed this trend. We identified low amplitude after 1 month of recording as compared to 3 months after implant, which decreased 6 months after implantation (Fig. 2c).

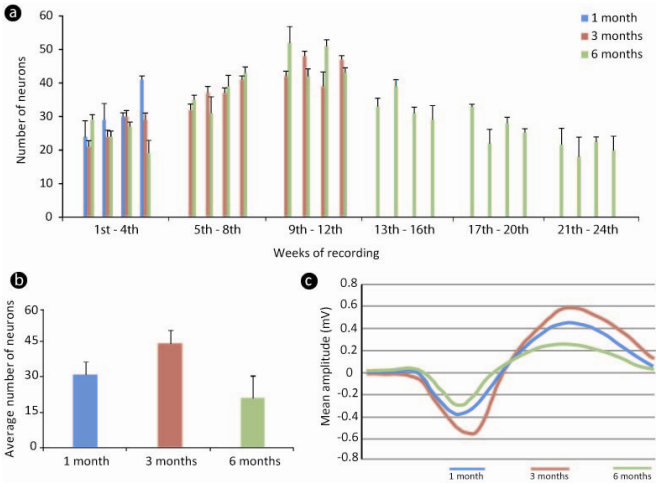

Fig. 2. Variation of the amplitude and the number of neuronal units over time. (a) Temporal variation of total number of recorded neurons month by month. Notice the peak of neuronal units 3 months after electrode's implantation. (b) There was an increase in the mean number of neuronal units recorded 3 months after implant when compared to 1 month of implant. In latter survival time (6 months after implant) the mean number of recorded cells decreased. (c) Mean amplitude (mV) of the units recorded in each time point evaluated. The amplitude increased 3 months after implant as compared to 1 month of recording, then decreasing 6 months after implantation.

B. *LFP power analysis*

The relative LFP power in different bands varied consistently in both groups of channels (Fig. 3), suggesting that neuronal signal remained effective even in the latter survival time (6 months of implant). Normally the LFP signal is very robust and changes little over months. In fact, it is very common that one can still record LFP from a given

channel even after no spike signal can be detected. Nevertheless, LFP can be used to detect variations in recording quality over time.

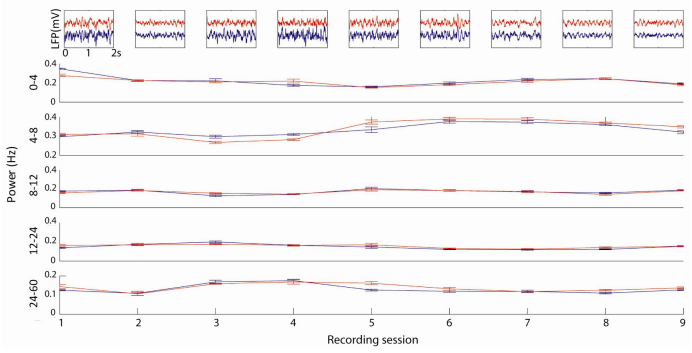

Fig. 3. Local field potential (LFP) power across distinct frequency bands over time. Top panels show raw LFP signals recorded across distinct time points, collected from channels with of without spike signals (red and purple lines, respectively). Bottom panels show the temporal variation of the LFP power within standard spectral bands. LFP bands varied consistently in both groups of channels, indicating that neuronal signal remains effective even in the latter survival time evaluated.

C. *c-Fos expression in chronic implanted sites*

A normal pattern of c-Fos expression was identified across implanted sites in all groups evaluated, with a similar pattern in the contralateral hemispheres (Fig. 4a). There was no significant difference among contralateral and implanted regions until 6 months of electrode implant, with cell profiles varying from intensely to weakly reactive (Fig. 4b). Additionally, quantitative analysis revealed no difference between the amount of c-Fos-reactive cells when regions near and far from electrode implant were compared in all time points examined (Fig. 4c). Test sections used as non-reactive control (replacement of primary antibody by normal serum) did not show nuclear labeling, indicating the absence of unspecific labeling or contamination stain.

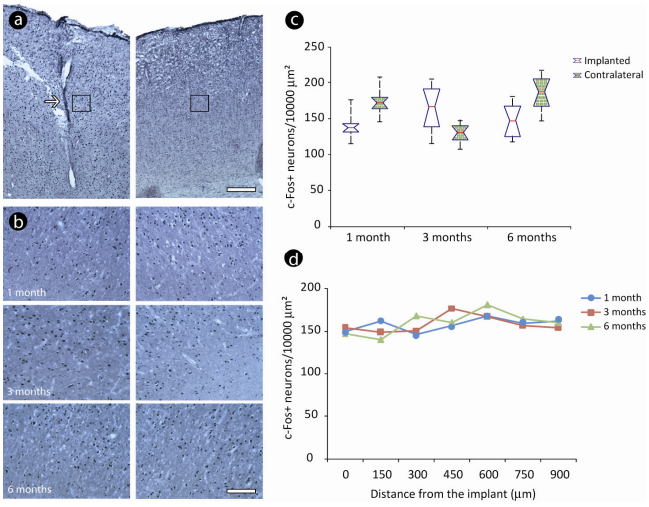

Fig. 4. c-Fos expression across multielectrode implanted sites. (a) It was possible to identify a similar pattern of c-Fos-labeling in both implanted (arrow) and contralateral hemispheres, with profiles varying from weakly to intensely reactive in all groups evaluated (b). (c) There was no significant difference between contralateral and implanted regions among distinct survival times. (d) The number of c-Fos-reactive cells did not differ significantly when regions near and far from implanted regions were compared. Values expressed as mean ± SEM. Scale bars: (a) 300 µm; (b) 100 µm. Black squares in (a) indicate the regions where high power pictures were obtained in all groups.

