Understanding the role of tendon in pediatric neuromuscular contracture following neonatal sciatic denervation in mice

Varun Arvind[1], Joshua E. Hyman[1], Alice H. Huang[1]
[1]Columbia University, New York, NY

DISCLOSURES: Varun Arvind (N), Joshua E. Hyman (N), Alice H. Huang (N)

INTRODUCTION: Cerebral palsy (CP), neonatal plexus injuries, and spinal muscular atrophy are common neuromuscular diseases that result in fixed myotendinous contracture(*1–3*). The development of fixed neuromuscular contractures (NC) is debilitating with no known etiology or definitive treatment. Existing research has demonstrated increased muscle inflammation and satellite cell dysregulation with muscle fibrosis/ shortening as causes of contractures(*4*). Despite this, muscle targeted treatments have demonstrated limited long-term improvement of NC(*5*, *6*). More recently, ultrasonography of Achilles tendon in CP patients demonstrated tendon lengthening suggesting a previously unexplored contribution of tendon specific changes to contracture formation(*7*). In this study, we perform neonatal sciatic denervation to determine tendon specific changes that may contribute to NC formation. Neonatal brachial plexus injury has previously been shown as a reliable model of NC formation with muscle phenotype resembling CP, however tendon specific changes have not been reported(*8*, *9*). We hypothesize that sciatic denervation will result in Achilles tendon lengthening with increased inflammation marked by the recruitment of macrophages and T-cells.

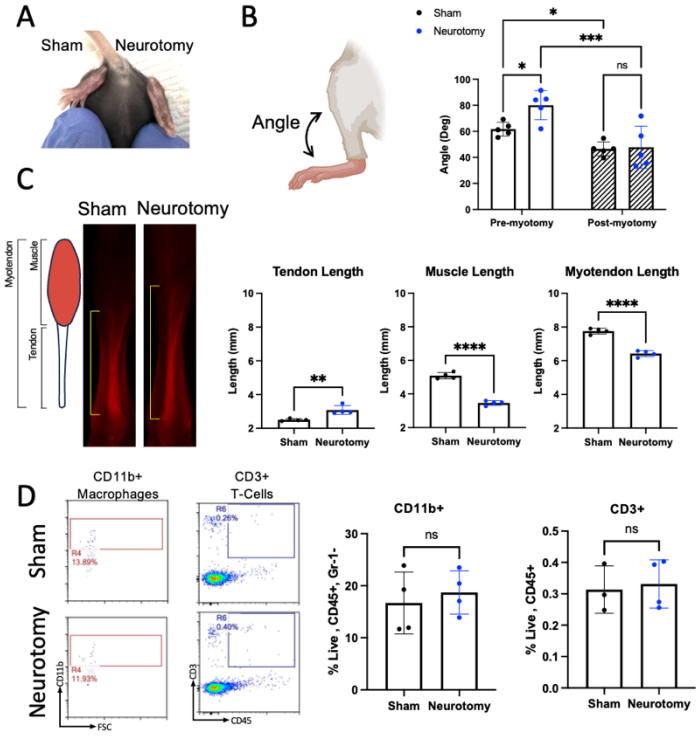

Fig 1. Neonatal sciatic denervation results in fixed equinus contracture. A. Claw deformity observed at 28DPN. **B.** Fixed equinus contracture that is correctable with myotomy (N = 5, two-way ANOVA). **C.** Tissue lengths measured at 14DPN (N=4, t-test). **D.** Flow cytometric profiling at 14DPN (N=4, t-test). *$p<0.05$ **$p<0.01$, ***$p<0.001$, ****$p<0.0001$

METHODS: Neonatal contracture model. At postnatal day 5 (P5), neonatal mice were anesthetized with isoflurane and sciatic nerve was identified and transected proximally in accordance with IACUC. In contralateral limbs, sham operations were performed where the nerve was identified and left intact. Range of motion (ROM): ROM was assessed under anesthesia with mice positioned laterally and limbs tensioned in dorsiflexion using a 5g mass. The angle subtended between the forefoot and tibia was measured digitally (ImageJ). Flow cytometry: Tendons were enzymatically digested (5mg/mL collagenase I, 1mg/mL collagenase IV) for 2hrs at 37C; Ly-6chi (inflammatory) and Ly-6clo (anti-inflammatory) macrophages were gated on DAPI-, CD45+, Gr1–, CD11b+ macrophages and T-cells were gated on DAPI-, CD45+, CD3+ cells. All gates were defined based on autologous spleen samples. Tissue length measurement: Tissue lengths were measured using whole mount microscopy following calibration (Leica). Tendon borders were demarcated using Scx-mCherry. Muscle borders were demarcated by tendon border distally and muscle origin proximally. Myotendon length refers to the sum of muscle and tendon length. Leg length measurement: Limbs were pinned in extension/ abduction and leg lengths were calculated as the sum of tibia and femur lengths measured by x-ray (25kV, intensity 50μA). Statistics were carried out by t-tests or two-way ANOVA (Graphpad Prism).

RESULTS: At 28 days post-neurotomy (28DPN) mice exhibited claw toe deformity (**Fig 1A**) with fixed equinus contracture. To confirm that contracture was not due to peri-articular capsular fibrosis seen following immobilization, we transected the gastrocnemius muscle proximally and observed correction of ROM to sham controls (**Fig 1B**). We also observed no significant differences in leg lengths at 28DPN (not shown). To determine tendon specific changes following contracture, whole mount microscopy of limbs was performed and demonstrated tendon lengthening with muscle and myotendon shortening (**Fig 1C**) at 14DPN. Analysis of immune cell recruitment by flow cytometry of tendons at 14DPN showed no differences in macrophage and T-cell recruitment (**Fig 1D**), with no difference in Ly6c-hi vs -lo macrophage polarization (not shown).

DISCUSSION: Here we establish sciatic denervation in the neonatal mouse as a novel model of hindlimb fixed neuromuscular equinus contracture. To differentiate between peri-articular capsular fibrosis versus myotendinous contracture we performed proximal gastrocnemius myotomy and observed complete correction of ROM to sham controls. This is distinguished from uncorrectable arthrogenic contractures resulting from cast immobilization, suggesting a unique mechanism of NC(*10*). Notably, this model resembles characteristics of neuromuscular contracture in children including claw toe deformity with muscle and myotendon shortening. Using the tendon reporter Scx-mCherry, we observe tendon lengthening consistent with Achille tendon lengthening in CP patients(*7*). While increased inflammation is a hallmark of muscle fibrosis and shortening in NC, we observed no difference in immune cell recruitment to the tendon as a result of denervation. These results suggest tendon specific responses to denervation that contribute to contracture are distinguished from muscle and raise open questions regarding tendon specific changes in ECM, proliferation, and maturation as a result of denervation.

SIGNIFICANCE: Elucidation of cellular and molecular mechanisms that regulate tendon responses to denervation is critical for developing novel therapies for neuromuscular contracture.

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