NRG/ErbB signaling regulates neonatal muscle growth but not neuromuscular contractures in neonatal brachial plexus injury

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Neonatal brachial plexus injury (NBPI) causes disabling and incurable muscle contractures that are driven by impaired growth of denervated muscles. A rare form of NBPI, which maintains afferent muscle innervation despite motor denervation, does not cause contractures. As afferent innervation regulates various aspects of skeletal muscle homeostasis through NRG/ErbB signaling, our current study investigated the role of this pathway in modulating contracture development. Through pharmacologic modification with an ErbB antagonist and NRG1 isoforms, we discovered that NRG/ErbB signaling does not modulate the development of contractures in neonatal mice. Instead, ErbB inhibition impeded growth in nondenervated skeletal muscles, whereas increased ErbB activation exacerbated denervation-induced skeletal muscle atrophy. This potential regulatory effect of NRG/ErbB signaling on neonatal muscle growth warrants deeper investigation.

Keywords: denervation; ErbB; muscle atrophy; muscle development; muscle growth; neonatal brachial plexus injury; neuregulin; neuromuscular contractures

Injury to the brachial plexus during childbirth is a common cause of pediatric paralysis, and it leads to the formation of secondary muscle contractures in the upper limb that further complicate the muscle weakness. [1–3] These contractures severely limit the passive range of motion of the involved limbs, resulting in dysfunction and deformity. [4] Unfortunately, current strategies are not effective for restoring muscle function or joint range of motion once these contractures have developed. [5–7] Therefore, it is imperative to establish greater insights into the pathophysiology of contracture development and design effective strategies to prevent them.

Utilizing a mouse model of postganglionic neonatal brachial plexus injury (NBPI) to induce contractures, we previously discovered that neonatal muscle denervation impairs longitudinal muscle growth. [8–11] More recently, our findings established that the deficit in functional muscle length, as characterized by a reduction in the number of sarcomeres, is driven by

elevated levels of proteasome-mediated protein degradation in denervated muscles. [12,13] Despite these advances, the precise mechanistic link(s) between denervation, proteasome activity, sarcomerogenesis, and contractures is presently unclear and warrants further elucidation. In particular, we need to better understand the molecular interactions between neurons and skeletal muscle, and the underlying signaling mechanism(s) by which neural input governs longitudinal muscle growth and contracture formation.

We can gain mechanistic insights into contracture development through a rare form of NBPI observed in children. In contrast to the more common version of NBPI in which postganglionic nerve root rupture causes complete muscle denervation, NBPI can also involve preganglionic nerve root avulsion in which the nerve roots are injured proximal to the dorsal root ganglion. [14] This type of injury not only disrupts the efferent (motor) innervation of the muscle in a manner similar to postganglionic injury, but also preserves the muscle's afferent (sensory) innervation, as the connection between the dorsal root ganglion containing the afferent neuron cell bodies and the affected muscle remains intact. Clinically, it has been noted that children with the preganglionic version of NBPI do not develop contractures, despite identical motor paralysis as occurs in postganglionic NBPI, which does cause contractures. [14] This observation challenges the long-held notion that contractures are an inevitable result of muscle paralysis and/or limb immobility, [4] and instead suggests that there are unique features of preganglionic NBPI that can protect against contractures even in paralyzed muscle. We have recapitulated both injury models in mice and found that preganglionic injuries indeed preserve afferent innervation and do not cause contractures, whereas postganglionic injuries result in complete denervation and the formation of contractures. [11] It is thus possible that preservation of muscle afferent innervation in preganglionic injuries sustains the muscle's longitudinal growth and protects against contractures despite motor paralysis. Interrogating this potential protective effect of afferent innervation could augment our understanding of its role in skeletal muscle growth and contracture development, and potentially lead to ways to recapitulate the protective effect of preganglionic NBPI against contractures in completely denervated muscle.

Afferent innervation is known to impact several aspects of muscle development, including muscle spindle formation and maintenance, through the NRG/ErbB signaling pathway. [15–18] This interaction is mediated by the binding of neuregulin-1 (NRG1) secreted by afferent axons to the ErbB family of tyrosine kinase receptors expressed on muscle fibers, [18,19] which promotes the differentiation of myofibers into intrafusal spindle fibers. [19,20] Our earlier work reported that spindles are preserved in preganglionic injury, but completely degenerated following postganglionic NBPI. [11] As ErbB activity is altered in the degenerated spindles following complete denervation, we speculate that NRG/ErbB signaling could be a mechanism by which preserved afferent innervation protects against contractures in muscles denervated by preganglionic NBPI. We further speculate that the formation of contractures following postganglionic NBPI is attributed to perturbed NRG/ErbB signaling due to loss of afferent innervation. A potential role for NRG/ErbB signaling in muscle growth and contractures is further supported by the prior findings that NRG/ErbB signaling is also important in regulating many key aspects of muscle homeostasis, including myogenesis and myoblast differentiation, [21–24] and muscle regeneration after injury. [15,25]

Hence, in this present study, we deciphered the involvement of the NRG/ErbB pathway in modulating muscle contractures. Specifically, we investigated whether the protective effect of preganglionic injury is conferred by NRG/ErbB signaling and whether restoration of NRG/ErbB signaling in postganglionic injury could prevent contractures. We utilized pharmacologic tools to manipulate ErbB activity following different models of NPBI that preserved afferent innervation or led to complete denervation, and found that NRG/ErbB signaling does not modulate the development of contractures. Instead, we discovered that alterations in this pathway resulted in impaired muscle length, cross-sectional area, and volume of both denervated and nondenervated muscles, suggesting a novel regulatory role of NRG/ErbB signaling for promoting proper growth and preventing injury-induced atrophy during neonatal muscle development. These collective findings rule out NRG/ErbB signaling as a potential mechanism and pharmacologic target for modulating contractures following NBPI, but identify a role for NRG/ErbB signaling in developmental muscle growth.

