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Electrical stimulation enhances the acetylcholine receptors available for neuromuscular junction formation

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ABSTRACT

Neuromuscular junctions (NMJ) are specialized synapses that link motor neurons with muscle fibers. These sites are fundamental to human muscle activity, controlling swallowing and breathing amongst many other vital functions. Study of this synapse formation is an essential area in neuroscience; the understanding of how neurons interact and control their targets during development and regeneration are fundamental questions. Existing data reveals that during initial stages of development neurons target and form synapses driven by biophysical and biochemical cues, and during later stages they require electrical activity to develop their functional interactions. The aim of this study was to investigate the effect of exogenous electrical stimulation (ES) electrodes directly in contact with cells, on the number and size of acetylcholine receptor (AChR) clusters available for NMJ formation. We used a novel *in vitro* model that utilizes a flexible electrical stimulation system and allows the systematic testing of several stimulation parameters simultaneously as well as the use of alternative electrode materials such as conductive polymers to deliver the stimulation. Functionality of NMJs under our co-culture conditions was demonstrated by monitoring changes in the responses of primary myoblasts to chemical stimulants that specifically target neuronal signaling. Our results suggest that biphasic electrical stimulation at 250 Hz, 100 μs pulse width and current density of 1 mA/cm^2 for 8 h, applied via either gold-coated mylar or the conductive polymer PPy, significantly increased the number and size of AChRs clusters available for NMJ formation. This study supports the beneficial use of direct electrical stimulation as a strategic therapy for neuromuscular disorders.

Statement of Significance

The beneficial effects of electrical stimulation (ES) on human cells *in vitro* and *in vivo* have long been known. Although the effects of stimulation are clear and the therapeutic benefits are known, no uniform parameters exist with regard to the duration, frequency and amplitude of the ES. To this end, we are answering several important questions on the parameters for ES of nerve and muscle monocultures and co-cultures by probing the effects on the enhancement of acetylcholine receptors (AChR) clustering available for neuromuscular junction formation using a conductive platform. This work opens the possibility to combine electrical stimulus delivered via conductive polymer substrates, from which biomolecules could also be delivered, providing opportunities to further enhance the therapeutic effect.

1. Introduction

Contractile muscle activity is controlled by the motor neuron-muscle system [1]. The regulation of this system involves the transmission of action potentials from the central nervous system to peripheral nervous system then to muscle fibers via neuromuscular junctions (NMJs) [2,3]. This complex system relies on dynamic interactions of signaling molecules and cell membrane proteins [4,5] to release neurotransmitters from motor neurons into the synaptic cleft, followed by neurotransmitter binding to specific receptors (AChR) that are located within the plasma membrane of muscle fibers [6,7]. There are many factors to be considered when investigating NMJ formation, maturation and function,

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however recent data reveals that clustering and maintenance of high densities of AChRs are key elements of synaptogenesis at the NMJ [8–11].

Recent reviews support the idea that dysfunction of these junctions may play a key role in several neuromuscular diseases, for example growing evidence supports the "dying-back" hypothesis of amyotrophic lateral sclerosis (ALS) suggesting that the survival of NMJs is essential to delay the progression of ALS [12]. It has also been suggested that stabilization of NMJs is a promising approach to attenuate the development of muscle wasting disorders, indicating that NMJs are good markers of motor neuron health [13]. Therefore, therapeutic treatments aimed at maintaining NMJs may be an effective approach to slowdown the progression of these diseases.

Recent literature reviews suggest that during development neurons target and form synapses driven by dynamic interactions of biophysical and biochemical cues, whilst electrical activity, in the form of ion transients, plays a role in neuronal development both before and after synapse formation [4,14,15]. Many *in vitro* and *in vivo* studies have been conducted using external electrical stimulation (ES) to control cell characteristics [7,16], indicating that ES has positive benefits in many areas such as wound-healing [16], bone growth [17], pain relief, muscle restoration [18,19], proliferation and differentiation of stem cells [20], as well as in nerve guidance and growth [21,22]. In addition, it has recently been shown that the formation and architecture of NMJs can be influenced by electrical stimulation (ES) *in vitro* [23] and *in vivo* [24,25], however, most of these stimulations relied on direct current which has been shown to generate faradic reactions allowing charge leakage through the electrodes, and compromising the safety of cells and tissues [26]. Therefore establishment of a system that delivers efficient and safe electrical stimulation to cells and tissues is needed. The system should deliver optimized parameters such as stimulation time, current amplitude, stimulus mode and electrode material to achieve the desired outcomes for a range of excitable tissues.

