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Increased Performance in Genetic Manipulation by Modeling the Dielectric Properties of the Rodent Brain *

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Abstract— **Genetic approaches to control DNA expression in different brain areas have provided an excellent system to characterize gene function in health and disease of animal models. With respect to others, *in utero* electroporation of exogenous DNA into progenitor cells committed to specific brain areas is the optimal solution in terms of simplicity and velocity. Indeed, this method entails one quick and easy surgical procedure aimed at DNA injection in the embryonic brain followed by brief exposure to a strong electric field by a bipolar electrode. Nevertheless, the technique is still lacking the necessary control and reliability in addressing the field. Moving from a theoretical model that accounts for the morphology and the dielectric properties of the embryonic brain, we developed here a set of novel and reliable experimental configurations based on the use of three electrodes for electroporation in mouse. Indeed, by means of a full 3D model of the embryonic brain and the surrounding environment, we showed that the distribution of the electric field can be finely tuned in order to target specific brain regions at a desired temporal window by proper placement of the three electrodes. In the light of this theoretical background, we manufactured a three-electrode device and performed model-guided experimental sessions. The result was an increased spatial control, extended time frames and unprecedented reliability of the genetic manipulation, with respect to the current state of the art. In particular, the outcomes of this method applied into the mouse model are reported here for the first time.**

I. Introduction

In the last few years, *in vivo* genetic manipulation of specific cell types at different brain areas has become a crucial step to investigate the role of particular genes in the regulation of the morpho/functional organization of neuronal networks in health and diseases [1]. Typically, exogenous nucleic acids are introduced into the cells for the up-regulation or downregulation of genes coding for specific proteins of interest [2]. Moreover, with the recent advent of inducible systems, a fine temporal regulation of gene expression is currently possible [3]. Among these genes, genetically encoded, light-based proteins (e.g. green fluorescent proteins and optogenetic probes) have been successfully engineered to monitor and also interfere with the functional activity of the complex organization of neuronal networks [4]. Thus, all these procedures have greatly extended the possibility for *in vivo* investigations of ongoing dynamics within brain circuits. Currently, information propagation can be potentially detected with spatial resolution at the single-cell level over temporal windows lasting months. To this aim, it is essential that the procedure for genetic manipulation ensures the optimal preservation of circuit integrity over long periods with minimal structural and functional alterations of cellular and network functionality. In this perspective, *in utero* electroporation of exogenous nucleic acids presents several distinct advantages compared to more common techniques like virus-mediated DNA delivery and generation of genetically modified animals. Most conveniently, it presents almost absent cellular toxicity, straightforward procedure and quick experimental approach. The *in utero* electroporation technique is based on the direct injection of plasmidic DNA encoding the molecule of interest into the brain of embryos (at the level of the ventricular system), followed by application of a properly addressed electric field by means of two extra-uterine forceps-type electrodes. This allows targeting embryonic progenitors of specific populations of neurons committed to defined brain areas. By destabilizing the structure of the lipophilic cell membrane, electrical pulses induce the generation of transient pores, which allow negatively charged molecules of DNA to flow into the cell driven by the electric field [5]. With expertise, success rate attest to 70-90 % of born pups, if electroporation is performed in its classical configuration (targeting of progenitors of neurons committed to the somatosensory cortex by two forceps-type electrodes) [6]. Unfortunately, the number of brain regions where *in utero* electroporation can be effectively performed and the temporal window useful for the procedure are currently very limited. This is not due to the physiology of the brain, as in theory one could address all brain regions by simply targeting their specific neuronal progenitors. Failure of electroporation to brain regions other than the somatosensory cortex, is rather due to technical constraints in the placement

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of the electrodes on the embryo's heads while inside *the uterus*, and the physics behind proper targeting of the electric field by two parallel electrodes.

To extend the spatio-temporal window of genetic manipulation achievable by means of *in utero* electroporation, our group developed a computational approach that took into account the geometrical and dielectric properties of the system [6], starting from a model of the embryo brain at specific ages. This innovative approach enabled for a better understanding of the distribution of the electric field according to the brain morphology, and also guided the design of an optimized hardware apparatus extending the performances of the standard available electroporation tools. The outcome in mouse of this novel method is presented here as a fundamental demonstration of the potentiality of the technique and it extends the initial proof of principle demonstrated in rat [6].