Materials and methods

Ethical statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All mice were handled according to approved Institutional Animal Care and Use Committee (IACUC) protocols (#2017-
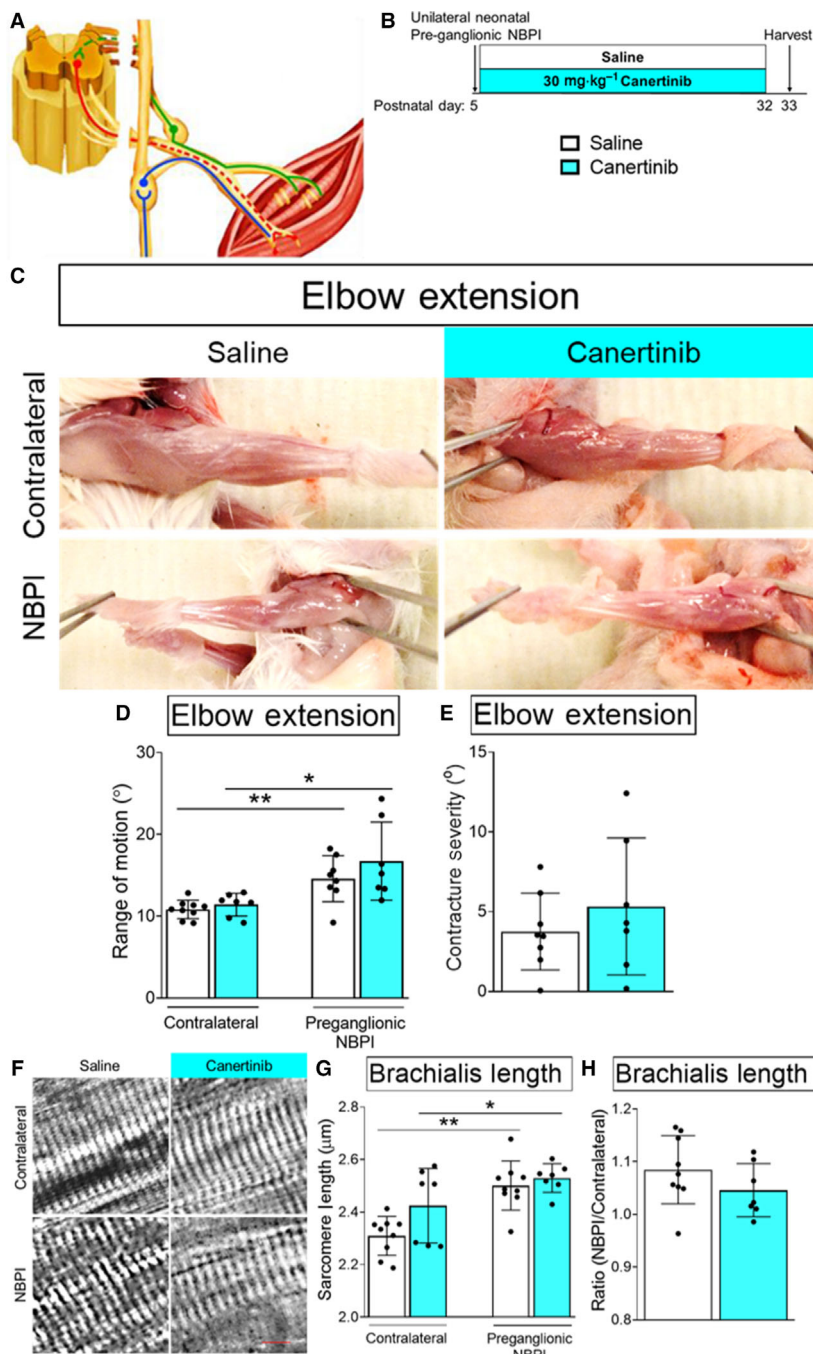
Fig. 1. Inhibition of ErbB activity does not induce contractures after preganglionic injury. (A) Schematic diagram of preganglionic NBPI, which preserves muscle spindle function, with efferent (red), afferent (green), and sympathetic (blue) neurons depicted. Dashed lines indicate the Wallerian degeneration of axons (denervation). (B) Schematic depicting saline or canertinib treatment during neonatal muscle development (P8–P32), and assessment of function at 4 weeks following a mouse model of preganglionic NBPI. (C) Representative images of forelimbs, and quantitation of both (D) elbow joint range of motion and (E) elbow contracture severity revealed that loss of ErbB activity does not promote contracture formation. (F) Representative DIC images of sarcomeres, and (G) quantitation of sarcomere length showed that preganglionic NBPI reduces brachialis muscle length. (H) Inhibition of ErbB activity does not further impair functional length in denervated muscles, as shown by analysis of normalized sarcomere length between control and denervated limbs. Data are presented as mean ± SD. Statistical analyses: (D), (G) unpaired, two-tailed Student's *t*-test between groups and paired, two-tailed Student's *t*-tests between limbs of mice in each group, (E), (H) unpaired two-tailed Student's *t*-tests. *P < 0.05, **P < 0.01. Scale bar: 10 µm.

0084) of the Cincinnati Children's Hospital Medical Center, and every effort was made to minimize suffering.

