Nanosecond Pulsed Electric Field Interactions with Microglia and Astrocytes

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ABSTRACT

Pulsed electrical fields (PEFs) that weaken the integrity of cellular membranes have been used for a variety of biomedical applications. Recent work suggests that nanosecond PEFs (nsPEFs) might be more beneficial than longer duration PEFs due to their slightly different mechanism of interaction with the cells. The nervous system presents an attractive, yet underutilized, biological target for nsPFE applications. In this paper we describe an *in vitro* primary mixed cortical cell system to test the effects of nsPFEs.

1 Introduction and Motivation

The stimulation of biological tissue and cells using pulsed electrical fields (PEFs) has many biomedical applications. Appropriate selection of pulse parameters can either irreversibly [1,2] or reversibly [3,4] permeabilize cell membranes a phenomenon called electroporation. Practical applications include electrochemotherapy[5], irreversible electroporation[6], and gene therapy[7].

While typical electroporation pulses have durations of microseconds to milliseconds, there might be benefits to shortening the pulse duration to approximately tens to hundreds of nanoseconds and increasing the field intensity to maintain similar energy levels [8]. These nanosecond PEFs (nsPEFs) can create nanopores in the cellular membrane[9] or permeabilize intracellular structures with minimal impact on the cellular membrane [8,10,11].

The aforementioned characteristics of nsPEFs are of special interest for neuroscience applications. In the nervous system, signal transduction occurs through action potential propagation through ion channels [12]. During an action potential, the voltage across the membrane of a neuron rises and falls rapidly due to changes in membrane ion flow. Modulating neural activity has been used to treat a variety of neurological and psychological disorders, including Parkinson's disease [13], dystonia [14], epilepsy [15], and depression [16]. Recent modeling studies indicate that nsPEFs can impact ion flow through neurons [17], and could thus provide an alternative stimulation paradigm for neuromodulation.

In addition to neurons, other supporting cells in the nervous system exhibit sensitivity to ion flow. Microglia are the resident immune cells in the central nervous system[18], and can be either neuroprotective or neurodestructive based on the pathologic context [19]. Studies of Bradykinin induced microglial migration indicate that intracellular and extracellular Ca^{2+} are important for microglial migration [20]. The potential to us nsPEFs to control Ca^{2+} flow, and subsequently, microglial function, could enable therapeutic applications.

To this goal, we propose using primary mixed cortical cultures to test the effects of nsPEFs on neurons and glial cells. In vitro testing allows for rapid screening of pulsing parameters compared to in vivo tests. Primary cells taken directly from living tissue more closely mimic physiological conditions, and can thus generate more relevant data.

In the following sections, we describe the methods for extracting and maintaining these primary mixed cortical cultures, the equipment setup for performing nsPEF, in addition to potential experimental nsPEF parameters and measurable outputs of interest.

2 Cell Dissociation

Complete growth medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY), 10% Fetal Bovine Serum (HyClone, Logan,UT), 10% Horse Serum (Sigma, St. Louis, MO), and 1% Penicillin-Streptomycin (Invitrogen, Grand Island, NY) is made before starting the cultures. 35mm polystyrene petri dishes are coated with poly-L-lysine (Sigma, St. Louis, MO) and incubated at 37 C for 2 hours, then rinsed with PBS, and put aside for plating the cells.

Forebrains from E17 embryonic rat pups are received in a 50 ml conical tube with 5 ml of Solution 1 (constituents) and mechanically dissociated by trituration in the following manner: After adding 18 µl of trypsin (Sigma, St. Louis, MO) to the tissue, the mixture is passed through a serological pipette several times until cloudy, then incubated in a 37°C water bath for twenty minutes, shaking intermittently to facilitate trypsin digestion. Following this incubation, a mixture of trypsin inhibitor and DNAase is added to halt trypsinization, and the tissue is centrifuged at 100 rpm for five

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minutes. The supernatant is removed by aspiration, and the pellet is resuspended in 16 ml of Hibernate-E (BrainBits, Springfield, IL), then passed through a 70 µm mesh filter (BD Biosciences, Bedford, MA) to remove undigested tissue, keeping only cells. The filtered cell suspension is centrifuged again at 1500 rpm for 5 minutes. The supernatant is removed by aspiration, and the cell pellet is resuspended in the complete growth medium.