An extensive series of materials has been used as electrodes to deliver electrical stimulation including stainless steel, titanium nitride, gold, platinum, platinum-iridium alloys and tungsten. These are materials that have been identified as safe, however according to previously published studies, electrical stimulation using some of these metallic materials can generate unwanted by-products commonly called "faradaic products" due to oxidation–reduction of components in the surrounding media [27]. Some metal electrodes are also prone to dissolution due to corrosion processes making it difficult to evaluate the true effect of the ES on cells [28].

Conducting polymers (CP) offer the possibility to improve the interaction of electrodes with biological systems by improving cell biocompatibility as well as avoiding the issues associated with electrolysis and corrosion [29,30], while providing a sufficiently low impedance electrode for cell stimulation. Furthermore, these "smart materials" as they have been called [29] offer many more advantages over metal electrodes, due to their physical, chemical and electrical properties which can be custom designed to fit specific applications [29,31,32]. CPs as electrode coating materials facilitate enhanced integration of electrodes with cells and tissues [20,33–36]. This is achieved by increased surface area, reduced impedance as a result of improved charge transfer and reduced inflammatory responses due to the modification of surface roughness [37]. In addition, CPs offer the capability to incorporate biological molecules, such as growth factors, enzymes, antibodies and DNA [38,39] into the polymer and release them locally in a controlled manner [38,40–42].

Since it was first described by Bolto in the 1960s [43], polypyrrole (PPy) is one of the CPs most extensively investigated for tissue engineering applications [43]. PPy is an amorphous and opaque material that has high electrical conductivity, ion exchange capacity, good environmental stability [34,37,39,44–46], but most importantly, it can be synthesized and modified in many ways, making it attractive for a wide range of applications [19,41]. One of the many remarkable benefits of this polymer is its electrical properties which can be attributed to the fast, facile ability to switch between different oxidation states [39]. PPy doped with dodecyl benzene sulphonate (DBS) has previously been shown by our group to enhance neuronal stem cell and muscle cell differentiation [19,20] as well as facilitate the controlled release of growth factors as treatments for nerve injuries to prevent nerve degradation and promote nerve protection [40].

In this study we propose an innovative *in vitro* model to investigate effects of ES on NMJ formation by exposing primary myoblast/motor neuron co-cultures to electrical stimulation, utilizing the conductive polymer polypyrrole doped with DBS to deliver the stimulus. The polymer properties were characterized using atomic force microscopy (AFM), scanning electron microscopy (SEM) and impedance measurements. Immunohistochemistry and confocal microscopy were employed to determine the increase in number and size of AChR clusters, which was further supported by analysis of cell lysates for NMJ-associated proteins by Western blotting. We demonstrated the functionality of the NMJ model by monitoring the responses to neuronal stimulation using calcium imaging as well as observations of muscle twitching. This *in vitro* model provides a tool for further investigation of the delivery of either direct or field electrical stimulation to the cells, and allows many different stimulation strategies to be assessed simultaneously. This model was used to establish a positive effect of ES using the conductive polymer PPy/DBS at 250 Hz/1 mA/cm^2 current density for 8 h using biphasic 100 μs pulses on NMJ formation, increasing the number and size of AChR clusters, as well as increasing the expression of the NMJ-associated proteins Rapsyn and Synapsin.

2. Material and methods

2.1. Preparation of polymer films

Pyrrole (Py) monomer was obtained from Sigma-Aldrich and distilled before use. The dopant dodecyl benzene sulfonate (DBS) was obtained from Sigma-Aldrich. Gold coated mylar (Solutia Performance Films) was prepared for polymerization by cleaning with isopropanol and rinsing with distilled water. Distilled Py (0.2 M) was mixed with DBS solution (0.05 M) in Milli-Q water, and PPy films were polymerized galvanostatically from this solution using a standard three-electrode electrochemical cell. Gold coated mylar films were used as the working electrode (WE), a platinum mesh as a counter electrode (CE), and a Ag|AgCl reference electrode (RE) were connected to an eDAQ EA161 potentiostat. The polymer was galvanostatically grown at 0.1 mA/cm^2 current density for 10 min according to a previous report from our group [20]. After polymerization, the films were rinsed with Milli-Q water and allowed to dry before use.

2.2. Atomic force microscopy

AFM images were taken using JPK NanoWizard II BioAFM (JPK, Germany) with samples submerged in phosphate buffered saline (PBS) solution. Images were taken using a silicon nitride cantilever with a spring constant of 0.42 Nm^{-1}in AC mode. Scans of 10 and 1 μm square areas were taken at 0.5–1 Hz rate and sampling sizes of 512 × 512 pixels. The root mean square (RMS) roughness (R_q) and the average roughness (R_{ave}) values were obtained using JPK image processing software.