Mice

Two models of neonatal brachial plexus injury (NBPI) were surgically created in postnatal day (P) 5 CD-1 mice (Charles River) under isoflurane anesthesia. To recapitulate the form of NBPI that does not induce contractures, preganglionic NBPI was generated by unilateral intraforaminal transection of the C5–C7 dorsal and ventral rootlets, which preserves afferent and sympathetic innervation to the muscle (Fig. 1A). [11] In contrast, postganglionic NBPIs were achieved by unilateral extraforaminal excision of the C5–T1 nerve roots, which causes complete efferent and afferent denervation and induces contractures (Fig. 3A). [11] The different nerve root levels selected for preganglionic (C5–C7) and postganglionic (C5–T1)

surgeries were chosen due to mortality encountered with C5–T1 preganglionic injury, as well as potential for spontaneous recovery of C5–C7 postganglionic injury, complicating the contracture phenotype. Nonetheless, both surgeries denervate the elbow flexor muscles, which are used as the model for contracture investigation in this study. Postsurgery, mice were returned to their mums and housed in standard cages on a 1:1 light/dark cycle, with nutrition and activity *ad libitum*.

Pharmaceutics

To inhibit ErbB function following preganglionic surgery, mice were treated with canertinib [26] (LC Laboratories, #CI-1033) at 30 mg/kg body weight through daily intraperitoneal injections. Conversely, separate groups of mice were treated with different isoforms of NRG1 [27,28] at 0.12 mg/kg via daily subcutaneous injections to stimulate ErbB activity after postganglionic surgery. The following isoforms were tested: alpha-extracellular domain (α-ECD; Roche, #5898-NR-050), beta-1 extracellular domain (β1-ECD; Roche, #377-HB-050/CF), beta-1 epidermal growth factor domain (β1-EGF; Roche, #396-HB-050/CF), sensory and motor neuron-derived factor (SMDF; Roche, #378-SM-025/CF), and glial growth factor 2 (GGF2; Reprokine, #RKQ022979). All pharmacologic treatments were started following the respective NBPI surgeries at P5 and continued for 4 weeks postsurgery. Saline (Hospira) and Dulbecco's phosphate-buffered saline (Gibco®) served as controls for canertinib and the NRG1 isoforms, respectively. Daily body weights were recorded for all mice to monitor toxicity.

Assessment of contractures

At 4 weeks post-NBPI (P33), mice were euthanized by CO_2 asphyxiation. Bilateral elbow range of motion was measured using a validated digital photography technique postsacrifice with blinding to treatment groups. [12,13] The presence of an elbow flexion contracture within a mouse is indicated by a discrepancy in range of motion that is $\geq 10°$ between its denervated and contralateral limbs. Representative images of forelimbs shown within Figs 1C and 3C have been processed to reflect comparable levels of sharpness, brightness, and contrast for illustrative purposes, although no image manipulation was performed prior to measurements.

Muscle collection

Following image photography, bilateral biceps muscles were then embedded in OCT compound and frozen in liquid nitrogen-cooled 2-methylbutane for cryosections, and bilateral triceps muscles were flash-frozen in liquid nitrogen and stored at –80°C for gene expression analysis. The

remaining forelimbs were imaged by digital X-ray for humerus length, and bilateral brachialis muscles were subsequently removed and processed for microcomputed tomography (micro-CT) to assess whole-muscle size. [12,13] Postscanning, muscle bundles were isolated and imaged for sarcomeres via differential interference contrast (DIC) microscopy. [12,13]

Ex vivo high-resolution studies

micro-CT was performed using a Siemens Inveon PET/SPECT/CT Scanner (Siemens Medical Solutions, Malvern, PA, USA). The cone-beam CT parameters were as follows: 360° rotation, 1080 projections, 1300-ms exposure time, 1500-ms settle time, 80-kVp voltage, 500-μA current, and effective pixel size 17.67 μm. Acquisitions were reconstructed using a Feldkamp algorithm with mouse beam-hardening correction, slight noise reduction, and 3D matrix size 1024x1024x1536, using manufacturer-provided software. Protocol-specific Hounsfield unit (HU) calibration factor was applied.

Morphometrics

Average sarcomere length of the brachialis muscle was determined by measuring a series of 10 sarcomeres from 6 representative DIC images with the AxioVision program. As previously described, the presence of elongated sarcomeres is associated with a fewer number of sarcomeres in series, thereby indicating a reduction in functional muscle length of the brachialis. [12,13] Representative images of sarcomeres shown within Figs 1F and 3F have been cropped to identical sizes and processed to reflect comparable levels of sharpness, brightness, and contrast for illustrative purposes. No image manipulation was performed prior to measurements. Humerus lengths were recorded from the proximal humerus physis to the distal articular surface on digital X-rays with AxioVision as well. Fiji programs were used to process micro-CT scans into digital imaging and communications in medicine (DICOM) images to quantify whole-muscle cross-sectional area and volume, which were subsequently normalized to humerus length of the corresponding limb. For illustrative purposes, raw DICOM files were processed in IMARIS software (Bitplane, Zurich, Switzerland) to create whole-muscle images presented in Figs 2B and 4B.

