Title: Myostatin as a potential target for treatment of muscle contractures
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Capsule:
Background: Neonatal muscle denervation impairs longitudinal muscle growth, and leads to the formation of disabling contractures.
Results: Pharmacologic inhibition of myostatin, a negative regulator of muscle size, reduced contracture formation and preserved longitudinal muscle growth only in neonatal female mice.
Conclusions: Myostatin signaling regulates contracture formation in a sex-specific manner.
Significance: Sexual dimorphism is a critical aspect of neurological deficits.

Introduction: Injury to the brachial plexus at birth (Neonatal Brachial Plexus Injury – NBPI) is the most common cause of upper limb paralysis in children,[1] occurring in approximately 1.5 of every 1,000 live births.[2] It leads to the formation of secondary muscle contractures, or limb stiffness, which are disabling and incurable. These contractures severely impede range of motion of the involved limbs, thereby impairing activities of daily living, and ultimately resulting in skeletal deformity and dysfunction.[3] However, current strategies are insufficient in restoring muscle function and joint range of motion once contractures have developed. It is therefore important to establish greater insights into the pathophysiology of contracture development in order to develop effective treatment strategies.

Using a mouse model of NBPI, we previously discovered that contractures result from impaired longitudinal muscle growth.[4] This deficit in muscle length is mediated by increased levels of proteasome-mediated protein degradation in the denervated muscle, as inhibition of the proteasome markedly reduces contracture development.[5] However, this novel pharmacologic strategy cannot be easily translated to humans. Prolonged administration of proteasome inhibitors results in potential cumulative toxicity as these drugs nonspecifically block degradation and cause tissue damage to many organs, such as the brain,[5] and even impede musculoskeletal development.[6] As a result, our lab seeks to identify safer strategies to prevent contracture development by targeting muscle-specific regulators of the balance between protein degradation and synthesis.

One such signaling pathway specific to skeletal muscle is the myostatin pathway.[7] A secreted growth differentiation factor, myostatin binds to Activin A through Activin Type 2 receptors (primarily ACVR2B) to increase protein degradation, decrease protein synthesis, and ultimately attenuate muscle mass.[7] As a negative regulator of protein balance and muscle mass, it is possible that myostatin is a signaling pathway by which denervation impairs muscle growth. Specifically, we hypothesize that neonatal denervation induces contractures through myostatin-dependent impairment of postnatal longitudinal muscle growth. If contractures are indeed mediated through myostatin signaling, it could lead to a potential breakthrough in identifying a muscle-specific target for contracture prevention. Hence, our primary aim is to elucidate the role of myostatin in the formation of denervation-induced muscle contractures. In the current study, we specifically investigated whether pharmacologic inhibition of the myostatin pathway preserves longitudinal muscle growth and prevents contractures after NBPI.

Materials and Methods: To test our hypothesis, we utilized our surgical mouse model of NBPI whereby injury to the brachial plexus at postnatal day (P) 5 induces contractures within 4 weeks after denervation.[4,5,6] Beginning one day prior to surgery and continuing for four weeks after surgery, we pharmacologically inhibited the myostatin pathway by treating wildtype neonatal mice with a soluble decoy receptor fused to an Fc domain (ACVR2B-Fc).[8] The binding of myostatin to the Fc domain inhibits myostatin from binding to Activin A, which blocks myostatin activity in the muscle, ultimately leading to increased muscle protein synthesis and robust muscle growth.[9] This

soluble decoy receptor was administered weekly at 10 mg/kg via intraperitoneal injections, whereas Dulbecco's phosphate-buffered saline (PBS) was used as a control in a separate litter of mice (**Figure 1A**). All mice were handled according to approved institutional animal care and use committee (IACUC) protocols (#2017-0084) of the Cincinnati Children's Hospital Medical Center.

Mice were sacrificed 4 weeks post-NBPI, whereupon passive range of motion of the elbow and shoulder joints were assessed to determine contracture severity as previously described.[4,5,6] We then harvested and weighed bilateral biceps muscles, which were subsequently assessed for total protein via Bradford assay. The remaining forelimbs were imaged by digital x-rays to determine humerus length, after which bilateral brachialis muscles were processed into muscle bundles and imaged for sarcomere length as a readout of functional whole muscle length.[5,6] All measurements were performed with blinding to treatment, and statistical tests were performed with Prism 8 software (GraphPad).

