

# Apolipoprotein E3 Modulates Motor Endplate Formation

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**INTRODUCTION:** Despite only comprising 0.1% of the myofiber surface area, a healthy motor endplate is densely packed with acetylcholine receptors (AChRs) responsible for mediating activity between the connected motor neuron and skeletal muscle via the neurotransmitter, acetylcholine. Gradual loss of the endplate, better known as fragmentation, is caused by traumatic nerve injury typically caused by lacerations or by old age. In both cases, fragmentation proceeds to muscle wasting and leads to decreased mobility, muscle strength, and quality of life. In injury models, fragmentation is recoverable if the motor nerve connection is re-established within an 18-month regenerative window. However, most patients will still face long term functional deficits due to dampened neurotransmission caused by fragmented remodeling. Our long-term goal is to create a treatment that preserves the motor endplate by slowing the remodeling process. The aim of the present study was to evaluate the role of Apolipoprotein E (Apoe) during muscle nerve cross-talk to increase the number of synapses formed on myotubes.

**METHODS:** *In vivo* study, a muscle injury model was produced in male Sprague Dawley rats (n=8/group) with a 1.5x1 cm volumetric muscle loss injury. A decellularized muscle matrix material was implanted into the defect site followed by nerve transfer enabled neurotization using the peroneal or tibial nerve. Injuries healed for 8 weeks and RNA was isolated post-mortem within the injury site. RNA was analyzed by nanostring technologies (Seattle, WA) for a homologous rat neuropathology and muscle panel. These Nanostring data were analyzed using the nSolver™ software. For our *in vitro* co-culture study, C2C12 myoblasts were plated onto a Corning TCPS 24-well plate at 10,000 cells/cm<sup>2</sup> in  $\alpha$ -MEM 10% FBS 1% P/S media cultured at 37C 5% CO<sub>2</sub>. NSC34 motor neurons were plated in parallel on Corning 6.5 mm 0.4  $\mu$ m pore polycarbonate transwell membranes at 10,000 cells/cm<sup>2</sup> in DMEM 10% FBS 1% P/S media cultured at 37C 5% CO<sub>2</sub>. Co-culture began once myoblasts reached 100% confluence. The cells were allowed to co-culture for 3 days in Neurobasal media 4 mM L-glutamine 1% P/S. Over 3 days, cells were harvested, RNA isolated, and RT-qPCR was used to measure levels of myogenic markers myoblast differentiation factor 1 (Myod), myogenin (Myog), Myh1 (Myosin heavy chain 1) in addition to synaptic markers acetylcholine receptor subunit-  $\gamma$  (Chrng), acetylcholine receptor subunit-  $\epsilon$  (Chrme), low-density lipoprotein receptor-related protein 4 (Lrp4), muscle specific kinase (MuSK), rapsyn (Rapsn), and Apoe. We next, tested whether Apoe3 or Apoe4 were involved in myogenesis and motor end plate development. Treatment with exogenous Apoe 3 or 4 started at 100% confluence. The Apoe proteins were sourced from Acro Biosystems with poly-his tags. C2C12 cells received either Apoe3 or Apoe4 at 10<sup>-7</sup>, 10<sup>-8</sup>, or 10<sup>-9</sup> molarity mixed in with the  $\alpha$ -MEM full media (n=6/group), RNA was isolated, and our gene panel tested.