Histology

Cryosections were fixed in 4% PFA, permeabilized with 0.2% Triton X-100, subjected to antigen retrieval (2N HCL), and blocked in 1% BSA/10% donkey serum. Samples were then incubated overnight at 4°C with primary antibodies against ErbB2 (1 : 100; R&D Systems, #AF5176, Minneapolis, MN, USA), or phosphorylated
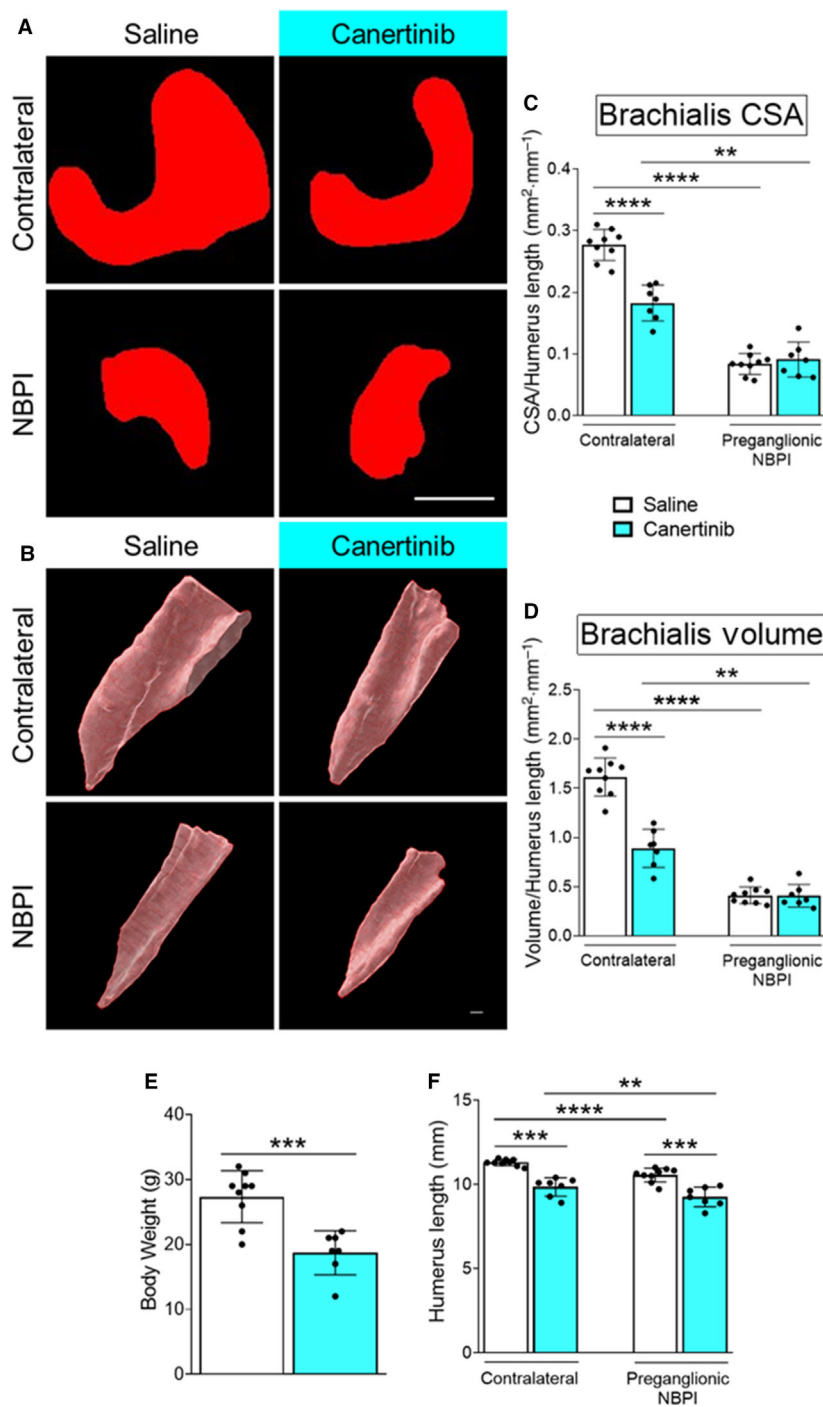
Fig. 2. Inhibition of ErbB activity impairs neonatal skeletal muscle development. Visual comparisons of representative micro-CT images in (A) transverse and (B) 3-dimensional views revealed smaller brachialis muscles in the absence of denervation with canertinib treatment. Analyses of (C) brachialis cross-sectional area and (D) muscle volume confirmed that skeletal muscle growth in control forelimbs is blunted with inhibition of ErbB activity. (E) Body weight and (F) humerus lengths of both control and denervated forelimbs were impeded after 4 weeks of canertinib treatment. Data are presented as mean ± SD. Statistical analyses: (C), (D), (F) unpaired, two-tailed Student's *t*-test between groups and paired, two-tailed Student's *t*-tests between limbs of mice in each group, (E) unpaired two-tailed Student's *t*-tests. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. Scale bar: 1000 μm.

ErbB2 (Tyr1248) (1 : 50; Sigma-Aldrich, #SAB4300061, St. Louis, MO, USA), and co-stained with anti-neurofilament (1 : 5000; Novus, #NB300-217, Littleton, CO, USA) and anti-myosin heavy chain (1 : 100; DSHB, clone S46). [11] Following 1 h of incubation with a secondary Alexa Fluor antibody (1 : 200–800; Invitrogen, Waltham, MA, USA), slides were mounted with ProLong™ Gold Antifade Mountant and visualized on a Nikon A1R Confocal System.

RNA analysis

Whole-muscle gene expression analyses were performed as previously described. [29,30] Briefly, total RNA was extracted from triceps muscles with TRIzol (Invitrogen), and cDNA was synthesized using the MultiScribe™ Reverse Transcriptase with random hexamer primers (Applied Biosystems, Waltham, MA, USA). Expression of NRG/

ErbB pathway genes was assessed using standard qPCR approaches with the PowerUp™ SYBR™ Green Master Mix (Applied Biosystems). [29,30] Analysis was performed on a 7900HT Fast Real-Time PCR Machine (Applied Biosystems) with the following primers: ErbB2 (forward, 5'-GCAAGCACTGTCTGCCATGC-3' and reverse, 5'-GGGCACAAGCCTCA CACTGG-3'); Egr3 (forward, 5'-CCGGTGACCATGAGCAGTTT-3' and reverse, 5'-TAA TGGGCTACCGAGTCGCT-3'); and GAPDH (forward, 5'-TGCGACTTCAACAGCAACTC-3' and reverse, 5'-GCCTCTCTTGCTCAGTGTCC-3') primers served as internal controls.