For cell counting, 14 µl of the cell suspension is added to 14 µl of Trypan Blue (Sigma, St. Louis, MO), and 20 µl of this mixture is placed into a hemocytometer to ascertain the number of live cells yielded by the dissociation procedure. An appropriate dilution with complete culture medium is performed and the cells are plated in the prepared petri dishes at $5x10^5$ cells/cm^2, and maintained in at 37°C in a 5% CO_2 environment.

3 CELL MAINTAINENCE

After plating, the cells are allowed to grow undisturbed for 48 hours to facilitate cell attachment. Following these initial 48 hours, the growth medium is replaced three times per week for seven to ten days, until a near-confluent layer of astrocytes has been established, and a dense network of neuronal connections is observed. Figure 1 shows a bright field image of primary mixed neural cultures exhibiting neuronal connections and glial supporting structures.

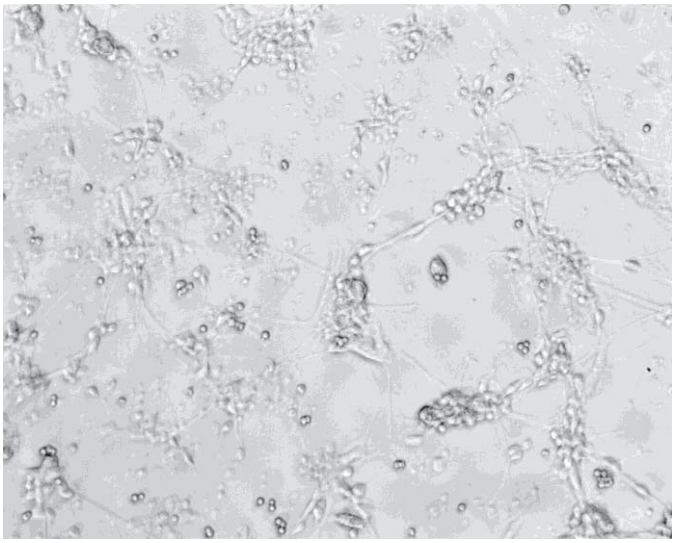

Figure 1. Bright field image at 10x magnification showing a primary mixed cortical culture at 5 days *in vitro*. Widespread neuronal connections can be observed, in addition to glial supporting structures.

4 NSPEF GENERATION AND APPLICATION

A custom-designed pulse generating system (Tanger Electronics, Norfolk, VA) based on a Marx generator topology is used for generating nsPEFs. This pulse generator is capable of achieving 10-100 ns pulses up to 1.5 kV in conjunction with a high voltage power supply (Hewlett Packard). A function generator (Quantum Composers 9612 Pulse Generator Plus) is used to trigger the pulse, and the applied voltage pulses are measured using an oscilloscope (vendor, location). For the electrodes, we used pairs of tungsten rod electrodes with diameters between 0.02 and 0.08 inches. A micromanipulator system (Narishige, East Meadow, NY) was used to hold the electrodes and to precisely position them into the petri dishes, which in turn were placed under a microscope with bright field and fluorescent capabilities. Figure 2 shows the complete setup