D. *Astrocytic activation around electrode tracks*

Activation of astrocytes was observed around the electrode tracks mainly after 6 months of implant, appearing as a stripe of labeling circumscribing the implant site (Fig. 5), defined by the presence of cells displaying hypertrophic amoeboid cell bodies and shorter and thicker processes. In the contralateral hemisphere, conversely, astrocytes possessed a non-activated morphology.

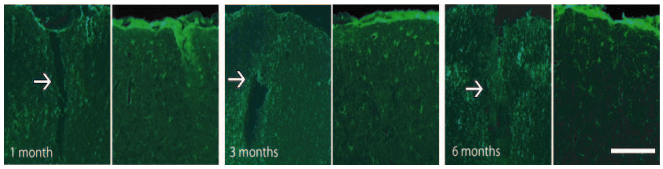

Fig. 5. Astrocytic immunoreactivity across electrode tracks. GFAP-reactive astrocytes with a hypertrophic morphology were observed only in the vicinity of implants (arrows), as a stripe of labeling. Activated astrocytes were not observed far from implant sites neither in contralateral hemispheres. Scale bar: 100 µm.

IV. DISCUSSION

We investigated the pattern of neuronal recordings in the motor cortex of chronically implanted rats with tungsten multielectrodes. Also, we evaluated the impact of the implant in the basal physiology by the analysis of c-Fos immunoreactivity in implanted regions, comparing them to the contralateral non-implanted hemisphere and also the degree of inflammatory response induced by electrodes. Our results pointed out that chronic microelectrode arrays remained viable for 6 months after implant, exhibiting a peak of high signal quality after 3 months of implantation. Moreover, both distribution and general reactivity of c-Fos-reactive cells were not affected by chronic implants. A small and circumscribed inflammatory response was observed only around implanted regions.

Currently, the development of stable and compatible devices to be incorporated to the human body is one of the major challenges in the field of neuroengineering. Distinct types of materials have been tested to develop a biocompatible multielectrode array as most efficient and tolerable by the nervous tissue as possible [19]-[22]. Other elements to be taken into account besides the material are the size, shape, texture and tip geometry of the array [23]-[26].

Tungsten electrodes have been used successfully to recording neuronal activity by long-lasting periods in both rodents and non-human primates [27]-[29]. Nonetheless, some questions related to the impact of the long-term presence of the electrodes in neuronal parenchyma remain to be elucidated. In a previous study our group described a small degree of tissue alteration induced by chronically implanted tungsten microelectrode arrays, including a small loss of tissue reactivity limited to the sites of electrode

implant, and a reduced cell death around electrode tracks [30]. Such alterations, nevertheless, did not involve any measurable functional loss since normal physiological neuronal responses were obtained at the implanted sites [30]. We hypothesize that this can be associated to the high flexibility of the microelectrode's shaft and the shape of its tip, since tungsten arrays are rigid to allow tissue penetration, but are flexible enough to preclude widespread mechanical injury due to their relative movement inside the nervous tissue. Following the same line, [31] described small and circumscribed alterations in tissue milieu after chronic presence of tungsten electrodes, especially related to its progressive encapsulation. Such event is related to gliosis, with consequent formation of a glial scar, a process widely involved with the variation of electrodes' impedance, mainly in the first weeks of implant [31]. So, inflammation seems to be the main event associated to decrease of electrode functionality [30].

Our present results corroborate our previous report related to a decay of neuronal activity across time [30]. However, even in latter time of implant (6 months), we still were able to record a significant number of functional neurons, indicating that the cellular environment remains preserved. Such notion is reinforced by the results of c-Fos immunohistochemistry. Immediate early-genes (IEGs) are a class of genes that respond rapidly and transiently to a variety of stimuli, being useful markers of neuronal activity in both normal and altered states [32]-[34]. In order to evaluate c-Fos baseline levels of activity, we focused in the normal amount of stimulation, induced by the spontaneous movement of the animals in their cages. There was not significant change in c-Fos expression over time, a result similar those described previously using other markers of cell metabolism, such as cytocrome oxidase [30]. Since the physiology of the nervous tissue was not broadly affected, the decaying of neuronal units should be associated rather to the insulation of electrodes by glial response than by tissue failure across implanted regions. In addition, the normal activation of IEGs in implanted regions can be actively involved in the process of cortical plasticity required to neuroprosthetic skills [35], [36].

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