Results: While pharmacologic inhibition of myostatin with the soluble decoy receptor has been shown to induce robust muscle growth in adult mice, it has not been validated in neonatal mice. We therefore began by verifying the effects of neonatal myostatin inhibition on developmental growth. Furthermore, as sexual dimorphism in body and muscle mass occurs in mice during postnatal growth, we analyzed the effect of myostatin inhibition on musculoskeletal development according to individual sexes. Our results revealed that neonatal myostatin inhibition impacts developmental growth in a sex-specific manner. Specifically, whole body weight gain was observed only in female mice (**Figure 1B**), whereas skeletal growth as measured by humerus length of non-denervated forelimbs was attenuated in male mice (**Figure 1C**). Importantly, neonatal myostatin inhibition enhanced muscle growth in both female and male mice, as evident by the increase in overall muscle mass and elevated protein content of non-denervated biceps muscles (**Figures 1D & 1E**). Despite this, the increase in muscle weight and total protein was greater in females than males when compared to respective controls (47-49% vs. 28-35%) (**Figure 1F**). These results indicate that inhibition of the myostatin pathway facilitates greater muscle protein anabolism and neonatal muscle growth in female mice.

Due to this differential effect on muscle growth, we speculate that myostatin signaling may mediate contracture formation in a sex-dependent manner. Indeed, we discovered that neonatal myostatin inhibition improved passive range of motion in both elbow and shoulder joints only in female mice after 4 weeks of NBPI (**Figures 2A, 2B, 3A, & 3B**). This improvement corresponded to a reduction in both elbow flexion and shoulder rotation contracture severity in female, but not male mice (**Figures 2C & 3C**). To gain insights on this sexual dimorphism, we next assessed the role of neonatal myostatin inhibition on preserving longitudinal growth in denervated muscles. Here, we observed that myostatin inhibition reduced sarcomere overstretch in the denervated brachialis muscles of female mice (**Figures 4A, 4B, & 4C**), indicating an improvement in functional muscle length.[4,5,6] Conversely, sarcomeres in the denervated brachialis of male mice remained overstretched, an indication of impaired longitudinal muscle growth. This sex-specific improvement in functional length of the denervated brachialis muscle was further associated with an increase in muscle mass and total protein content of the denervated biceps muscle in female mice (**Figures 4D & 4E**). Hence, our results suggest that myostatin inhibition preferentially reduces contracture formation in female mice by increasing protein levels and preserving longitudinal growth of denervated muscles.

Discussion: Our overall findings from this study demonstrate a sex-dependent role for the myostatin pathway in mediating contracture formation following neonatal muscle denervation. This discovery establishes a critical link between denervation and impaired longitudinal muscle growth, which helps guide translation of safer strategies for preventing contractures by targeting a skeletal muscle-specific signaling pathway. To decipher additional mechanistic insights on

sexual dimorphisms in myostatin-mediated contracture formation, future studies will explore the relationships between sex hormones, such as testosterone and estrogen, and myostatin signaling in NBPI. Another significant discovery is that myostatin inhibition during the neonatal period enhances normal muscle growth, which may have potential implications in other pediatric muscle disorders. To further validate this strategy, additional studies will assess the temporal expression of myostatin during neonatal development.

While the role of sex in neonatal muscle denervation is unknown, we speculate that sex mediates divergent pathways that ultimately lead to contracture formation. While our findings indicate that contractures are regulated by the myostatin signaling pathway in females, it is unclear what signaling mechanism(s) govern contracture formation in males. As such, exploring the role of sex hormones as mentioned above may lead to the identification of key pathways involved. It is also possible that the pharmacodynamics of the myostatin decoy receptor employed in this study elicits different responses in females and males. We speculate that the concentration, frequency, and duration of ACVR2B-Fc administration could potentially induce distinct therapeutic and adverse effects between the sexes. To eliminate these confounding factors and verify the potential sexual dimorphisms observed here, future studies will compare our pharmacologic findings against a genetic model of myostatin deletion.

