Fine-Tuning Neuroblastoma Differentiation and Extracellular Vesicle Secretion through Electrical Stimulation Time Components

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Introduction: Recent advancements in electrobiology research have unveiled the potential of low voltage electrical stimulation (ES) in the range of endogenous electric field as a modulatory tool for promoting pro-regenerative cellular responses, including neural differentiation[1]. Previously we showed that direct ES trigger regenerative responses, such as altering stem cell fate, motility and function[2]. Accordingly, ES of mesenchymal stem cells has been shown to upregulate the expression of several regenerative, neuroprotective, neurodegenerative and angiogenic markers (such as VEGF, BDNF, NGF, etc.)[3].

One of the challenges in bioelectrical stimulation is the estimation and optimization of the effective parameters. Amplitude of the voltage and/or current, signal shape, frequency, and duty cycle play important role in communicating effective signal to the cells.

In this study, we maintained a constant field amplitude of 25 mV/mm, which represents a relevant endogenous field. Our primary focus was to investigate the impact of temporal factors, such as frequency and duty cycle, on neural differentiation within a human neuroblastoma (SH-SY5Y) model. The impact of ES, in the absence of additional essential neurotrophic factor, i.e., brain-derived neurotrophic factor (BDNF), has been compared across different time components and equated with the group stimulated by BDNF.

Moreover, we studied the effect of ES parameters on modulation of extracellular vesicles (EVs) secretion. EVs are the insoluble fraction of the cell secretome, which consists of membraned micro/nano particles (50-150 nm for exosomes and 100-1000 nm for microvesicles). These vesicles facilitate the transport of bioactive lipids, proteins, various RNA and DNA subtypes, thus serving a vital role in cell-cell communication. Additionally, recent discoveries have highlighted their potential benefits in tissue regeneration[4]. In this study we explored the effect of electrical stimulation parameter on production and cargo of the EVs from SH cells and assess the size and concentration of particles as well as protein content.

Methodology: SH-SY5Y cell were expanded in growing medium (DMEM/F12 + 10% FBS + 1% Antibiotics). Cells were seeded at the density of $3x10^4/cm^2$ in ES chambers developed in our lab (8-well pate setup; each well with surface area of 10 cm^2, 3 plates used per condition per experiment). The day after seeding, cells were exposed to pre-differentiation medium consist of Neurobasal, B27, glutamate and retinoic acid for 5 days. The cells were subjected to either pulsed electrical stimulation of 25 mV/mm (ES1: 1Hz, 500µs (0.05% duty cycle); ES2: 50Hz, 500µs (2.5% duty cycle); ES3: 1Hz, 250 ms (25% duty cycle)), or 5ng/ml BDNF treatment.

One day post treatment, cells were fixed and assessed for the expression of neural markers (MAP-2 and Tau-5) using the immunofluorescent technique (IF). Metabolic activity of the cells was assessed using the alamar blue assay. Finally, the cell secretome was purified through centrifugation at 300xg and 2000xg to eliminate dead cell debris and apoptotic bodies. The purified secretome was then subjected to ultracentrifugation at 100,000xg using a 30% sucrose in PBS cushion. EVs were obtained and purified through an additional washing step with PBS and ultracentrifugation, resuspended in PBS and stored at -80°C. EVs were assessed using Nanoparticle Tracing Assay to determine their size and concentration. The morphological quality is assessed with transmission electron microscopy. The protein content is assessed by means of mass spectroscopy.

Results and Discussion: It is observed that low-intensity electrical stimulation (25 mV/mm) induces neural differentiation in neuroblastoma cells, even in the absence of BDNF, and this effect is comparable to the gold standard chemical stimulation using BDNF. Figure 1 A shows expression of Tau-5 in all ES and BDNF stimulated groups. Morphological evaluation of neurite out growth and complexity of the network reviled that there is a significant difference between cell treated with ES protocols and BDNF. Our comprehensive assessment (results are not shown) showed that although BDNF treatment might benefit the number of the primary neurite, electrical stimulation, significantly increases the number of branching, nodes and secondary and tertiary neurite. There are meaningful morphological differences between the cells in the stimulation groups corresponding to the temporal component of the stimulation protocol. As the duty cycle increases, the complexity of the neurite network also grows.