Statistics

For all data sets, outliers were first detected by Grubbs' test and excluded. All data were subsequently tested for normality with the Shapiro–Wilk test. Normally distributed data were compared with two-tailed Student's t-test, paired where parameters were compared between forelimbs (NBPI versus contralateral) in individual animals, and unpaired when parameters were compared between animals. Non-normally distributed data were compared using the Mann–Whitney U-tests for unpaired data or Wilcoxon's signed-rank tests for paired analyses where parameters were compared between forelimbs. All data are presented as mean ± SD. The degree of significance between data sets is depicted as follows: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$. *A priori* power analyses based on prior work determined that 6 mice per group were required to detect a 10° difference in contractures and a 0.2 µm difference in sarcomere lengths at 80% power between experimental conditions.

Results

Loss of NRG/ErbB signaling does not elicit contractures in a preganglionic injury model

To investigate the role of NRG/ErbB signaling in contracture formation, we first asked whether this signaling pathway is responsible for the absence of contractures in muscle denervated by preganglionic NBPI. As described previously, preganglionic NBPI does not cause contractures, despite motor paralysis of affected muscles. If this protective effect against contractures in the paralyzed muscles is governed by NRG/ErbB signaling, we would expect that inhibition of NRG/ErbB signaling after preganglionic NBPI would lead to the formation of contractures. Following unilateral preganglionic surgery (Fig. 1A), neonatal P5 mice were subjected to daily treatments of canertinib for 4 continuous weeks to block ErbB activity (Fig. 1B). This strategy inhibited whole-muscle gene

expression of Egr3 (a downstream transcription factor of the NRG/ErbB pathway) [31,32] in both control and denervated forelimbs, as well as phosphorylated ErbB levels in muscle spindles of denervated forelimbs (Fig. 1A–C). Despite successful pharmacologic inhibition of NRG/ErbB signaling, there were no signs of contracture formation at the elbow joint upon assessment at P33 (Fig. 1C–E). Preganglionic surgery alone did restrict range of motion in the elbow joint by ~ 5°, which is below the 10° threshold of clinically relevant contractures. Nonetheless, this subtle change in range of motion corresponded to an elongation of sarcomeres (sarcomere overstretch) in brachialis muscles, indicating reduced functional muscle length with fewer sarcomeres in series (Fig. 1F,G). [12,13] Importantly, loss of ErbB activity did not further worsen sarcomere overstretch after preganglionic surgery (Fig. 1G,H), which, along with an absence of contractures with canertinib treatment, demonstrates that the protective effect of preganglionic surgery is not conferred by NRG/ErbB signaling.

Loss of NRG/ErbB signaling impairs neonatal muscle growth

Despite seeing no effect of canertinib treatment on longitudinal growth or contractures following preganglionic injury, we investigated the contribution of this pathway to other aspects of musculoskeletal development. Following preganglionic injury, canertinib treatment reduced both cross-sectional area and volumetric growth in control but not denervated brachialis muscles (Fig. 2A–D), when measured relative to humerus length. These deficits in neonatal muscle growth were accompanied by reductions in body weight and humerus lengths of both limbs (Fig. 2E,F). These collective findings indicate that while inhibition of NRG/ErbB signaling does not induce contractures after preganglionic injury, it is detrimental to neonatal musculoskeletal development.

Enhanced NRG/ErbB signaling in postganglionic injury does not prevent contractures

