Potential Role of Non-Canonical Myostatin Signaling Pathways ERK and JNK in Neuromuscular Contractures after Neonatal Brachial Plexus Injury

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INTRODUCTION: Neonatal Brachial Plexus Injury (NBPI) is the most common cause of upper extremity paralysis in children. Although the paralysis can recover, secondary muscle contractures that limit functional use of the upper limb cannot be prevented or cured with existing therapies. Previous work in animal models has shown that contractures form due to impairments in muscle longitudinal growth following denervation. This growth impairment is associated with increased proteasome-mediated protein degradation, and systemic administration of proteasome inhibitors following NBPI can rescue longitudinal muscle growth and prevent contractures. However, the potential for off-target toxicity of proteasome inhibition calls for investigation of muscle-specific therapies. To that end, we found that inhibition of myostatin (MSTN), a muscle-specific inhibitor of muscle growth, reduced proteasome activity, restored longitudinal muscle growth, and rescued contractures. However, the therapeutic effect of MSTN inhibition was only seen in female mice and was not associated with alterations in its canonical signaling pathway (SMAD 2/3, Akt). Thus, we must examine the role of non-canonical myostatin signaling pathways, including Extracellular Receptor Kinase (ERK) and c-Jun N-Terminal Kinase Signaling (JNK), in contracture pathophysiology and as targets for contracture prevention across sexes. We hypothesized that JNK and/or ERK signaling would be disrupted in neonatally denervated muscle, and that inhibition of JNK and ERK would restore longitudinal muscle growth and prevent contractures.

METHODS: With IACUC approval, NBPI was surgically created in postnatal day (P) 5 mice (Charles River) by unilateral extraforaminal excision of the C5–T1 nerve roots under isoflurane anesthesia. This model reliably produces elbow flexion contractures within 4 weeks following denervation. To characterize signaling activity during contracture development, mice were harvested at P19, 2 weeks post-NBPI surgery, when contractures are beginning to form. Brachialis and bicep muscles were homogenized, and RNA was extracted for qPCR and protein for Western blots, respectively. In separate mice following NBPI surgery, ERK and JNK activity were inhibited with Mirdametinib at 5mg/kg (Selleckchem) and SP600125 at 15mg/kg (Selleckchem) respectively. Drug treatment was administered via IP injections beginning immediately after surgery at P5 and then every other day until harvest at 4 weeks post-operatively. 10% DMSO/PBS solution was injected in separate litters of mice as controls for both inhibitors. Four weeks post-NBPI at P33, mice were sacrificed, whereupon bilateral elbow passive range of motion was assessed through a validated digital photography technique to determine contracture severity. Elbow flexion contracture severity is calculated as the difference in passive elbow extension between the denervated and contralateral limbs. All measurements were performed blinded to treatment. We performed 2-way Analysis of Variance (ANOVA) tests with a Bonferroni correction of multiple comparisons using GraphPad Prism 8. A total of 29 mice were used in this study.

RESULTS: We began by characterizing ERK and JNK gene and protein expression in denervated muscles. Gene analysis via qPCR reveals a 5-fold increase in relative ERK2 gene expression in muscles of male mice after NBPI compared to contralateral controls (6.737±4.161 vs. 1.325±0.2591) (**Fig. 1A**). These results are mirrored in relative ERK2 expression in denervated female muscles, and JNK1 expression in denervated male muscles (**Fig. 2A**), though data is underpowered in this preliminary analysis. No differences were observed with protein expression of ERK and JNK in denervated muscles of either sex, nor with the ratio of phosphorylated to total ERK and JNK. We then manipulated ERK and JNK signaling via pharmacologic inhibitors. In comparison to vehicle controls, JNK inhibition reduces elbow flexion contractures in male mice only (63.41.5±6.702º vs. 30.23±14.19º) (**Fig. 2B-C**). No changes in contracture severity were observed with ERK inhibition in either sex (**Fig. 1B-C**).

DISCUSSION: Our current preliminary findings reveal that the MSTN noncanonical pathway JNK may have a role in mediating contracture formation following NBPI, potentially in a sex-specific manner, as JNK inhibition rescues elbow flexion contracture formation in male mice, corresponding to a potential, though currently underpowered, increase in JNK1 mRNA levels in male denervated muscle following NBPI. Conversely, the increased ERK2 expression was not reflected in a rescue of contractures with ERK inhibition, although these preliminary analyses may have failed to find more subtle differences. Increasing sample size in ongoing experiments in additional litters will be able to detect or rule out more subtle differences in gene expression, protein levels/activity, and contracture phenotype for both ERK and JNK signaling. Additionally, future experiments will examine ERK and JNK signaling after myostatin inhibition and after genetic manipulation of myostatin signaling. Nonetheless, our preliminary data suggest a potential role for noncanonical myostatin signaling pathways in muscle growth and contractures. Furthermore, the sex dimorphisms seen here underscore the need to consider sex as a biological variable while dissecting molecular mechanisms of contracture pathophysiology.

SIGNIFICANCE/CLINICAL RELEVANCE: This study identifies further clues to the molecular basis of neuromuscular contracture formation, potentially leading to additional targets for medical contracture prevention and treatment strategies.

Figure 1: (**A**) NBPI alters ERK transcription in neonatal skeletal muscle. (**B**) Representative images of forelimbs, and (**C**) quantitative analysis of passive elbow extension revealed that inhibition of ERK signaling with Mirdametinib does not prevent the formation of elbow flexion contractures. Data are presented as mean ± SD. Statistical analyses: 2-way ANOVA for sex and denervation (repeated measures between limbs) (**A**), or for sex and treatment (**C**), with a Bonferroni correction for multiple comparisons. *P<0.05.

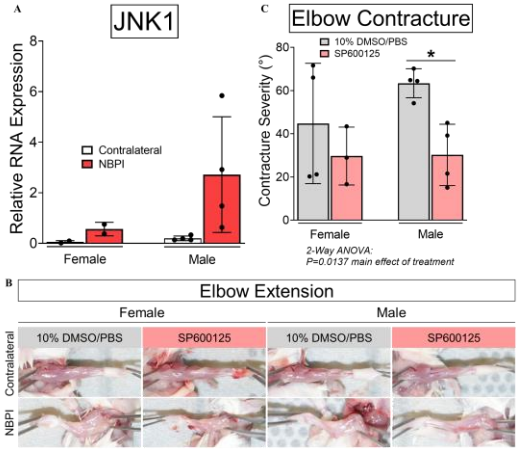

Figure 2: (**A**) NBPI alters JNK1 transcription in neonatal skeletal muscle. (**B**) Representative images of forelimbs, and (**C**) quantitative analysis of passive elbow extension revealed that inhibition of JNK signaling with SP600125 prevents the formation of elbow flexion contractures in male mice. Data are presented as mean ± SD. Statistical analyses: 2-way ANOVA for sex and denervation (repeated measures between limbs) (**A**), or for sex and treatment (**C**), with a Bonferroni correction for multiple comparisons. *P<0.05.