II. Material and Methods

A. Brain model design and parametrization

The full 3D volumetric model of the embryo brain at embryonic day 16 (E16) was rendered from serial sections (spaced 0.2 mm) commonly available in brain atlases [7]. Then, the rendering was imported into the platform COMSOL multiphysics. Care was provided to ensure proper definition of the boundaries corresponding to the ventricular system, where exogenous DNA was injected experimentally. A simplified mesh corresponding to the brain was grouped with that of the environment surrounding the brain and that of the electrodes for the application of the electrical field. Finite element method was utilized to solve the electromagnetic problem by calculating both the electric field distribution and the current density. In order to set the correct dielectrical configuration of the system, distinct permittivity and conductivity values were recovered from dispersion data available in the literature for the 1-100 Hz frequency band of the different electrical domains and materials [8] (permittivity and conductivity of ventricular environment: epsilon = 80, sigma = 1.6 S/m; average permittivity and conductivity of brain environment: epsilon = 5x10^7, sigma = 0.16 S/m; average permittivity and conductivity of external environment: epsilon = 80, sigma = 1.6 S/m). The number, the positions, the sizes and the proper polarities of the electrodes were set as boundary conditions at the beginning of the simulation runs, according to distinct experimental configurations aiming at the genetic manipulation of different brain regions (distance between forceps-type electrode = 6 mm; distance between the plane perpendicular to the forceps-type electrodes and the third electrode = 4 mm). After reaching convergence, the steady state configuration of the whole system was computed and the maps for the intensity and the orientation of the electric field were generated for the whole brain. Although not designed with a specific optimization algorithm, such a simplified model was already able to confirm empirical considerations on the field. Moreover, the simulation offered new insights into the features of the electrical field distribution and intensity within the system. Indeed, it first explained the high reliability of the experimental outcomes by the standard electroporation procedures by the two forceps-type electrodes placed at the sides of the brain to target the somatosensory cortex located at the parietal region of the brain nearby the position of the positive electrode [1], [2]. Second, the simulation with an additional third electrode clearly showed effects on increased intensity of the electric field, and precise spatial distribution aimed at targeting the genetic manipulation to neurogenic areas of the brain never or only rarely achieved before (i.e., the hippocampus, motor cortex, visual cortex, prefrontal cortex and cerebellum) [6].

B. Hardware apparatus

In the light of the simulations, a novel layout for the hardware apparatus was designed and applied for the experimental validation of the approach. With respect to the traditional configuration based on two oppositely biased electrodes, a novel third electrode was introduced with the freedom of arbitrary motion from the frontal to the posterior part of the brain, while keeping the other two electrodes on the sides of the brain. A first handcrafted version of this device with three independent electrodes was used for first experimental validation [6]. Here, a more sophisticated prototype was realized by means of rapid prototyping using a plastic commercial mold (fullcure870, Vero Black) joining together the forceps-type bipolar electrode and an additional moving arm holding the third electrode (Fig. 1).

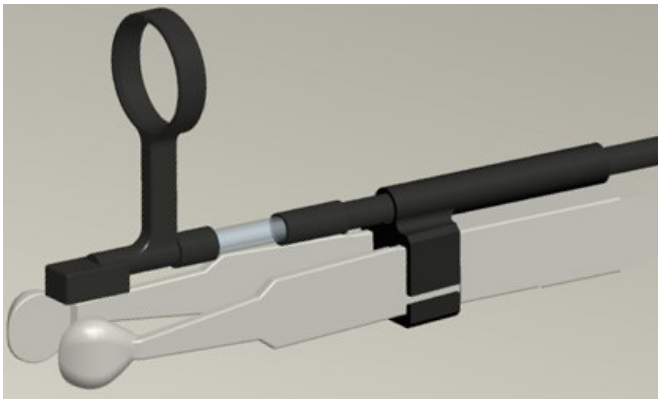

Fig. 1. Rendering of the novel configuration of the hardware apparatus for the electroporation, with the two circular electrodes of the standard configuration at the ends of the forceps and the additional arm holding the moving third electrode below a ring for convenient finger holding.

A voltage stimulator (CUY21Edit, Nepagene) was used to deliver square electrical pulses to the electroporation device via a custom-made switching board to change the polarities at the different electrodes.