One limitation in this study is that we did not analyze levels of muscle protein synthesis and degradation. As a result, we are currently unable to conclusively establish whether inhibition of myostatin signaling prevents contractures in female mice by correcting the dysregulation in muscle proteostasis. Hence, future studies will more clearly elucidate the role of myostatin inhibition in restoring muscle protein balance after NBPI by assessing synthesis and degradation levels.

In conclusion, the detection of sex-specific outcomes in our current study highlights the often overlooked importance of sex in disease pathology and treatment response. Moving forward, we are eager to dissect the mechanistic links between sex and contracture formation as we continue to investigate the pathophysiology of pediatric muscle contractures.

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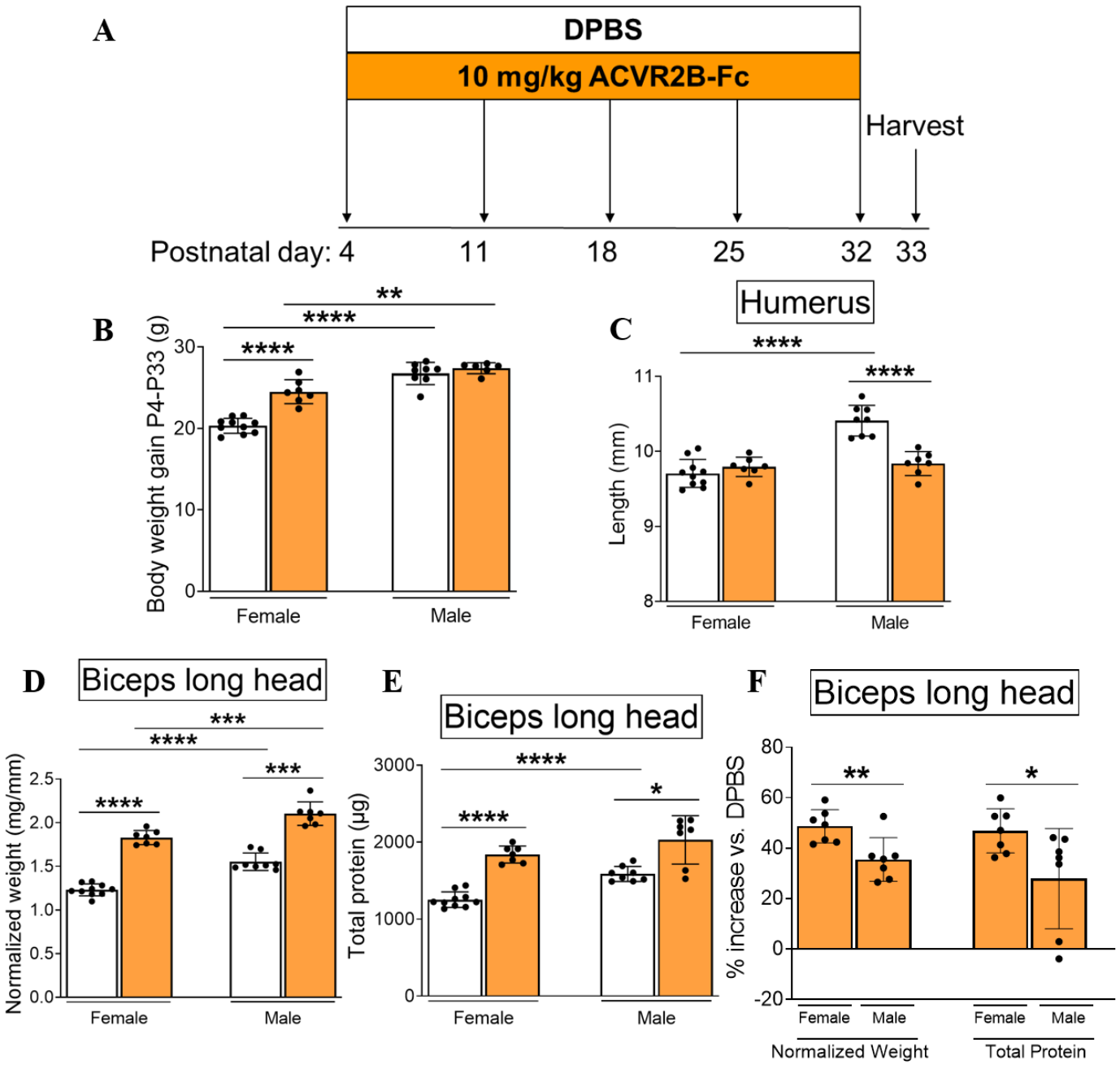

