Afferent Muscle Denervation And Contracture Formation Following Neonatal Brachial Plexus Injury

Athanasia Nikolaou, PhD\textsuperscript{1}, Liangjun Hu, MS\textsuperscript{1}, Christopher Wylie\textsuperscript{2}, Roger Cornwall\textsuperscript{1}.

\textsuperscript{1}Cincinnati Children’s Hospital Medical Center, Cincinnati, OH, USA, \textsuperscript{2}Cincinnati Children’s Research Foundation, Cincinnati, OH, USA.

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Introduction: Neonatal brachial plexus injury (NBPI) occurs in 1-3 per 1,000 live births, leaving permanent paralysis in 20-30\% of children. Secondary shoulder and elbow contractures (joint stiffness) are common in these children and are the most common reason for surgery following NBPI. However, no surgical or nonsurgical treatments have proven reliably and safely able to restore normal upper extremity function once contractures have developed, largely because the etiology of such contractures is incompletely understood.

We have previously demonstrated in a mouse model of NBPI that impaired growth of denervated muscle contributes to contracture formation. We have also demonstrated that denervation leads to contractures in a dose-dependent manner, and that early reinnervation restores muscle growth and prevents contractures. However, the relative contribution of motor (efferent) versus sensory (afferent) denervation and reinnervation to contracture pathophysiology is unknown. Clinical evidence suggests that preservation of afferent innervation protects against contractures, as children with neonatal pre-ganglionic nerve rootlet avulsion injuries do not develop contractures, whereas post-ganglionic injuries routinely cause contractures. In this investigation, we carried out pre-ganglionic NBPI via intraforaminal dorsal/ventral nerve rootlet rhizotomies and post-ganglionic NBPI via extraforaminal nerve root excision to test the hypothesis that maintenance of afferent innervation of neonatal muscle would be protective against contracture formation following NBPI, whereas mixed nerve denervation caused by a post-ganglionic NBPI would lead to contractures.

Methods: All protocols were approved by the Institutional Animal Care and Use Committee. Four litters of 5-day-old CD-1 mice were randomized to undergo unilateral pre- or post-ganglionic NBPI at 5 days of age under general anesthesia. In the pre-ganglionic group, the C5 and C6 dorsal and ventral rootlets were transected through cervical laminectomies and durotomies. The rootlets and dorsal root ganglia were reflected out of the foramen to prevent healing and then the dura mater was repaired with fibrin glue. In the post-ganglionic group, the C5 and C6 extraforaminal nerve roots were excised to prevent healing. Laminectomies were then performed and fibrin glue placed on the dura mater to control for the effect of spinal exposure and fibrin glue between the two groups. Appropriate neurological deficit was confirmed by motor function assessment post-operatively. Mice with insufficient paralysis (present elbow flexion) or excessive paralysis (global brachial plexus injury) were excluded from further study. Eight mice per surgical group remained.

At four weeks post-operatively, blinded to the surgery group, motor function was assessed pre-sacrifice, and then passive range of motion (PROM) was assessed post-sacrifice using a validated digital photography technique. The musculocutaneous nerve (MCN) was removed from operated and control limbs, fixed and processed in paraffin for immunohistochemical analysis of axonal content using anti-neurofilament-H (NF-H) to stain total axons and anti-parvalbumin (PV) to stain the muscle afferent axons (distinct from cutaneous sensory axons). Operated and control forelimbs were then harvested and fixed while secured to cork cubes at 0° shoulder abduction and 90° elbow flexion. Following fixation, the biceps and brachialis were removed, stained with 25\% Lugol’s solution in PBS overnight and imaged at 20μm resolution by MicroCT to determine muscle volume and cross-sectional area (CSA). Muscles were then recovered and processed in paraffin for hematoxylin and eosin staining and determination of sarcomere length by measurement of images taken under 40x oil DIC microscopy.

Continuous variables were compared using Student t-tests (paired when using the contralateral limb as control). Samples sizes for each experiment were set to provide at least 80\% power for each variable tested.

Results: Total axon counts in the MCN identified by anti-NF-H staining confirmed a similar level of denervation was achieved between the two surgical groups. However, anti-PV staining revealed the MCN of mice injured by pre-ganglionic NBPI to contain a 2.8-fold higher number of afferent axons than the post-ganglionic NBPI surgical group (P=0.03, unpaired t-test), indicating the preservation of some muscle afferent innervation following pre-ganglionic NBPI. Assessment of PROM at the elbow showed an absence of contracture development following pre-ganglionic NBPI (average 3.5° ± 4.1°), in contrast to post-ganglionic NBPI which generated elbow flexion contractures >10° (average 10.9° ± 8.7°; P=0.05, unpaired t-test). The denervated biceps and brachialis muscles were significantly smaller in volume and CSA than their contralateral controls in both groups (P<0.0001 each pair, paired t-test), although no difference existed between the two surgical groups, consistent with the similar efferent denervation. Sarcomeres were also significantly elongated in the brachialis muscles on the operated versus control sides in both groups (P=0.01 for pre- and P=0.0004 for post-ganglionic NBPI, paired t-test), although no differences in sarcomere elongation...
were noted between the two groups. Analysis of biceps sarcomere length is ongoing.

**Discussion:** The findings of the current study support the hypothesis that preservation of afferent innervation is protective against contractures following NBPI. Our surgical model of pre- and post-ganglionic NBPI caused similar denervation of the elbow flexors, as evidence by similar MCN total axon counts and muscle volume and CSA. However, pre-ganglionic NBPI, preserving a greater number of anti-PV stained afferent axons, failed to cause the contractures that were seen in the post-ganglionic group and that have been seen previously in our model of post-ganglionic NBPI. The sarcomere elongation we have seen in previous experiments with our model was not present to the same degree in either group in this study, possibly due to tissue processing differences that may have also been responsible for the lack of difference in sarcomere elongation between the two groups in this study. Thus, the effect of preserved afferent innervation on sarcomere elongation, and thus functional muscle length, remains to be determined in ongoing experiments. Similarly, while the muscles of the shoulder girdle were not examined in this study, an even more dramatic difference was seen between the two groups in the presence of shoulder internal rotation contractures, warranting ongoing study of this process at the shoulder as well. Nonetheless, the current study encourages further exploration of the role of afferent innervation in postnatal muscle growth.

**Significance:** Understanding the cellular and molecular basis of contracture formation following neonatal denervation will allow novel strategies to prevent and treat contractures as well as improve muscle functional recovery while it awaits reinnervation. Furthermore, understanding the link between innervation and postnatal muscle development will have implications in a wide variety of childhood neuromuscular and musculoskeletal disorders.

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**References:**

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