C. Experimental approaches

In accordance with the procedures licensed to the Italian Institute of Technology from the Ministry of Health, mice and rats at appropriate pregnancy stages were anesthetized with isofluorane, and a surgery was performed to expose the embryos. 2-5 microliters of a solution containing DNA (1-3 μg/μL) coding for the green fluorescent protein GFP together with the dye Fast Green (for visualization of the injection site) was injected into the ventricular region in both

hemispheres of the brain with a 30 gauge needle. While the embryo's head was carefully held between the forceps-type circular electrodes, the third electrode was accurately positioned at different locations (distance between forceps-type electrode = 7 mm; distance between the plane perpendicular to the forceps-type electrodes and the third electrode = 5 mm). For rat electroporation, five electrical unipolar pulses lasting 50 ms, interleaved 150 ms and with amplitude of 50 V for hippocampus and cortices or 35 V for the cerebellum were applied to the electrodes according to a polarization scheme dependent on the neurogenic region to target [6]. Considering mouse embryo's size and age, applied voltages were reduced for mouse electroporation to 6 pulses of 30 V and 50 ms durations, interleaved 1 s. Polarities and positions of the three electrodes were differently adjusted to target the diverse brain regions, as previously described for rat [6]. After electroporation, embryos were returned to the abdominal cavity of the dam and allowed to continue their normal development.

D. Image Acquisition

Analysis of electroporated brain areas was performed on images acquired from coronal brain sections by means of a confocal laser-scanning microscope (10X oil immersion objective) or an epifluorescence microscope (10X air objective).

III. Results

A. Points emerged in the simulations

The theoretical simulation first suggested that, the spatial distribution of the electric field would become effectively controlled and focused in novel regions of interest by the introduction of a third electrode. This is highlighted by warm colors of the color-coded images at neurogenic target regions (asterisk) in Fig. 2 and Fig. 3. Second, the simulation showed that the same electroporation voltages used for traditional bipolar configuration resulted in higher electric field intensity locally achievable due to the change in the geometryof the electrode configuration (see [6] for direct comparison with standard bipolar configuration).

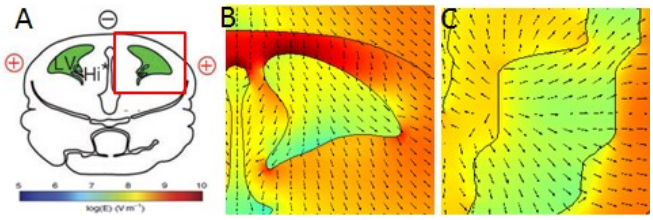

Fig. 2. A coronal view of the embryonic brain at the hippocampal level with the ventricular system filled with DNA in green, the electrode positions and polarities, and the asterisk highlighting the location of hippocampal progenitors (A). Coronal (B) and horizontal (C) maps of the electric field distribution (in pseudo color log scale) and current density direction considering negative carriers (black arrows) obtained by the simulation. LV = lateral ventricle. Hi = hippocampus.

As a consequence of the possibility to substantially reduce the applied voltages, we speculated on the possibility to extend the temporal window for the experiments beyond the limitation dictated by the balance between intensity of the applied voltage and the survival rate of the electroporated embryos. In this scenario depicted by the simulation, unprecedented degrees of freedom for the genetic manipulation would be possible with the tripolar configuration both in terms of spatial localization and temporal frame of the developmental process. The third intriguing aspect emerging by the simulations of the tripolar configuration was that the whole physical system composed by the brain and the electroporation device acquired a high degree of symmetry. In comparison to the standard bipolar design, the electrical field generated by the tripolar configuration affected both brain hemispheres in the same manner (Fig. 3, see [6] for direct comparison with asymmetric electric-field distribution by the standard bipolar configuration).

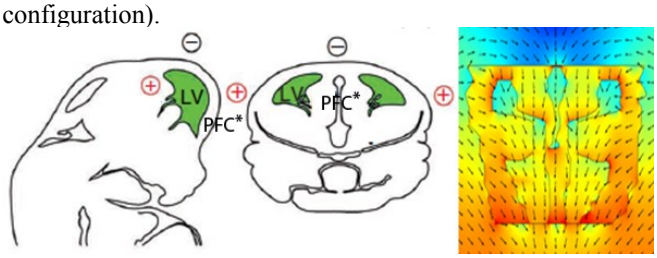

Fig. 3. Lateral and coronal views of the embryonic brain at the level of the prefrontal cortex with the ventricular system filled with DNA (green) in both hemispheres, the electrode positions and polarities, and the asterisk highlighting the location of prefrontal cortex progenitors (A). Horizontal map of the electric field distribution (in pseudo color log scale) and current density direction (considering negative carriers, black arrows) obtained by the simulation, clearly showing the current direction and the symmetry characteristic of the field distribution (B). LV = lateral ventricle. PFC = prefrontal cortex.

B. Experimental validation of the tripolar configuration

By usage of the configuration and device designed in the light of the simulations, we validated several experimental protocols for genetic manipulation in mouse. Successful electroporations of different regions of the brain (Fig. 4) confirmed the points of novelty emerged within the simulations.