Figure 1: Sexual dimorphisms in developmental growth with neonatal myostatin inhibition. A) Schematic depicting weekly administration of ACVR2B-Fc in neonatal mice. **B)** Myostatin inhibition increased body weight in female mice but **C)** decreased humerus length of the non-denervated forelimb in male mice. **D)** Myostatin inhibition increased the weight and **E)** total protein content of the non-denervated biceps muscles in both sexes. However, **F)** females had a greater increase in muscle weight and total protein than males when compared to respective controls (47-49% vs. 28-35%). All data are presented as mean ± s.d. n = 6-10. Statistical analyses: **(B)**, **(C)**, **(D)**, **(E), (F)** unpaired two-tailed Student's t-tests. The degree of significance between data sets is depicted as follows: *P<0.05, **P<0.01, ***P<0.001 ****P<0.0001.

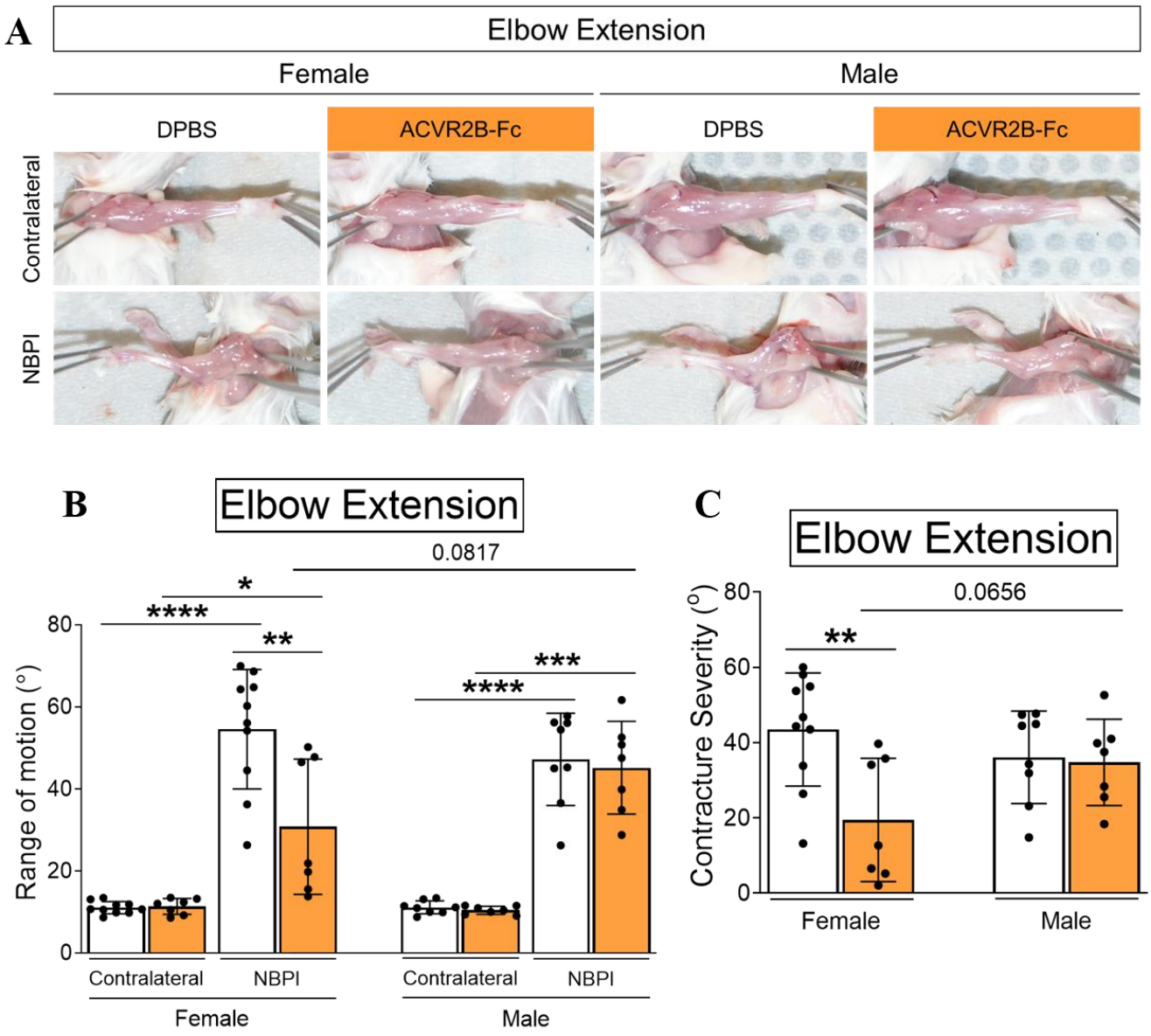

Figure 2: Neonatal myostatin inhibition reduces formation of elbow contractures in a sex-specific manner. A) Representative images of denervated (NBPI) and contralateral forelimbs reveal an improvement in elbow extension only in female mice treated with ACVR2B-Fc. Quantitative analyses further demonstrate **B)** a rescue in elbow joint range of motion in the NBPI forelimb and **C)** a reduction in elbow flexion contracture severity of female, but not male mice. Contracture severity is calculated as the difference in passive elbow extension between the denervated (NBPI) side and the contralateral control side. All data are presented as mean ± s.d. n = 7-10. Statistical analyses: **(B)** unpaired, two-tailed Student's t-test between groups and paired, two-tailed Student's t-tests between limbs of mice in each group, **(C)** unpaired two-tailed Student's t-tests. The degree of significance between data sets is depicted as follows: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