Furthermore, our observations revealed that the secretion of EVs is influenced by the frequency and duty cycle of the stimulation regimen. In summary, the particle count of EVs generated through electrical stimulation conditioning (ES-EVs) significantly exceeded that of the control group (No ES). Importantly, ES-EVs exhibited morphological

similarities to natural EVs, suggesting that electrical stimulation does not induce any abnormal effects on EVs. The proteomic assessment of EVs (results are not shown) indicated that all EVs isolated in this study express more than 90% of EVs signature proteins indexed in Exocarta. These results confirm that EVs from electrically stimulated groups contain large amount of pure exosomal fraction and do not express abnormalities. More importantly, we identified the significant abundance of 30 unique proteins that are only expressed in the ES-EVs: proteins in involved in cellular transport and membrane dynamics (RAB1B, TSPAN14, ATP2B4, RAP1A, TMED9); proteins involved in cytoskeletal regulation (TPM4, DYNLL2, RP2); proteins involved in RNA processing and transport: (STAU1, SNRPD3, PRPF19, ILF2, HNRNPH3); proteins associated with extracellular matrix and cell adhesion (ALCAM, HAPLN3, PCOLCE, SCPEP1); proteins with enzymatic functions (TPT1, PTP4A2, HACL, GBA3); proteins associated with neuronal function (DPYSL3, SUPT16H); proteins involved in signaling and cell regulation (IGFBP5, SSB, TPP1); Proteins with roles in metal ion transport (SLC39A14, ALAD). There are also significant differences in the abundance of some of these proteins between the ES conditions that are under analysis.

Conclusion: Pulsed electrical stimulation at 25 mV/mm emerges as a potent physical approach for inducing neural differentiation in neuroblastoma cells, even in the absence of the critical growth factor BDNF. Our study demonstrates that the effectiveness of this method is dose-dependent, with the frequency and duty cycle significantly influencing the morphological expression of neurite outgrowth. Moreover, these parameters also exhibit a meaningful impact on the production yield of EVs and their protein cargo. In summary, our findings underscore the viability of fine-tuning of cell behavior and cell secretion through the regulation of electrical stimulation parameters.

Acknowledgments

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Figures

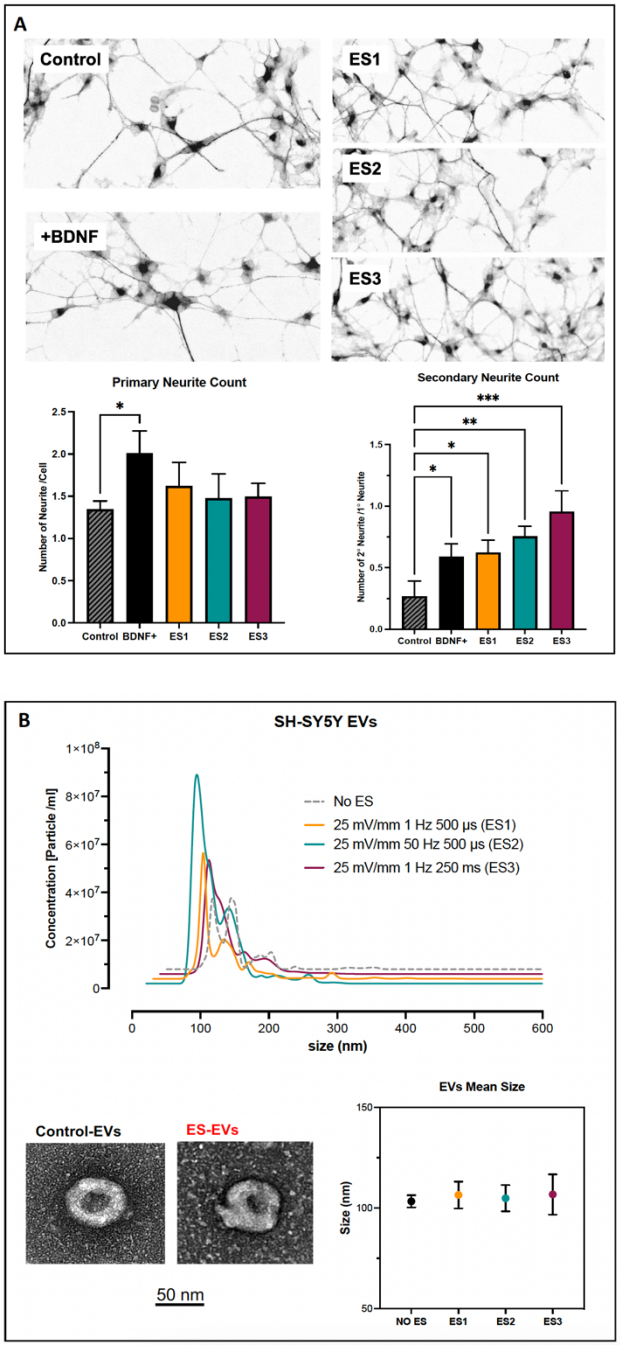

Figure 1. A) Influence of electrical stimulation on neural differentiation in SH-SY5Y cells. Immunofluorescent images depict the expression of Tau-5 (an axonal marker) in Control, BDNF+, ES1, ES2, and ES3 conditions. Quantification of primary and secondary neurites is based on three independent experiments (n=3). B) Impact of electrical stimulation on the secretion of extracellular vesicles from SH-SY5Y cells. Relative changes are illustrated through NTA analysis (top and bottom right) and TEM images (bottom left).