Brain region	Model	Age	Voltages	# of pups	% of positive pups
Hippocampus	Rat	E17.5	50V	150	86.2±2.7
Visual Cortex	Rat	E17.5	50V	59	92.9±4.0
Motor Cortex	Rat	E17.5	50V	37	91.4±4.4
Cerebellum	Rat	E14.5	35V	41	57.7±7.7
Hippocampus	Mouse	E14.5	30V	23	81.1±11.2
Prefrontal Cortex	Mouse	E15.5	30V	12	83.1±5.7
Visual Cortex	Mouse	E15.5	30V	45	98.5±1.5

Fig. 4. Percentage of successful electroporation for different regions of the brain targeted by the three-electrode configuration and device in rat [6] and in mouse, together with protocol details.

In terms of the spatial control of the electroporation process, we demonstrated that the innovative design was effective for the manipulation of mouse brain areas such as hippocampus (already from E14.5, Fig. 5), visual cortex and prefrontal cortex (Fig. 6). Success rates were very high, and increased significantly compared to the traditional bipolar design (rat:

86.2 % vs 18.5 % for hippocampus and 92.9 % vs 51 % for the visual cortex [6]).

Fig. 5. Confocal microscope images of a brain coronal section showing the results of the electroporation for the mouse hippocampus performed at E15.5 (A), and a higher magnification of the square box (B). Electroporated cells are in green due to expression of the green fluorescent reporter protein GFP. Scale bars, 200 μm.

The speculation about the possibility of reducing the voltages applied to the electrodes—while preserving the minimal electric field intensity necessary to effective electroporation—was confirmed by experiments aiming at genetic manipulation of the cerebellum. Indeed, reducing the voltages (to 35 V, Fig. 4) allowed the anticipation of the experimental window from E17.5 to E14.5 in rat, which would result in massive embryonic lethality with standard electroporation voltage (50 V, Fig. 4). E14.5 is the proper developmental stage to target Purkinje cells, and electroporation at this stage by the tripolar configuration resulted for the first time in reliable targeting of the rat cerebellum [6]. Finally, we also tested the capability of manipulating both brain hemispheres in the same electroporation step by the three-electrode novel design in mouse. Once we filled the entire ventricular system with DNA by passive diffusion of the solution, we found that a single electroporation step resulted in symmetric patterns of genetic manipulation into both brain hemispheres for the motor cortex, the hippocampus, the visual cortex and the prefrontal cortex (Fig. 6).

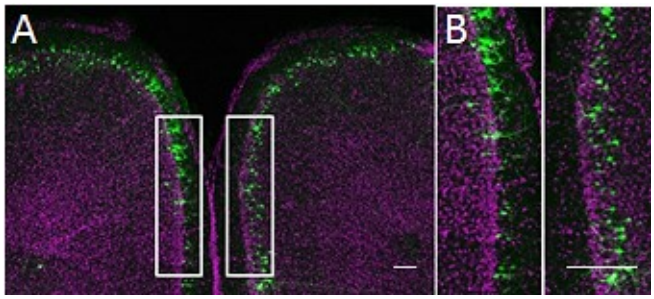

Fig. 6. Confocal microscope images of a brain coronal section showing the results of the electroporation for the mouse medial prefrontal cortex (A), with a higher magnification of the square boxes (B). Electroporated cells are in green due to expression of the green fluorescent reporter protein GFP. Scale bars, 100 μm.

This feature will possibly be fundamental for many practical experimental paradigms of research in neuroscience (e.g., behavioral testing and electrophysiological recordings). Before the development of the tripolar configuration this was only hardly and partially achievable by standard *in utero* electroporation or any other technique of quick genetic manipulation [5].

IV. Conclusions

From the initial proof of principle obtained in the rat model [6], we extended for the first time here the three-electrode *in utero* electroporation method to the mouse, a more common and characterized animal model for genetic manipulation and study of genetic diseases. These results in mouse represent important evidence that the proposed method—based on the development of a model guided hardware and experimental design—increases the spatio-temporal window for genetic manipulation by *in utero* electroporation. Our data also support the theoretical conclusion that more reliable electroporation procedures and more consistent yields can be obtained with a proper design of the electroporation device provided a better understanding of the physical system from the electrical point of view. This work represents a conceptual advance in the field of quick genetic manipulation by electroporation, as it paves the way to targeting new brain areas by theoretical electric-field optimization followed by experimental variation of the number, together with the polarities of the electrodes.

V. References

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Acknowledgments

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