To further dissect the contributions of NRG/ErbB signaling in contracture development, we subsequently tested the hypothesis that increased ErbB activation prevents contractures in a mouse model of postganglionic injury that does cause contractures. Following unilateral postganglionic surgery (Fig. 3A), separate groups of neonatal P5 mice were treated daily for 4 weeks with different isoforms of NRG1, the primary ligand for ErbB receptor binding (Fig. 3B). This
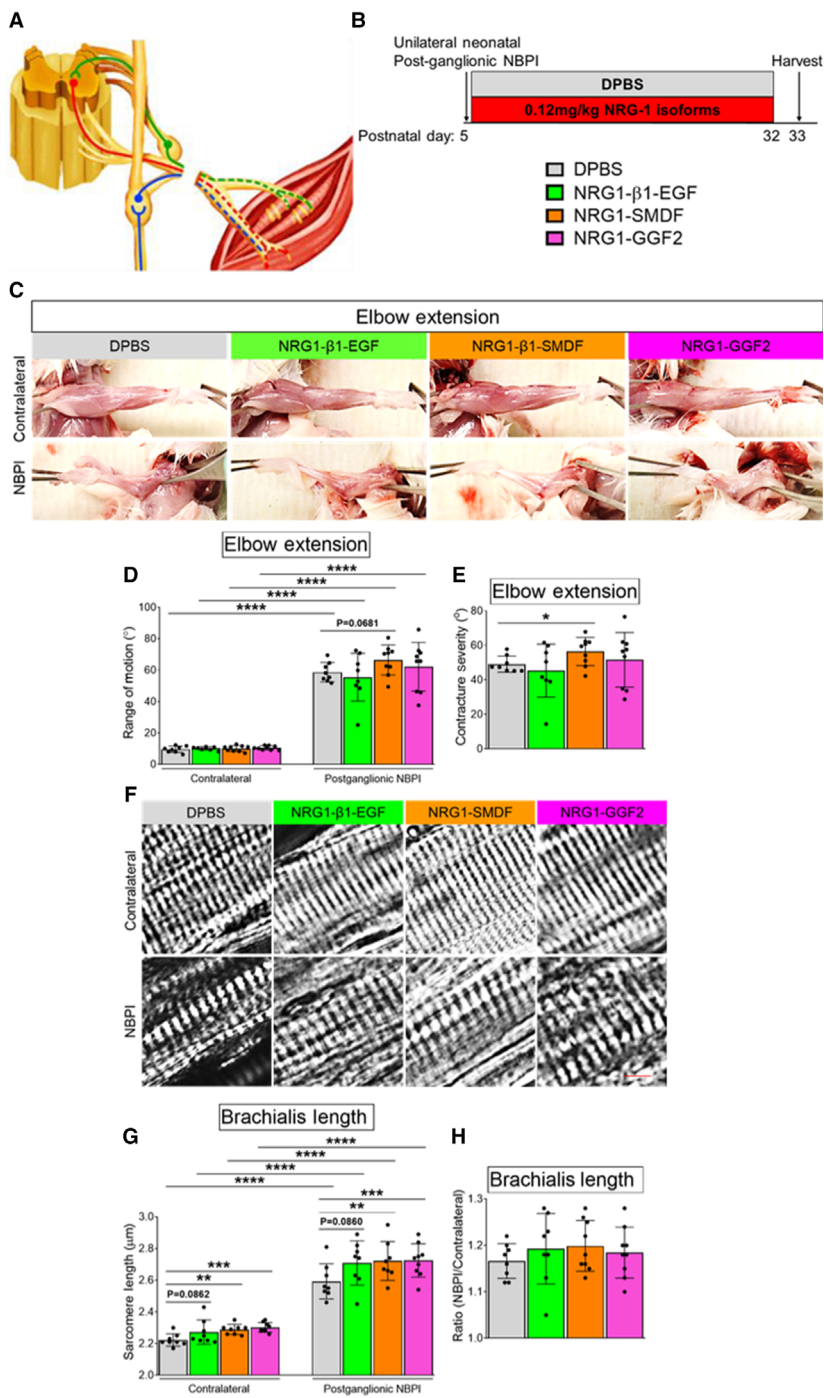
Fig. 3. Increased ErbB activation does not prevent contractures after postganglionic injury. (A) Schematic diagram of postganglionic NBPI, which denervates muscle spindles, with efferent (red), afferent (green), and sympathetic (blue) neurons depicted. Dashed lines indicate the Wallerian degeneration of axons (denervation). (B) Schematic depicting treatment with Dulbecco's phosphate-buffered saline (DPBS) or different NRG1 isoforms from P8–P32, and assessment of function at 4 weeks following a mouse model of postganglionic NBPI. (C) Representative images of elbow extension, and quantitative analyses of both (D) elbow joint range of motion and (E) elbow contracture severity demonstrated that increased ErbB activation does not prevent contracture formation. (F) Representative DIC images of sarcomeres and (G) quantitation of brachialis sarcomere length revealed reductions in functional muscle length of both control and denervated limbs with the SMDF and GGF2 isoforms. (H) Normalized sarcomere length between control and denervated limbs remained unchanged with the different treatments. Data are presented as mean ± SD. Statistical analyses: (D), (G) unpaired, two-tailed Student's *t*-test between groups and paired, two-tailed Student's *t*-tests between limbs of mice in each group, (E), (H) unpaired two-tailed Student's *t*-tests. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Scale bar: 10 μm.

pharmacologic strategy successfully increased ErbB and Egr3 expression in control muscles with the β1-EGF, SMDF, and GGF2 isoforms (Fig. S2A, S2B). In operated limbs, treatment with the β1-EGF and SMDF isoforms increased both ErbB expression and Egr3 expression beyond the elevated levels caused by complete denervation alone. However, the associated increase in ErbB signaling with these 3 isoforms failed

to prevent the development of elbow flexion contractures at 4 weeks postsurgery, and contracture severity was instead worsened by the SMDF isoform (Fig. 3C–E). Furthermore, the SMDF and GGF2 isoforms caused sarcomere overstretch in control and denervated muscles (Fig. 3F,G), without altering proportional muscle lengths between limbs (Fig. 3H). These results indicate that enhanced NRG/ErbB signaling is

ineffective in preventing contractures and rescuing sarcomere overstretch after postganglionic injury, but, instead, impairs sarcomerogenesis in both normal and denervated muscles.

