Muscle-Nerve Interaction Differentially Alters Extracellular Matrix Remodeling and Fibrosis

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INTRODUCTION: Within seven days following muscle denervation, extracellular matrix (ECM) remodeling begins. If the muscle remains denervated, the ECM becomes fibrotic with fatty tissue infiltrate as the tissue undergoes atrophy. Myofiber ECM is a highly organized heterogenous structure, which specialized zones such as the neuromuscular junction. Fibrotic changes to this ECM change how muscle responds to ECM-related signals. To better understand how the ECM remodels under different conditions of muscle paralysis, we compared two rat models. The first was a neurectomy, resulting in loss of volitional control and loss of parasympathetic and sensory signals. The second was a Botox induced muscle paralysis model that preserves the neuromuscular junction, parasympathetic, and sensory connections, but halts contractions by preventing acetylcholine release from the motor end terminal. The purpose of the present study was to determine differences in ECM and fibrosis between a denervation and Botox model of paralysis. We hypothesized that maintaining a neuromuscular junction (Botox induced paralysis) would not stimulate fibrotic ECM remodeling.

METHODS: Twenty male Sprague-Dawley rats were randomly assigned to either receive a sciatic-femoral neurectomy or Botox induced paralysis. (n=8/group determined by power analysis) Both methods were carried out while animals were under 2.5% isoflurane/400 mL/min O_2 with approval by the Virginia Commonwealth University Institutional Animal Care and Use Committee. The sciatic-femoral neurectomy was performed by making a ~0.5 cm^2 intramuscular window in the bicep femoris to expose the sciatic nerve. Two to three centimeters of nerve was removed with stumps sutured to prevent regeneration. The femoral neurectomy was then performed with a dorsomedial incision starting at the femoral mid-diaphysis exposing the femoral nerve before being carefully cut and sutured. Botox induced paralysis was performed by injecting 2 units/muscle-group of botulinum toxin type A into the hindlimb paraspinal muscle, quadricep, bicep femoris, and posterior crural muscles. Functional muscle recovery was assessed after fifty-two days of paralysis with muscle force testing using the 1300A Whole Animal System (Aurora Scientific). Following *in vivo* muscle force testing, animals were euthanized and tissue collected for downstream PCR and protein analysis. The presented data are shown as mean ±SEM analyzed with a using One-way ANOVA with a Tukey *post-hoc* test in GraphPad Prism 6.0 (GraphPad, La Jolla, CA). Groups not sharing letters are considered significant (p-value < 0.05).

RESULTS: Sciatic-femoral neurectomy (SFN) and Botox induced paralysis (BTX) treatments both resulted in increases in mRNAs for *Myh1* and *Myh3*, indicating atrophy induced slow to fast fiber switching and fetal fiber expression. *Pax7* also increased in both treatment groups indicating satellite cell activation. *Myog, Itga7, Myf5*, and *Myf6* were increased compared to control and contralateral limbs. However, Botox treated limbs had lower expression than the SFN treatment. Further, immature AChR-γ and mature AChR-ε were only increased compared against their contralateral in the SFN treated groups. In muscle force testing, only the SFN treated groups showed decreased tetanic maximum force output compared to their contralateral limbs despite the total time of initial contraction to relaxation increasing with both treatments. Additionally, only the SFN treated muscles had increased production of specific ECM proteins collagen type I, collagen type IV, collagen VI, laminin, and fibronectin compared to contralateral and control tissues (Fig. 1A-D). Only collagen type VI was increased in both SFN and Botox treated limbs. We then tested a fibrotic gene panel for all samples, and determined that interleukins IL1β, IL2β, and IL33 were significantly different between SFN and BTX. Next, we tested TGF-β related signaling factors and determined that Tgfb1, Akt1, and connective tissue growth factor (Ctgf) were all higher in SFN compared to BTX (Fig. 2A-E). Similarly, we determined that NF-kB signaling was modulated. Nfkb1 and Traf6 were elevated in SFN compared to BTX, while Ikbkg, Nfkbia, and Ikbkb were similar between SFN and BTX.

DISCUSSION: Previous literature has shown both Botox and neurectomy can induce atrophy, but this study's data show that despite similar increased mRNA expression of markers associated with atrophy, only the animal model losing muscle-nerve interaction and nerve feedback had fibrotic ECM remodeling. Increased collagen I, IV, and VI in SFN treated rats is a hallmark of fibrosis in skeletal muscle. Moreover, our data also showed increased laminin and fibronectin levels in SFN, while BTX muscles were similar to controls. These levels suggested that TGF-β might also be affected since fibronectin sequesters TGF-β in its latent phase. Thus, we tested TGF-β signaling and showed that mRNA levels for TGF-β1 and CTGF were elevated in SFN but not in BTX, supporting our hypothesis that maintaining a neuromuscular junction would limit fibrosis. Further research is needed to determine whether fibrosis directly affects motor endplate remodeling, and whether therapeutic targets can be identified to limit motor end plate breakdown following denervation. In summary, we demonstrated the differential effects that dissimilar paralysis models have on ECM remodeling and fibrosis. Research into this topic may yield therapeutic targets to reduce fibrotic development and increase likelihood of muscle innervation after a peripheral nerve injury.

SIGNIFICANCE/CLINICAL RELEVANCE: While fibrosis is well-described in denervation atrophy, understanding why fibrosis occurs is clinically significant. In this abstract, we report two paralysis models that compared intact neuromuscular junctions and denervated muscle. Our data demonstrated that maintaining a connection between muscle and nerve affected the surrounding ECM, and these findings could be a critical step in improving clinical outcomes for denervation atrophy.

IMAGES AND TABLES:

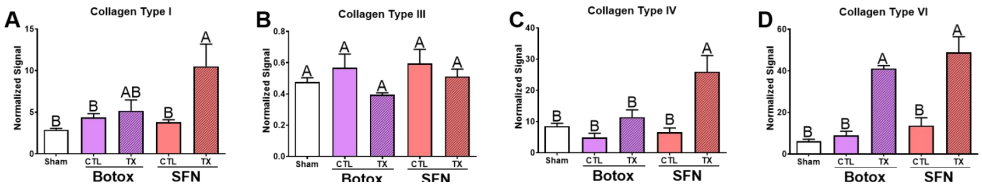

Fig. 1. Fibrosis increases in SFN muscles compared to BTX. Letters not shared indicate a significant differences $p < 0.05$.

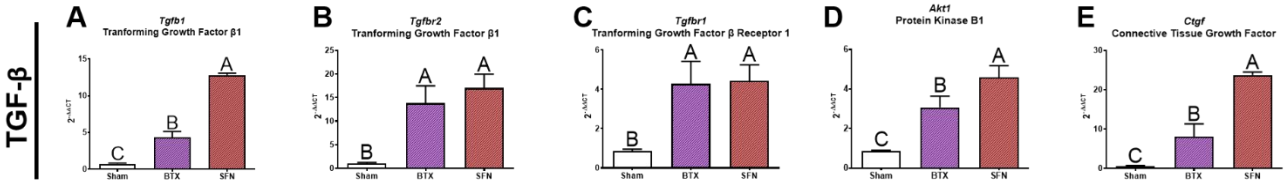

Fig. 2. Gene expression for fibrotic mRNA were elevated in SFN compared to BTX. Letters not shared indicate a significant differences $p < 0.05$.