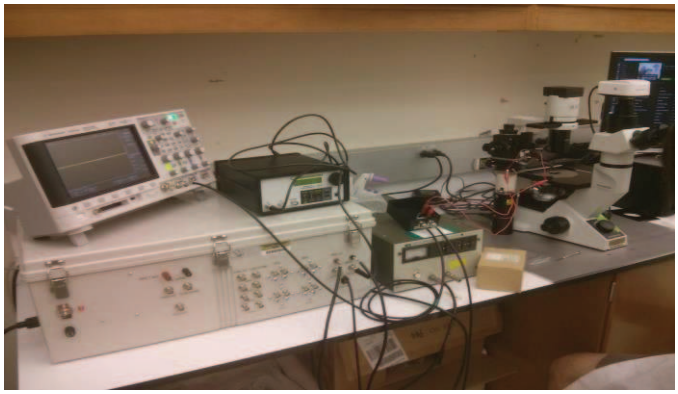

Figure 2. Setup showing Oscilloscope, Function Generator, Pulse Generator, HV Power Supply, MicroManipulator and Microscope system

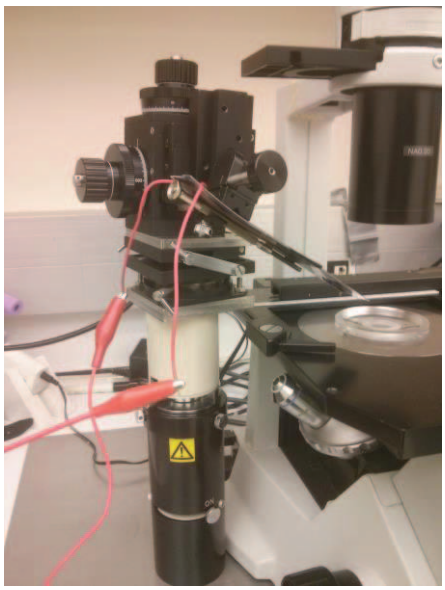

Figure 3. A close-up view showing the manipulator system, tungsten electrodes, and a petri dish.

5 PROPOSED EXPERIMENTAL PARADIGM

Using the materials and methods described in the previous sections, we can test multiple nsPEF parameters, including pulse duration, pulse amplitude, number of pulses. The effects of various nsPEFs can be quantified through by using actively and passively uptaken molecular dyes that are commonly used to assess membrane integrity and cell viability, such as Cell Tracker, Propidium iodide, and Yo-Pro 1. A combination of carefully chosen markers can be used to differentiate apoptotic and necrotic cells, as well as determine overall cellular

viability. A more sophisticated approach would involve using a 5% CO_2/37°C chamber mounted on the microscope, which would enable time lapse imaging over an extended period of time to monitor the movement of microglia in response to nsPEFs. A time dependent analysis of microglial migration can determine whether nsPEFs can induce electrotaxis on motile cells, such as microglia.

REFERENCES

[1] W. A. Hamilton and A. J. H. Sale, "Effects of high electric fields on microorganisms: II. Mechanism of action of the lethal effect," Biochim. Biophys. Acta (BBA)-General Subj., vol. 148, no. 3, pp. 789–800, 1967.

[2] A. J. H. Sale and W. A. Hamilton, "Effects of high electric fields on micro-organisms: III. Lysis of erythrocytes and protoplasts," Biochim. Biophys. Acta (BBA)-Biomembranes, vol. 163, no. 1, pp. 37–43, 1968.

[3] J. C. Weaver and Y. A. Chizmadzhev, "Theory of electroporation: a review," Bioelectrochem. Bioenerg., vol. 41, no. 2, pp. 135–160, 1996.

[4] J. Teissie, N. Eynard, B. Gabriel, and M. P. Rols, "Electropermeabilization of cell membranes," Adv. Drug Deliv. *Rev.*, vol. 35, no. 1, pp. 3–19, 1999.

[5] D. Miklavčič, G. Serša, E. Brecelj, J. Gehl, D. Soden, G. Bianchi, P. Ruggieri, C. R. Rossi, L. G. Campana, and T. Jarm, "Electrochemotherapy: technological advancements for efficient electroporation-based treatment of internal tumors," Med. Biol. Eng. Comput., vol. 50, no. 12, pp. 1213–1225, 2012.