Enhanced NRG/ErbB signaling exacerbates denervation-induced muscle atrophy

Besides impairing sarcomerogenesis, ErbB activation with exogenous administration of the β1-EDF and SMDF isoforms led to diminished cross-sectional area and volume of denervated brachialis muscles after postganglionic injury (Fig. 4A–D), when measured relative to humerus length. Mild attenuations in the size of control muscles were also observed with the β1-EDF isoform (10–14% vs. DPS), though they were not statistically significant. Nonetheless, prominent disruptions in muscle size with these ectopic ligands are observed mainly in denervated muscles. Unlike pharmacologic inhibition of ErbB activity, the deficits in denervated muscle size with enhanced ErbB signaling did not correspond to reduced body weight and humerus length (Fig. 4E,F). However, deficits in longitudinal muscle growth with the GGF2 isoform were associated with a reduction in humerus length of both limbs. Therefore, these results reveal that enhanced NRG/ErbB signaling is not only ineffective for preventing contracture development, but also it is instead detrimental to sarcomerogenesis and exacerbates denervation-induced skeletal muscle atrophy. Taken together, our overall findings highlight the importance of tight regulation of NRG/ErbB signaling in neonatal muscle development and protection against muscle wasting from denervation injury.
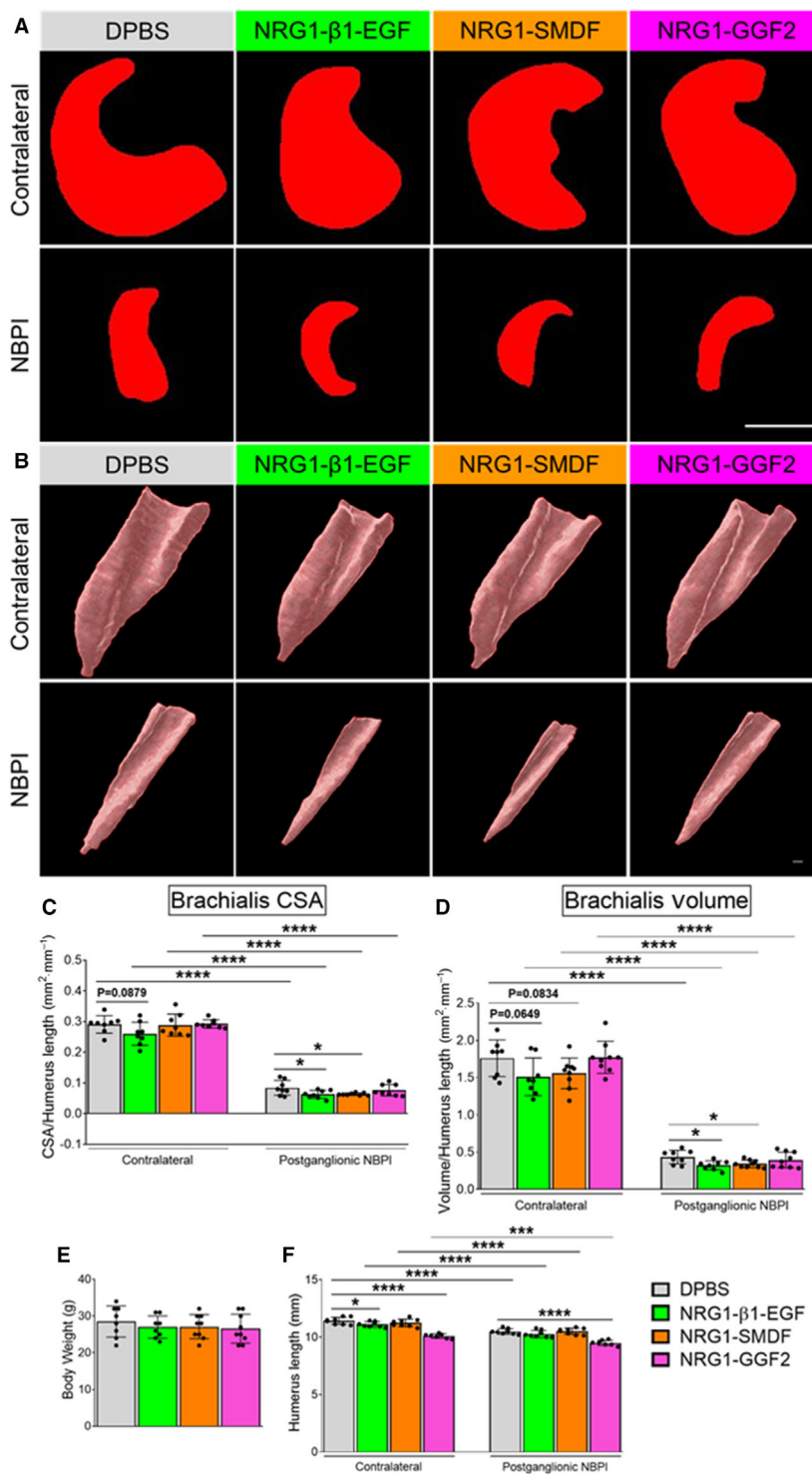
Discussion

The pathophysiology of muscle contractures stemming from brachial plexus birth injury has yet to be fully elucidated, and such limitation in knowledge severely hampers the effectiveness of existing strategies to restore function in denervated limbs. To address this limitation, we have successfully created different murine models of neonatal denervation. With these models, we previously identified that in a preganglionic injury, which disrupts only efferent axons, preservation of afferent innervation potentially protects against contracture development. [11] Therefore, identifying the molecular mechanisms underlying the absence of contractures following preganglionic NBPI could allow pharmacologic recapitulation of this protective effect in the more common postganglionic NBPI that does cause contractures. In the current study, we investigated

whether the protective effect of preganglionic injury is mediated through NRG/ErbB signaling, the predominant pathway governing antegrade afferent neuromuscular transmission, and whether recapitulation of this pathway in completely denervated muscles could prevent contractures. Our results demonstrated that treatment with an ErbB antagonist, canertinib, did not lead to contracture formation following preganglionic injury. We also discovered that increased levels of ErbB activity, following exogenous administration of different isoforms of NRG1, were ineffective for preventing contractures after postganglionic injury. These results rule out the NRG/ErbB signaling pathway as a mechanism in contracture formation, an important step in elucidating the unknown mechanism(s) by which neural input regulates longitudinal muscle growth and contractures, thus narrowing the search for pharmacologic targets to prevent contractures.

However, canertinib impaired cross-sectional area and volume in the normally innervated neonatal muscles, suggesting a regulatory role for NRG/ErbB signaling in governing muscle growth during neonatal development. In addition, certain NRG1 isoforms worsened sarcomere overstretch and reduced cross-sectional area and volume of denervated muscles, indicating a role for NRG/ErbB signaling in denervation-induced muscle atrophy as well. Overall, our findings establish that a precise regulation of ErbB activity is required to facilitate neonatal muscle development and limit muscle loss after denervation. This cautionary finding is particularly relevant in light of recent interest in the NRG/ErbB pathway as a potential target to ameliorate the deleterious effects of muscle denervation. [33]

Since our results demonstrate that the protective effect of preganglionic NBPI against contractures is not conferred by NRG/ErbB signaling, we must consider other potential mechanisms. First, the protective effect of preganglionic injury may be conferred through an alternate molecular cross-talk pathway between afferent neurons and skeletal muscle. Second, following preganglionic injuries, both dorsal root and sympathetic ganglia remain connected to the muscle, thereby preserving both afferent innervation and sympathetic innervation. [11] Therefore, the protective effect of preganglionic injury could instead be conferred primarily by sympathetic innervation. Sympathetic neurons directly innervate muscle spindles, and sympathetic innervation of skeletal muscle is required for the function and maintenance of the neuromuscular junction. [34,35] This relationship is exerted through β-adrenergic signaling, [35] which also prevents denervation-induced muscle wasting, and promotes muscle growth and regeneration after injury. [36] Future efforts should therefore discern