**RESULTS:** Decellularized muscle treatment using neurotization showed increased maximum tetanic force, increased numbers of myosin labeled fibers, and Nanostring analysis showed that Apoe increased specifically in neurotized groups (Fig. 1). *In vitro* model of muscle-nerve crosstalk using C2C12s and NSC34 motor neurons. Control myoblasts increased Apoe gene expression levels in a time-dependent fashion over 4 days in culture, where Apoe increased over the early stages of myogenesis with a 20-fold increase in day 4 compared to day 0 and 1. C2C12 myoblasts co-cultured with NSC-34 motor neurons increased myogenic markers and acetylcholine receptor  $\gamma$  and  $\epsilon$  levels (Fig 2A) with a concomitant increase in Apoe (Fig. 2B). These results were correlated with  $\alpha$ -bungarotoxin staining that revealed increased clusters of dispersed AChRs in myoblasts treated with NSC-34 cells. The effect of treatment different Apoe isoforms (Apoe3 and Apoe4), and determined that proliferation in C2C12 myotubes did not change and was not dose dependent. Similarly, myogenic markers Myh1, Myog, and MyoD did not change between doses or isoform of Apoe. However, Apoe3 had a dose dependent effect on Chrng expression (Fig. 2C) with no effect of Apoe4 on Chrng expression (Fig 2D). The expression for Lrp4, a known receptor for Apoe, was unaffected by both treatments. No changes in synaptic expression were measured in Apoe4 treated C2C12 cells.

**DISCUSSION:** Following peripheral nerve injury, the specialized cross-talk between motor nerve and muscle breaks down leading to gradual changes in motor endplate morphology that reduce muscle function even if the nerve is successfully reconnected. Big data analysis show increase in Apoe level and indicated Apoe may play a role in synaptic development, interacting with Lrp4. The co-culture experiment determined that Apoe not only increased in myotubes following differentiation, but that it correlated with synaptic development and was influenced by the presence of a motor neuron. A myoblast time-course study showed Apoe is expressed intrinsically in C2C12 cells entering myogenesis, treatment with Apoe3, not Apoe4, further increases acetylcholine receptor expression indicating its potential as a therapeutic to provide neuro-protection at the synapse. Further research is needed to understand how Apoe3 signaling is affecting synaptogenesis and how dosing *de novo* myotubes with exogenous Apoe3 may change the myotube's capacity to communicate with motor neurons.

**SIGNIFICANCE/CLINICAL RELEVANCE:** Improving clinical outcomes of denervation injuries relies on better understanding of signaling pathways responsible for motor endplate formation and protection. Our data show Apoe may play an important role in this process and may have a potential therapeutic effect to protect motor endplates following denervation. The correlative increases in Apoe and Chrng may suggest the activation of a previously understudied feedback loop involving other key neuroprotective factors that we are continuing to investigate.

## IMAGES AND TABLES:

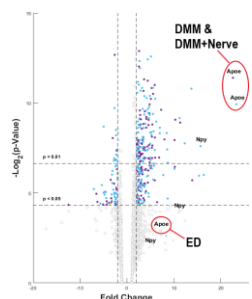


Fig. 1 Volcano plot of Nanostring™ results with Apoe highlighted.

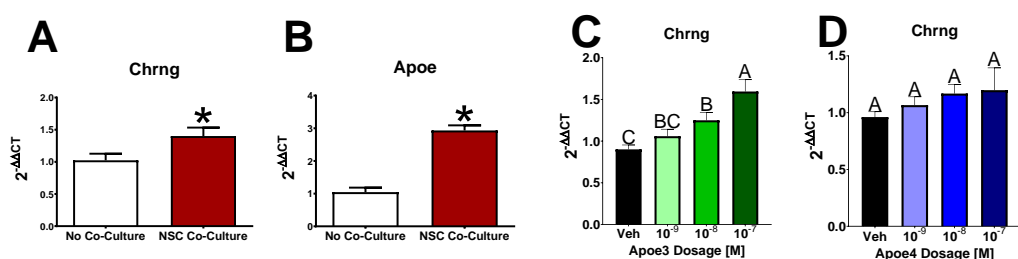


Fig. 2 *in vitro* C2C12 myoblast experiments showing Apoe and Chrng (Acetylcholine receptor subunit  $\gamma$ ) increases in response to co-culture with NSC-34 motor neurons (A-B). Chrng dose dependently increases with exogenous Apoe3 delivery (C), but the effect is lost dosing with exogenous Apoe4 (D).