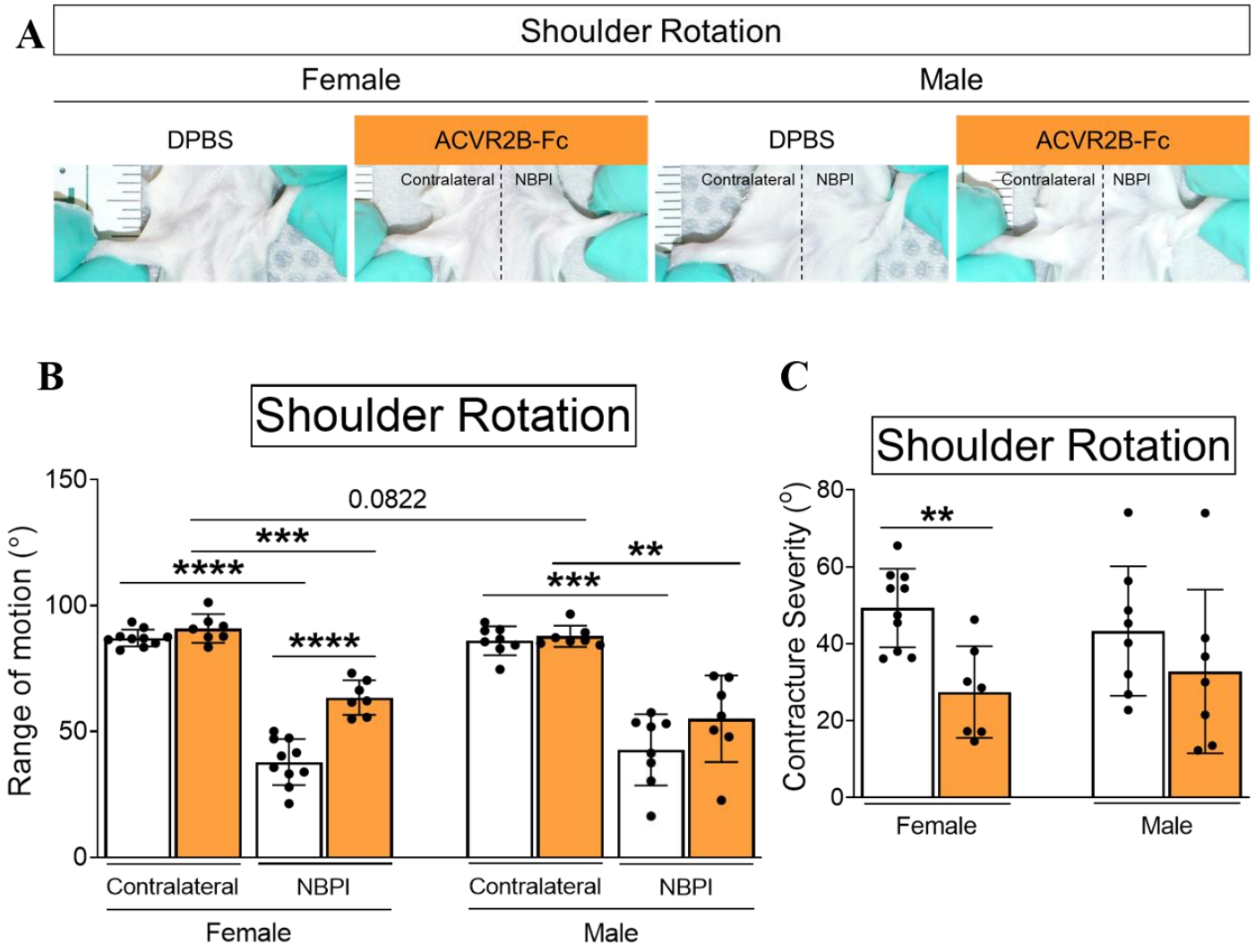

Figure 3: Neonatal myostatin inhibition reduces formation of shoulder contractures in a sex-specific manner. A) Representative images of denervated (NBPI) and contralateral forelimbs reveal an improvement in shoulder rotation in female mice treated with ACVR2B-Fc. Quantitative analyses further demonstrate **B)** a rescue in shoulder joint range of motion in the NBPI forelimb and **C)** a reduction in shoulder rotation contracture severity of female, but not male mice. Contracture severity is calculated as the difference in passive shoulder rotation between the denervated (NBPI) side and the contralateral control side. All data are presented as mean ± s.d. n = 7-10. Statistical analyses: **(B)** unpaired, two-tailed Student's t-test between groups and paired, two-tailed Student's t-tests between limbs of mice in each group, **(C)** unpaired two-tailed Student's t-tests. The degree of significance between data sets is depicted as follows: **P<0.01, ***P<0.001, ****P<0.0001.