Fig. 4. Increased ErbB activation exacerbates denervation-induced muscle atrophy. Representative micro-CT images in (A) transverse and (B) 3-dimensional views (C) revealed smaller brachialis muscles after denervation with treatment of the β1-EGF and SMDF isoforms. Quantitative analyses of cross-sectional area and (D) whole-muscle volume confirmed further size reductions in denervated brachialis muscles following treatment with the β1-EGF and SMDF isoforms. (E) Body weight was not altered by treatments with any of the NRG1 isoforms. (F) Humerus lengths of both control and denervated forelimbs were reduced after 4-week treatment with the NRG1-SMDF isoform. Data are presented as mean ± SD. Statistical analyses: (C), (D), (F) unpaired, two-tailed Student's *t*-test between groups and paired, two-tailed Student's *t*-tests between limbs of mice in each group, (E) unpaired two-tailed Student's *t*-tests. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$. Scale bar: 1000 μm.

precisely whether preservation of sympathetic innervation alone is sufficient for preventing contractures, or whether the synergy of afferent and sympathetic innervation is responsible for the protective effect of preganglionic NBPI.

A paradoxical finding in this study is the reduction in functional muscle length of both control and denervated muscles with enhanced ErbB activity, despite the absence of any modifications in contracture development. As we previously reported that contractures

arise from impaired longitudinal growth of denervated muscles, [8–11] our current results posit a threshold-dependent role for sarcomere length whereby contractures only occur following a critical magnitude of growth deficit. Alternatively, additional mechanisms may drive contracture formation after denervation injury. It is therefore paramount to continue deciphering the pathophysiology of contracture development to identify appropriate therapeutic targets. Nonetheless, our findings suggest a possible role for NRG/ErbB signaling in longitudinal muscle growth. Muscle length is an underappreciated aspect of skeletal muscle development and adaptation. Increases in myofiber length after surgical methods of mechanical overload and exercise models of muscle hypertrophy are associated with increased myofiber cross-sectional area, [37,38] whereas limb immobilization reduces the number of sarcomeres. [39] Despite the relevance of such adaptations, underlying mechanisms that regulate the length of a growing muscle, specifically the addition of sarcomeres, are poorly understood. In light of this knowledge gap, NRG/ErbB signaling serves as a potential candidate for dissecting mechanisms associated with muscle length and sarcomerogenesis.

While the expression of NRG1 and ErbB receptors has been reported to be upregulated in skeletal muscles after denervation, [33,40] their roles in this context are not well-defined. In an *in vitro* cell culture model of injury, NRG1 activity has recently been shown to elicit a modest rescue in C2C12 myotube diameter following dexamethasone treatment. [33] In contrast, we observed that stimulation of ErbB activity with certain NRG1 isoforms is unexpectedly deleterious to denervation-induced muscle atrophy in neonatal mice. This discrepancy in findings may be partially attributed to differences in the models utilized. As elegantly described by Morano et al, the detection of the complete effects of NRG1 might be hindered by assessing myotubes as opposed to myofibers, and by the presence of a subpopulation of undifferentiated myoblasts. [33] In addition, our *in vivo* mouse model induces a neurological deficit, whereas dexamethasone impairs skeletal muscle regeneration postinjury primarily by undermining the activation and differentiation of muscle stem cells (satellite cells). [41,42] Nevertheless, we cannot discount the possibility that the differential effects observed with NRG1 treatment may be dose-dependent, and future studies are needed to conclusively verify the function of NRG/ErbB signaling in muscle atrophy with denervation and/or injury.

Besides exacerbating muscle loss after neonatal denervation, our study also revealed that NRG/ErbB signaling contributes to neonatal skeletal muscle development. In mice, fusion of satellite cells to existing myofibers (myonuclear accretion) drives the initial stage of neonatal muscle growth, whereas subsequent gains in muscle mass are mediated by protein synthesis. [43,44] As ErbB activity is enhanced in satellite cells during developmental myogenesis, [24] NRG/ErbB signaling could serve as a potential mechanism through which myonuclear accretion is regulated during neonatal muscle development. Beyond developmental growth, as satellite cells upregulate NRG1 during postnatal adaptive processes such as muscle regeneration, [25] NRG1/ErbB signaling may potentially underlie how myonuclear accretion facilitates muscle hypertrophy after exercise or increased mechanical load. [29,30] Future investigations should therefore dissect feedback mechanisms and identify specific downstream targets of NRG/ErbB signaling to develop novel strategies for preventing low muscle mass during childhood, as well as treating muscle wasting disorders in pediatric patients.

Although NRG/ErbB signaling does not modulate contracture development following NBPI, our discovery allows us to focus on more effective targets for novel pharmacologic therapies. Moreover, our findings identify novel roles for NRG/ErbB signaling in neonatal skeletal muscle during developmental growth and atrophy after denervation injury, thereby establishing greater mechanistic insights into the regulation of skeletal muscle mass.

Acknowledgements

Author contributions

RC conceived and supervised the study; BLH, QG, SN, and RC designed experiments; BLH, QG, SN,

LH, KSW, and RC performed experiments and analyzed the data; and QG wrote the manuscript with assistance from all authors.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig S1. Pharmacologic inhibition of ErbB activity after preganglionic NBPI.

Fig S2. Pharmacologic stimulation of ErbB activity after postganglionic NBPI.