[6] G. Onik, P. Mikus, and R. B, "Irreversible electroporation: a new ablation modality--clinical implications," Technol. Cancer Res. Treat., vol. 6, no. 1, pp. 37-48, 2006.

[7] L. M. Mir, "Nucleic acids electrotransfer-based gene therapy (electrogenetherapy): past, current, and future," Mol. Biotechnol., vol. 43, no. 2, pp. 167–176, 2009.

[8] K. H. Schoenbach, R. P. Joshi, J. F. Kolb, N. Chen, M. Stacey, P. F. Blackmore, E. S. Buescher, andd S. J. Beebe, "Ultrashort electrical pulses open a new gateway into biological cells," Proc. IEEE, vol. 92, no. 7, pp. 1122-1137, 2004.

[9] A. G. Pakhomov, J. F. Kolb, J. A. White, R. P. Joshi, S. Xiao, and K. H. Schoenbach, "Long-lasting plasma membrane permeabilization in mammalian cells by nanosecond pulsed electric field (nsPEF)," Bioelectromagnetics, vol. 28, no. 8, pp. 655–663, 2007.

[10] E. S. Buescher and K. H. Schoenbach, "Effects of submicrosecond, high intensity pulsed electric fields on living cells-intracellular electromanipulation," IEEE Trans. Dielectr. Electr. Insul., vol. 10, no. 5, pp. 788–794, 2003.

[11] T. B. Napotnik, Y.-H. Wu, M. A. Gundersen, D. Miklavčič, and P. T. Vernier, "Nanosecond electric pulses cause mitochondrial membrane permeabilization in Jurkat cells," Bioelectromagnetics, vol. 33, no. 3, pp. 257–264, 2012.

[12] D. Purves, *Neuroscience*. Sinauer Associates, Sunderland, Mass., 1997.

[13] J. M. Bronstein, M. Tagliati, R. L. Alterman, A. M. Lozano, J. Volkmann, A. Stefani, F. B. Horak, M. S. Okun, K. D. Foote, P. Krack, and others, "Deep brain stimulation for Parkinson disease: an expert consensus and review of key issues," Arch. Neurol., vol. 68, no. 2, pp. 165-171, 2011.

[14] J. K. Krauss, J. Yianni, T. J. Loher, and T. Z. Aziz, "Deep brain stimulation for dystonia," J. Clin. Neurophysiol., vol. 21, no. 1, pp. 18–30, 2004.

[15] J. A. Cramer, E. Ben Menachem, and J. French, "Review of treatment options for refractory epilepsy: new medications and vagal nerve stimulation," Epilepsy Res., vol. 47, no. 1, pp. 17–25, 2001.

[16] D. A. Groves and V. J. Brown, "Vagal nerve stimulation: a review of its applications and potential mechanisms that mediate its clinical effects," Neurosci. Biobehav. Rev., vol. 29, no. 3, pp. 493–500, 2005.

[17] R. P. Joshi, A. Mishra, J. Song, A. G. Pakhomov, and K. H. Schoenbach, "Simulation studies of ultrashort, high-intensity electric pulse induced action potential block in whole-animal nerves," IEEE Trans. Biomed. Eng., vol. 55, no. 4, pp. 1391–1398, 2008.

[18] F. Vilhardt, "Microglia: phagocyte and glia cell," Int. J. Biochem. Cell Biol., vol. 37, no. 1, pp. 17–21, 2005.

[19] J. M. Pocock and H. Kettenmann, "Neurotransmitter receptors on microglia," Trends Neurosci., vol. 30, no. 10, pp. 527–535, 2007.

[20] M. Ifuku, K. Färber, Y. Okuno, Y. Yamakawa, T. Miyamoto, C. Nolte, V. F. Merrino, S. Kita, T. Iwamoto, I. Komuro, and others, "Bradykinin-induced microglial migration mediated by B1-bradykinin receptors depends on Ca2+ influx via reverse-mode activity of the Na+/Ca2+ exchanger," J. Neurosci., vol. 27, no. 48, pp. 13065–13073, 2007.