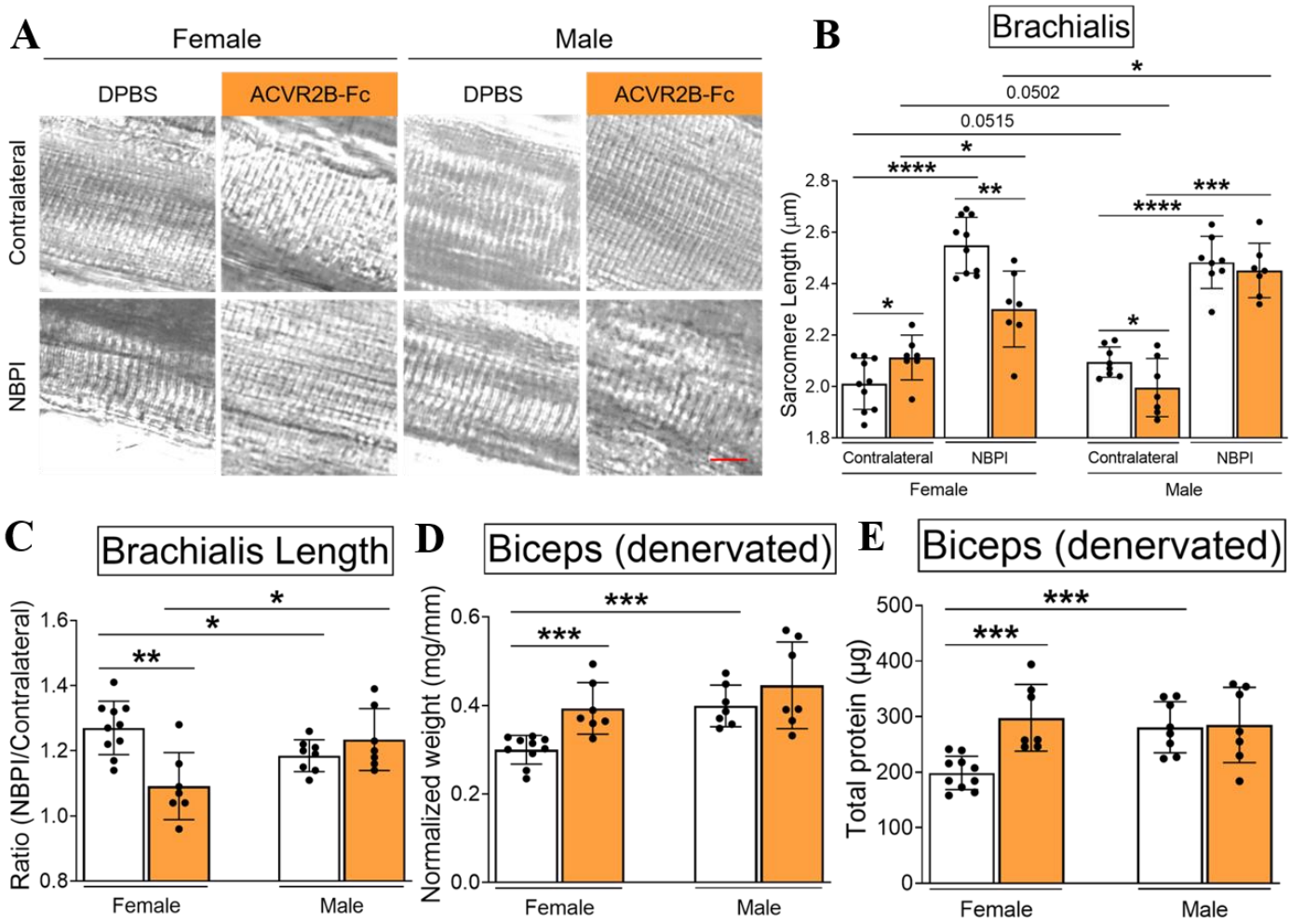

Figure 4: Neonatal myostatin inhibition preserves longitudinal muscle growth and reduces denervation-induced muscle atrophy in a sex-specific manner. A) Representative DIC images of sarcomeres in denervated (NBPI) and contralateral brachialis muscles and **B,C)** quantitation of sarcomere length reveal that ACVR2B-Fc treatment reduced sarcomere overstretch after NBPI in female mice, indicating an improvement in functional length of denervated muscles. However, the sarcomeres of denervated muscles in male mice remain elongated following ACVR2B-Fc treatment, indicating functional muscle shortening. The restoration in longitudinal muscle growth with myostatin inhibition in female mice is accompanied by an increase in **D)** muscle weight and **E)** total protein content of denervated biceps. In (**C**), sarcomere length on the NBPI side is normalized to the contralateral side, with a normal sarcomere length ratio of 1.0, and any value over 1.0 indicating sarcomere overstretch on the NBPI side, and thus, functional muscle shortening. All data are presented as mean ± s.d. n = 7-10. Statistical analyses: **(B)** unpaired, two-tailed Student's t-test between groups and paired, two-tailed Student's t-tests between limbs of mice in each group, **(C)**, **(D)**, **(E)** unpaired two-tailed Student's t-tests. The degree of significance between data sets is depicted as follows: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Scale bar: 100 pixels.